# SOME ASPECTS OF
# SYMBIOTIC BIOLOGICAL FIXATION OF NITROGEN
## (with special reference to factors affecting nodule formation on excised roots and normal root systems).

being a thesis presented by
MOIRA P. McGONAGLE, B.Sc.
for the degree of Doctor of Philosophy of the
University of Glasgow.

March, 1949.

# CONTENTS.

## ACKNOWLEDGEMENTS.

The work described in the following pages was carried out in the Botany Department of the University of Glasgow during the tenure of a Robert Donaldson Research Scholarship, a Carnegie Research Scholarship, and latterly, under a grant from the Agricultural Research Council.

The author wishes to express her thanks to Professor John Walton for support in obtaining the Research Scholarships and for placing the facilities of his Department at her disposal.

Special gratitude is due to Dr. George Bond whose patient supervision and guidance have been much appreciated throughout the course of the investigations. The writer is also grateful for his kindness in lending text books and papers from his private collection and in taking some of the photographs which illustrate this thesis. The remaining photographs are the work of Mr. W. Anderson to whom thanks is also offered.

# GENERAL INTRODUCTION.

Despite centuries'-old recognition of the importance of leguminous plants in agriculture, and the large amount of research into their characteristics performed during the past half century, many problems concerning the symbiotic relationship between legumes and nodule bacteria remain unsolved. The present author has attempted to apply to some of them the relatively new technique of tissue culture.

In 1902 the botanist Haberlandt first suggested the possibility of cultivating isolated portions of an organism in an artificial environment containing all the essentials of their natural habitat. His choice of material, however, was unfortunate and his results unsuccessful, but five years later, Harrison, a zoologist, using similar methods, successfully cultivated the neuroblast of a frog in clotted lymph and thus provided the starting point for the extensive work on tissue culture carried out in subsequent years. As we shall see in the following section, the culture of excised roots of plants was achieved in due course and the investigator now had a unique opportunity of studying the requirements and responses of one particular organ unhindered by reactions taking place simultaneously in neighbouring regions of the plant. The apices of roots proved to be especially suitable for in vitro culture due to "their meristematic character, their ease of excision and consequent relative freedom from trauma, and the considerable variety of their normal physiological responses". (White, 1943b)

It seemed likely that the technique of root culture could be profitably applied to the investigation of the development and function of leguminous root nodules and might facilitate the study of several aspects of nodule physiology. So far as is known to the author, there has been no previous attempt to secure nodules on roots cultured from small excised portions of apical meristem, although, as we shall see, the nodulation of young root systems excised in their entirety has been investigated, and a small number of nodules obtained. In the following pages, however, the term 'excised root', unless otherwise stated, always refers to an organ grown in vitro from an original fragment approximately 0.5 cm. in length. The primary and main object of the present work has been to get nodules to form on such excised roots and although this object has not yet been achieved, results have been obtained which will be of guidance in further experiments along these lines. Arising directly from the work with excised roots a number of experiments have been carried out to determine the effect of various factors on the nodulation of whole leguminous plants. Despite their intimate relationship, it has been thought best to separate in the thesis the investigations relating to whole plants from those concerned with excised roots. This seemed especially desirable since in some instances the enquiries into certain aspects of the nodulation of whole plants have been developed further than was strictly necessary for the purpose of giving guidance in procedure with root cultures. A brief report on some of the earlier findings has already appeared (McGonagle, 1944), while a more detailed account of several of the experiments with whole pea plants has been accepted for publication by the Royal Society of Edinburgh.

If it proved possible to secure nodules on the excised roots of legumes, the part normally played by the cotyledons and shoot in the process of nodule formation and function could be more accurately assessed than with whole plant cultures. For example, it might be possible to determine the origin and nature of the specific stimulus which causes the nodule bacteria to invade the root hairs of the appropriate host. It has been stated that a water-soluble substance which causes bacterial multiplication and eventual invasion of the plant is excreted from the roots of intact legumes at a time co-incident with the opening of the first true leaves (Thornton, 1929). Therefore, if the presence of such an excretion could be detected with excised roots, it would be obvious, either that the root itself must be the region of its synthesis, or that it had been transferred from the cotyledons to the root tip in the short period preceding excision. It would also be interesting to note whether nodules which might form on excised roots would contain haemoglobin, the red pigment present in all nodules which contain bacterial strains effective in fixing nitrogen. Some further knowledge of the actual mechanism of the fixation process might then be gained. Moreover, all the substances essential for healthy development of excised roots are present in the culture medium, and it is thus possible to determine the effect of these on the bacteria themselves, and on the various aspects of their symbiotic relationship with the plant, commencing with invasion of the root hairs. Yet another advantage of the root culture technique is that the presence of light becomes unnecessary and therefore it is possible to experiment with equal facility at any season of the year. The whole plant methods at present widely employed in legume studies usually restrict investigations, in this climate at least, to the late spring and summer months.

# SECTION I.

## Culture of excised leguminous roots and attempts to secure nodule formation on such roots.

# INTRODUCTION.

Theophratus, in his philosophical dissertations, put forward the idea that the plant or animal body could not be considered as a whole, but was subdivided into innumerable regions each with its own particular function, regions which our modern nomenclature now terms "tissues". However, with the passage of time, his early observations came to be forgotten, and it was only a little over a century ago that the present concept of the structure of living organisms was formulated. This so-called Cell Theory stated that plants and animals were aggregates of small units, or cells, containing the 'life' substance, protoplasm, capable of modification, and grouped together according to function into larger cell masses known as tissues. Previous to the formulation of this theory the naturalist had studied the characteristics, behaviour and requirements of entire plants or animals, but now interest was stimulated in their smaller component parts.

To investigate the structure and function of single cells, highly specialised techniques had to be evolved and the possibilities of one such method were realised by Haberlandt in 1898 and the results of his experiments published in 1902. He maintained that it should be possible to grow single somatic cells of the higher plants in vitro by supplying them with all the requisites contained in their original surroundings.

For his investigations he chose highly specialised chlorophyll-bearing cells e.g., those from the palisade layer of the leaf of Lamium, pith cells of petioles, etc., since such cells might be expected to carry on photosynthesis and thereby supplement the materials provided in the medium. The culture solutions employed were:- ordinary tap water, 1 - 5 per cent. sucrose, Knop solution, Knop solution with the addition of glucose (1 - 4 per cent.), glycerine, asparagine, and peptone, and his results indicated that certain cells increased in size but there was no evidence of cell division.

Since Haberlandt (1902) was the first to attempt to grow fragments of an organism in artificial media, he may be regarded truthfully as the first in the field of tissue culture. However, some years previously, Rechinger (1893), experimenting with isolated buds and roots, grew them in sand without nutrient solution, relying on the materials stored in the fragment itself. He claimed that the sections "planted" had to be at least 20 mm. thick before growth would occur and hence concluded that the presence of a certain amount of vascular tissue was essential.

After this preliminary work by botanists the problem of tissue culture was taken up not only by plant physiologists, but also by those interested in the workings of the animal cell, and the investigations of this latter group met with immediate and signal success. The classic work carried out by Harrison (1907) with nerve cells has proved to be the precursor of a vast number of investigations whose results have had widespread influence upon our knowledge of animal metabolism.

Although plants possess powers of regeneration far exceeding those of animals, and hence should be more readily cultured in vitro, the advance of the botanical side of the technique has been both slow and arduous. In the years immediately following the initial steps taken by Haberlandt, the problem was tackled by numerous workers who used a variety of tissues such as embryos, palisade cells, spongy parenchyma and fragments of leaves. In some cases no culture media were supplied, in others, mineral solutions or mineral solutions plus various sugars and amino compounds, and in still others, the fragments were cultivated in sap from their own species of plant. With embryos, embryo sac fluid was employed. The cells in some of these experiments apparently remained alive for as long as four months and enlarged slightly, but no cell divisions were noted and therefore no true growth had occurred.

Knudson (1919) made some interesting observations concerning the root cap cells of corn and Canada field pea. The plants were grown in Pfeffer's nutrient solution with and without one half per cent.

sucrose, and the root cap cells as they were sloughed off collected at the base of the culture vessels as a slimy mass. He reports that examination of such cells in sucrose cultures of Canada field pea showed that many were still alive after 71 days. They could be plasmolysed and subsequently de-plasmolysed. The corn cultures were examined when 45 days old and none of the root cap cells were dead.

Molliard (1921) attempted to grow fragments of roots, hypocotyls, and cotyledons in a Knop solution plus glucose, but once more only enlargement was obtained and no evidence of cell division.

The first real step forward was taken almost simultaneously in 1922 by Kotte in Germany and Robbins in America. They both used exclusively the meristematic tissues of higher plants as exemplified by root tips and shoot tips. Kotte (1922) cultivated tips as small as 1.0 mm. in length, excised from the roots of Vicia Faba and Zea Mays, in Knop's solution to which were added in various combinations, glucose, peptone, meat extract, etc. The best growth (up to 60 mm.) was achieved in 1 per cent. glucose plus 0.5 per cent. meat extract. Geotropic curvatures were in evidence and branch roots were produced. Some of the tips which grew satisfactorily did not contain rudimentary vascular strands, thus disproving the contention of Haberlandt and others that vascular tissue is a pre-requisite for the growth of plant fragments in culture.

The experiments of Robbins (1922a) were carried out with excised root tips and shoot tips of corn, peas and cotton, which he grew in the dark in a Pfeffer solution plus 2 per cent. glucose or 2 per cent. levulose. The results indicated that glucose is a more satisfactory source of energy than levulose, and when excised root tips of corn were grown in mineral solution plus glucose for ten days or two weeks and then transferred to fresh solution, the growth was less in the second period, and ceased entirely in a third such period. This led him to conclude that the seedling root contains some material derived from the seed (other than glucose, mineral salts, water and free oxygen) which is necessary for continued growth, and which the root cannot synthesise in the dark from the substances supplied. In a second series of experiments, Robbins (1922b) investigated the effect of various sources of nitrogen and found that the addition of peptone or autolysed yeast to the medium permitted corn root tips to grow through four to six two-weekly periods before growth ceased. The autolysed yeast was more beneficial than peptone but its effects were not evident during the first growth period, and whatever the cause of its beneficial action, its effect was limited in some way because varying concentrations of yeast produced no correspondingly increased benefits, and the solution plus glucose plus yeast would still not permit of continued growth.

Robbins and Maneval (1923) varied the concentrations of glucose added to their cultures of corn root tips and concluded that the 4 per cent. value was optimal. They also attempted to cultivate tips of beans, radish, morning glory, lupin, alfalfa, mustard, wheat, sunflower, flax, cress, buck-wheat and squash and found that flax gave by far the best growth. The root tips of this species at the time of excision varied in length between 2 and 60 mm. and no appreciable difference in their growth was observed. These same authors (1924) investigated the effect of light upon their cultures and concluded that it was beneficial. Under the conditions provided, one root was kept growing for a period of 150 days.

From this point onwards we shall confine ourselves to a review of that branch of tissue culture which deals with the cultivation of excised root tips, since a survey of the literature on the culture of other plant organs would not be strictly relevant to the thesis.

Chambers (1923), using the culture medium plus glucose and peptone which had been advocated by Robbins, but solidifying it with agar, grew isolated root tips of squash in hanging drop cultures at a temperature of 27° C. The larger initial fragments grew into normal differentiated roots, but those which originally measured

0.3 - 1.0 mm. showed a migration of cells comparable to that in an animal fibroblast culture. The next investigator to choose root tips as his material was Mayer (1929) and although he experimented with many types of solution his success was but indifferent.

Malyschev (1932) cultured the root tips of peas, beans and maize in media including 1.25 - 1.50 per cent. agar, full strength and half strength Knop solution, 2.5 - 5.0 per cent. sucrose, and asparagine, but he failed to obtain satisfactory results in similar media which were not solidified by the addition of agar.

This year of 1932 also marked the entry into the field of plant tissue culture of P.R. White of the Rockefeller Institute for Medical Research, who was to play a larger and more significant part in the further development of the technique than any other single investigator. In his preliminary observations (1932a) he used young embryos of Portulaca oleracea, root tips of Triticum vulgare and seed primordia of Antirrhinum majus, and in each case strong evidence of the feasibility of such cultures was obtained. As was observed by Kotte (1922) , the growth of root tips occurred in the absence of any differentiated vascular tissue in the original fragment. Vascular strands formed although they seemed unnecessary either for conducting or support, giving an idea of the very early regional determinism in such an organ.

In further experiments later in the same year, White (1932b) concluded that the optimum conditions for the growth of excised wheat root tips embraced:- redistilled water, the salts and salt proportions of the Uspenski (1925) solution, 20 gm. of dextrose per litre of solution, yeast extract from 0.1 gm. yeast per litre of solution, a final or equilibrium H ion concentration giving pH value about 5.0, a maintained temperature of about 26 - 27° C, continuous electric light, and at least 25 cc. solution per culture of a single root tip.

White (1933a) observed the effect of varying the ionic concentrations of the elements contained in the Uspenski solution, and in other experiments (1933b) the effect of the .SH radical on the growth of excised wheat root tips was considered. He also (1933c) compared his own results and those of Malyschev (1932a and 1932b) who advocated the use of a substrate solidified with agar. White used yeast extract instead of asparagine, but otherwise the solutions of the two investigators were chemically comparable, and he was able to state that "The ratio of daily increment to initial value, which appears to be the best measure of tissue activity, has been from 100 to 600 times as great in the writer's cultures in a liquid medium as in Malychev's cultures on solid media."

We see therefore that up until the time of

Kotte, Robbins, Robbins and Maneval, Malyschev and White, all that had been achieved was merely a retardation of the process of dying, a slowing down of the vital activities of the cell which enabled it to survive in vitro for relatively long periods, but no cell divisions were observed and hence no true growth had occurred. The work of these last mentioned authors, on the other hand, has proved that appreciable growth increments can be obtained in cultures of meristematic regions of the higher plants and that the constituents of the media contribute largely both to the quality and quantity of such growth. However, since the observed mean growth rates decreased rather consistently during the course of the experiments, White (1934a) says, "This would appear to indicate that the rate of increment might have been determined by the concentration of some unknown material furnished to the culture from the tissues of the original fragment and diluted in the multiplication of these tissues. If this be true, these cultures also were cases of survival, though for long periods".

White's aim therefore, was to discover a complete nutrient which would maintain a plant tissue in a state of active increase over a long period of time, and through a sufficient number of passages, to ensure a dilution of all the materials contained in the original fragment beyond the point where they could possibly be effective in determining growth rates. 1934 saw the realisation of this ambition since he was then able to publish (1934a) results which showed that he had achieved the potentially unlimited growth of excised root tips of tomato for over a year, through 52 passages, and in continuous active growth. The theoretical dilution of any substance contained in the original fragment was well beyond $10^{-40}$ so that the entire material of most of the ultimate cultures was derived from the nutrient provided. The growth rates showed no consistent tendency to fall off, in fact, an increase in rate was observed from the first to the twenty-sixth passages, after which time it remained constant. To the mineral salt mixture employed was added 2 per cent. by weight of sucrose, which for tomato was found to be superior to the dextrose used in previous experiments, and the filtered extract of 0.1 per cent. by weight of dried brewers' yeast. The cultures were grown in diffuse sunlight in a potting shed, subject during the summer months to the temperature fluctuations of the outdoor atmosphere.

White (1934b) used his technique as a method for cultivating the viruses of tobacco mosaic and aucuba mosaic in isolated tomato root tips grown in vitro. It provides a simple means of maintaining stocks of these two viruses in a state of active multiplication, yet free from contamination with other viruses,

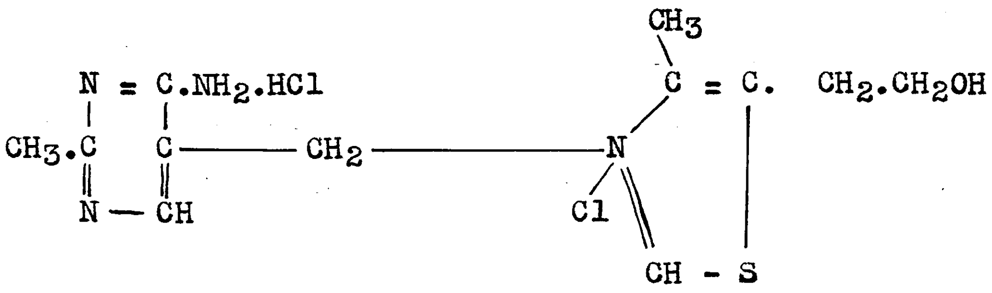
Galligar (1934) found that there was some correlation between the growth behaviouf of excised root tips and the type of food stored in the seed. All tips from seeds or grains high in starch reserves were able to grow well, but the growth of those from seeds selected for oil or sugar varied widely, while tips from seeds with high protein reserves e.g., pea (Pisum sativum) and Manchu soya bean (Soja hispida) were least able to maintain growth. This was true of all the members of the Leguminosae which were tested.

Robbins, Bartley and V.B. White (1936) showed that a decapitated root tip, 1 mm. or so in length, contains within itself all the essentials for regenerating a new apical meristem and root cap if it is grown on an agar medium containing mineral salts and 2 per cent. glucose. They suggest that the cultivation of such excised root tips may permit a more complete analysis of the physiology of regeneration than is possible when the root is attached to the plant, and supplied with all the materials which may be derived therefrom.

Since Robbins (1922) and White (1932b) had found that yeast extract was beneficial to the growth of isolated roots, interest was now aroused in discovering exactly what constituent of this organic complex was proving to be the active principle. Bonner (1937), using excised pea roots, came to the same conclusion already arrived at by Robbins (1922b) for the growth of root tips of corn, viz. that although additions of yeast extract had no stimulating effect during the first passage, when the third passage was reached, pea roots cultivated in nutrient medium without yeast ceased growth completely, whereas roots in the same medium but with the addition of 0.01 per cent. yeast extract, could be carried through many further subculturings with an average growth rate of 6 to 9 mm. per root per day. Therefore the root as cut from the seedling plant contained sufficient 'growth factor' to permit of growth for a limited time, but after two or more passages this initial supply was used up and the root then responded to the presence of yeast. Bonner next found that Vitamin $B_1$[*] concentrates were considerably more active as a source of this root growth factor than was yeast, and this suggested that Vitamin $B_1$ itself might be the active principle. Experiments showed that 0.2 gamma of the Vitamin per cc. of solution was able to replace the optimal yeast extract concentration, and in fact was superior to it. Even 0.002 gamma per cc. had a marked stimulating effect.

---

[*] Vitamin $B_1$ is also known as aneurin or thiamin and has the following formula (as its hydrochloride).



Robbins and Bartley (1937a), using the excised root tips of tomato confirmed the above results of Bonner with peas. Yeast ash was not capable of replacing yeast, but when it was replaced by natural or synthetic Vitamin $B_1$ excised tomato roots continued to grow, and the Vitamin was found to be effective at great dilution. "Our results demonstrate that the parasitic relationship of the tomato root to the top involves both carbohydrate and Vitamin $B_1$". Later in the year (1937b) they slightly modified this last statement in view of results which showed that 4-methyl-5-B hydroxyethyl thiazole was effective in the growth of excised tomato roots. Whether the thiazole actually replaces the Vitamin $B_1$ in the metabolism of the tomato root or whether the root produces sufficient of the necessary pyrimidine to permit synthesis of the Vitamin when the thiazole is supplied, was not known. These same authors (1937c) record observations in contrast to those made by White who stated that excised tomato roots require sucrose and are unable to utilise dextrose as their source of carbohydrate. They grew tomato roots for more than six months in a dextrose solution without noting any decrease in growth rate, and therefore felt justified in concluding that the tomato root tips were able to assimilate the dextrose.

Robbins and V.B. White (1936) investigated the limited growth and the abnormalities which developed in excised corn root tips growing in liquid media. It was thought that the poor growth was the result of a deficiency partly inorganic, in the solutions since it could be partially counteracted by the addition of agar, water extracts of agar, qualitative filter paper, water extracts of qualitative filter paper, soluble starch, autolysed or dried brewers' yeast, or a mixture of salts of manganese, boron and zinc. Proceeding still further with their experiments, they discovered (1937) that although milk from immature corn grains was injurious or without benefit to the growth of excised corn root tips, the diffusate from germinated grains was highly beneficial, and therefore they concluded that organic material in addition to dextrose is essential to good growth.

Meanwhile, P.R. White was continuing his researches and towards the end of 1937 recorded a number of interesting observations. He measured the growth rates (1937a) of a clone of isolated tomato root tips at weekly intervals over a period of three years and found that these rates showed a cyclic fluctuation correlated with the seasons of the year. Investigation into the effects of light showed that the seasonal changes in illumination were a neglible factor, but the cultures proved to be very sensitive to temperature differences and those recorded were sufficient to account for the seasonal variations in growth rate. He then studied the effects of temperature in greater detail and found (1937b) that with isolated tomato roots a temperature of 15° C. is more suitable than 8° C. or 22° C. for the maintenance of large numbers of stock cultures. Like other investigators, White (1937c) now turned his attention to determining the nature of the constituents in brewers' yeast which were of significance in promoting the growth of isolated root tips. Only 18 mg. of an 85 per cent, alcohol extract was needed for optimal results. Extraction of this material with 100 per cent alcohol separates it into two fractions, both of which are essential for satisfactory growth. The material insoluble in 100 per cent. alcohol contained considerable quantities of amino acids and White therefore suggests that these may play an important role as growth promoting substances for isolated tomato roots. In a further paper (1937d) he showed that this insoluble fraction can be replaced by a mixture of 9 amino acids without appreciably reducing the growth of the roots. Experiments then showed (1937e) that Vitamin $B_1$ is a probable constituent of the soluble fraction and that it is an important and perhaps indispensable factor in the nutrition of excised tomato roots. However White states that the growth promoting effects of the Vitamin are only detectable in the presence of twelve "Accessory salts" which are also indispensable. In the following year he (1938a) made an effort to determine the relative importance of the constituents of the accessory salt mixture. It was thought that the ions of its twelve salts might replace the unidentified but important ash constituents contained in the yeast extract. The results showed that now only four, viz. $MnSO_4$, $H_3BO_3$, $ZnSO_4$,,and KI appear to be essential for the growth of tomato roots under the conditions reported. When these four salts were added to a nutrient containing the standard salts of Pfeffer's solution, iron, sucrose, amino acids and Vitamin $B_1$ in proper proportions, the resulting nutrient was almost, if not quite, equal to a yeast extract medium as a source of materials essential for the growth of excised tomato roots.

Simultaneously with this work of White, Bonner and Addicott (1937) were devoting their attention to the detailed study of the environmental factors which affect the excised roots of pea (Pisum sativum). Although this species of root had been used in the culture experiments of Kotte (1922), Robbins, Bartley and V.B. White (1936) and Fiedler (1936), strikingly good results had not been obtained and the optimal culture conditions were still unknown. Bonner and Addicott took into consideration every possible aspect of the culture solution, and since pea roots were used to a great extent in the work of the present author, the results of their investigations merit some detailed study. In the first place, they discovered that the best growth was attained by roots which originated as fragments at least 3 mm. long, so it appeared that for the production of a normal root, both the 'initial zone' and a relatively large amount of the tissue immediately behind it were necessary. They next investigated the inorganic constituents of the medium and arrived at a formula which appeared to be best suited to peas. As regards the type of carbohydrate employed, Bonner and Addicott found that pea roots resemble tomato roots in a preference for sucrose, and that the 4 per cent. concentration is more beneficial than the 2 per cent. value previously employed. The growth of 3.5 mm. tips in one week in the standard medium with 4 per cent sucrose varied between 60 and 95 mm. and since the growth of similar pea roots attached to the seed is 75 mm. in the first week, the excised roots were considered normal as far as the growth rate was concerned. They recorded that yeast extract (0.01 per cent.) must be added to the basic medium if good growth in passages after the first is to be maintained, and stated that pea roots were kept in such cultures plus yeast, with weekly transfers, for a period of four months. Crystalline Vitamin $B_1$ was capable of partially replacing the yeast extract, but the Vitamin alone was not able to support the continued optimal growth of excised pea roots in later passages. However, it was found that a mixture of pure crystalline amino acids was able to replace that portion of the activity of yeast extract which was not due to Vitamin $B_1$. Therefore, with the use of both the Vitamin and the acids, a highly satisfactory and completely known medium for the cultivation of excised pea roots was obtained, which supported the growth of such roots as well, or better, than media containing yeast extract. In conclusion they state that substances such as Vitamin $B_1$ which are necessary in minute amounts for the growth of the isolated roots, but which are normally supplied by some other portion of the intact plant, are carriers of a growth correlation and hence are to be regarded as phytohormones.

In their next publication, Addicott and Bonner (1938) reported that a mixture of seven amino acids was capable of increasing the growth rate of isolated pea roots over that obtained with Vitamin $B_1$ alone, but was incapable of supporting growth at an undiminished rate for more than three weekly transfers. However, nicotinic acid in combination with Vitamin $B_1$ exerted a very striking effect upon the growth of the isolated pea root since in an experiment which was maintained through ten weekly transfers, pea roots which were supplied with nicotinic acid in addition to Vitamin $B_1$ and the amino acid mixture, showed an undiminished growth rate. The authors state that in the future it may be found that the amino acid mixture is not essential to the continued optimal growth of such roots but emphasise that nicotinic acid must be regarded as a factor quite as significant as Vitamin $B_1$.

In this same year Bonner (1938) published an article dealing still further with the role of thiamin in the metabolism of isolated pea roots, and some of his conclusions resemble the earlier findings of Robbins and Bartley (1937a) with tomato roots. He states that the isolated pea root is able to utilise an equimolar solution of the pyrimidine and thiazole portions of the thiamin molecule and that it it probable that these two portions are synthesised in vivo to the Vitamin molecule itself. As the thiazole component of such a mixture, the pea root can use a considerable number of substituted thiazole compounds, but it is essential that a hydroxyl group (or a group readily metaboliseable to such) be present in the molecule if the substance is to possess activity as a root growth factor.

Later in the year, investigations on the syntheses carried out by isolated pea roots were advanced a stage further by Bonner and Buchman (1938) who showed that such pea roots did synthesise Vitamin $B_1$ (or a substance indistinguishable from it by the Phytophthora bioassay) from a mixture of the pyrimidine and thiazole components of the Vitamin molecule. They suggest that specific enzymes, "thiaminase" and "thiazolase" may play a part in the natural synthesis of thiamin by the plant and it is hoped that the methods outlined will offer a new and more exact approach to the problems of the mechanism of biosyntheses.

van Overbeek collaborated with Bonner (1938) in an effort to establish whether or not auxin is present in isolated roots cultured in vitro. In 1933 it had been conclusively demonstrated by Boysen-Jensen (1933) that auxin is present in the normal root, but with respect to the isolated root Fiedler (1936) reported that auxin, although originally present, disappears completely and in general within 24 hours when the root is cultivated in vitro. Nagao (1936), on the other hand, showed that auxin could be recovered from isolated roots after 6 days cultivation. The present authors, van Overbeek and Bonner, found that isolated pea roots in culture contained auxin for at least three weeks after the original tip was removed from the germinating seed, and they also observed that roots which had been cultured for two weeks or more contained less auxin than did the initial root tips. van Overbeek (1939a) compared the amounts of auxin obtained from excised root tips of germinating peas by diffusion and by extraction with improved techniques. He grew tips of Alaska peas 3-4 mm. long on agar blocks containing 10 per cent. of dextrose and noted that diffusion into these blocks yielded less auxin than was obtained by extraction of freshly excised tips. It seemed therefore that under the given experimental conditions, the auxin given off by excised pea root tips was auxin which was already present in the tip at the time of excision and was not subsequently synthesised. However, as the result of further experiments, van Overbeek (1939b) was able to present evidence that auxin is produced in isolated pea roots cultivated in vitro under sterile conditions and in a complete medium containing Vitamin $B_1$ and nicotinic acid. The roots were grown in such a way that at the end of each week the culture was continued with the 10 mm. tip only, and the basal parts of the roots, which grew approximately 70 mm. per week, were discarded. The total amount of auxin extracted from these discarded bases during the 20 weeks of culture was 9 times as much as that extracted from the initial tip from which the cultures originated.

Meanwhile, Robbins and Schmidt (1938) investigated in even greater detail the environmental conditions affecting the growth of excised tomato roots. Their results verified many of the previous observations already mentioned. Vitamin $B_1$ did not replace inorganic nitrogen or inorganic sulphur in the growth of isolated tomato roots and such roots seemingly require no source of nitrogen other than nitrates for their unlimited growth. Cane sugar, glucose, levulose, maltose or cellobiose proved to be available sources of carbon, and the contaminants in supposedly "pure" sugars markedly affected the growth of the roots. White's mineral solution was not found to be adequate for the best growth of isolated tomato roots, but its effeciency was improved by the addition of boron, zinc, filter paper and filter paper ash. A still further improvement was obtained by substituting a modified Pfeffer's solution with 2.5 p.p.m. of $Fe_2(SO4)_3$ for White's solution when yeast extract was replaced by Vitamin $B_1$, and they also note that light was unnecessary for the unlimited growth of excised tomato roots.

White (1938d) attempted to grow the isolated root tips of 30 species of dicotyledonous plants in-vitro in various types of culture solutions. Of these, 18 grew successfully, while the remaining 12 proved refactory to the treatment used. He therefore concluded that although root tips of all species of dicotyledons can probably be grown under proper conditions, it appears that these conditions will in many cases differ widely, and thus apparently no generalisation can be drawn as regards the requirements of isolated roots which will apply satisfactorily to all species.

Addicott (1939), investigated the role of Vitamin $B_1$ in the metabolism of excised roots of pea. Since Bonner had shown that the freshly excised pea root tip contained sufficient of the Vitamin (and presumably of other growth factors) to maintain growth for the first week, the Vitamin was omitted from the medium during this period. After one week in culture, the roots showed a characteristically swollen base, but at the end of succeeding transfers the root diameter was more uniform although there was a gradual thickening towards the base. Those supplied with the Vitamin were smooth and white with a slightly yellowish meristematic region, whereas the remainder were short, brownish and irregularly swollen. The conclusion reached was that the action of Vitamin $B_1$ as a growth hormone of roots is through an effect on meristematic activity rather than on cell elongation which is the primary effect of the auxins. As far as could be observed, cell elongation, differentiation, and maturation proceeded normally in those roots to which Vitamin $B_1$ was not supplied, even though meristematic activity was greatly reduced.

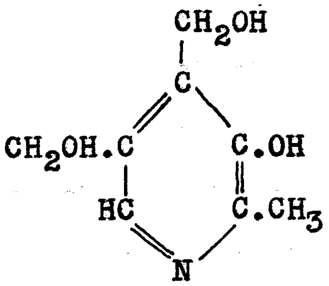
Since it had been suggested in the literature that the second growth factor for excised tomato roots might be found among the micro elements, (Robbins, White, McClary and Bartley, 1936; Robbins and Schmidt, 1938) or among the amino acids of yeast extract (White, 1937d) and since the preliminary experiments of Bonner and Addicott (1937) also indicated that amino acids might be a source of growth factors for pea roots, Addicott and Devirian (1939) now investigated both these types of substance. They concluded that the essential growth factor necessary in addition to Vitamin $B_1$ and present in yeast, was not to be found among the amino acids nor among the micro elements of plant nutrition. Nicotinic acid, however, could act as this factor and therefore it was stated that a culture medium containing salts, sugar, Vitamin $B_1$

and nicotinic acid could support the growth of excised pea roots for indefinite periods of time.

Following on this work, Bonner and Devirian (1939) studied the growth factor requirements of four species of isolated roots. They found that isolated pea roots can be cultured indefinitely in a nutrient medium containing Vitamin $B_1$ and nicotinic acid in addition to mineral salts and 4 per cent. sucrose, and the growth rate in this medium is 70 - 85 mm. per week. Numerous other substances, including vitamins and amino acids, are without effect in increasing the growth rate of isolated pea roots in the presence of Vitamin $B_1$ and nicotinic acid. As regards radish, once again both Vitamin $B_1$ and nicotinic acid were essential, while all other substances tested were without effect. Flax roots required only Vitamin $B_1$ as a growth factor, and tomato roots could be cultivated indefinitely at an average growth rate of 40 mm. per week in medium containing only Vitamins $B_1$ and $B_6$[*] and the rate could be increased to 60 mm. per week by the addition of nicotinic acid.

---

[*] Vitamin $B_6$ is also known as pyridoxine and has the the following formula:-



The experiments of Galligar (1939) on the growth behaviour of one millimeter excised root tips confirmed the view held by Kotte (1922) and White (1932a) that the presence of vascular tissue is not essential for the growth of such tips.

Robbins (1939) published a paper dealing with the part played by thiamin in plant growth. He found that although it is only required in small amounts, thiamin is not to be regarded merely as a stimulant since it plays a definite and significant role in the metabolism of carbohydrates and probably in other metabolic processes also. It appears that one of its functions is to serve as a precursor of a part of an enzyme system involved in respiration. Thiamin diphosphate is known as cocarboxylase which helps to catalyse the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide.

In the same year, Robbins and Schmidt (1939a) discovered yet another growth substance which they state to be essential to the growth of excised tomato roots. They found that when a light brown sugar was substituted for pure cane sugar growth was much improved, the average dry weight of individual roots reaching as much as 70 or 80 mg. The beneficial effects of the impure sugar were partly due to its minerals, but mainly to its content of Vitamin $B_6$. The addition of nicotinic acid nicotinamide, or White's mixture of amino acids to a solution of minerals, pure cane sugar and thiamin was of slight or no benefit to the growth of the roots, but Vitamin $B_6$ gave a marked stimulation. Vitamin $B_6$ is important for the growth of rats, and the writers claim that it is also important for the growth of plants and may prove to be as generally significant for living organisms as thiamin. Bonner and Devirian (1939) in the paper previously mentioned, agree as to the beneficial effects of Vitamin $B_6$ for the growth of isolated tomato roots but found that when nicotinic acid was added as well, the growth rate increased from 40 to 60 mm. per week.

In further experiments, Robbins and Schmidt (1939b) found that even though unlimited growth of excised tomato roots can be obtained in mineral solutions plus sugars to which thiamin or thiazole has been added, the rate of growth in the presence of these supplements is slow. This slowness they attribute to the limited ability of the roots to synthesise adequate amounts of a second growth substance needed in their development, viz. Vitamin $B_6$. They also conclude that amino acids are not essential since the sole source of nitrogen available to the tomato roots of their experiments was in the form of nitrates.

White (1939) found that glycine was capable of replacing the entire group of amino acids which he had used earlier as a constituent of the nutrient for the cultivation of excised tomato roots. He then continued his investigations and while still upholding the benefits of glycine (1940), he differed from Robbins and Schmidt (1939b) in concluding that Vitamin $B_1$, nicotinic acid, and pyrimidine are without value as growth promoting substances for the strains of tomato roots employed.

Bonner (1940a) summarised the growth substance requirements of ten species of isolated roots, viz. pea, radish, flax, alfalfa, clover, cotton, carrot, tomato, datura and sunflower. He also found that flax and clover were capable of synthesising small amounts of Vitamin $B_1$ and Biotin. No significant amount of secondary thickening was observed in any of the root cultures investigated during the experiments and it would seem therefore that the factors influencing secondary thickening are not inherent in the root itself, and are, moreover, different from the factors conditioning root growth in length. Special factors influencing the initiation of secondary roots were not observed except that the number of visible secondary roots per root was, in general, a direct function of the growth of the primary root. Each species retained its own branching peculiarities in cultivation.

Bonner (1940b) investigated the ability of 23 substances chemically related to nicotinic acid to replace the latter substance as a growth factor for isolated pea roots. Among those tested, only substances which yield nicotinic acid by simple hydrolysis were found to be active.

Bonner and Koepfli (1939) found that auxins inhibited the growth of excised pea roots cultured in a nutrient solution containing Vitamin $B_1$ and nicotinic acid, which is known to supply all the substances necessary for normal root development. Fifty per cent. inhibition of root growth was given by a solution approximately $10^{-7}$ molar in indoleacetic acid. Therefore it seems unlikely that auxin inhibition of root growth can be attributed to any kind of direct effect upon the availability of accessory growth factors. Rather it would appear necessary to assume that auxin acts upon the root in some as yet obscure manner, i.e., the effect seems to be directly upon the root cells rather than upon the mobilisation or transport of other root growth substances.

White (1946) gives a concise review of all the work done on root cultures from 1940 until his time of writing. Much of this has no direct bearing on the investigations of the present author, but it serves to round off the history of excised roots and must be briefly quoted. Some of the papers mentioned have been read by the author while in other cases the references are taken from White's account.

McClay (1940) succeeded in cultivating excised roots of a monocotyledon plant, Zea Mays, for extended periods for the first time. Five per cent. glucose and an agar substratum were used as compared with 2 per cent. sucrose and liquid medium which seem to be superior for the roots of most dicotyledon plants. Roots of a dicotyledon tree, Acacia melanoxylon, were cultivated for the first time by Bonner (1942a). The observed increment rates (about 1.0 mm. per day) were very much less than those recorded for most herbaceous plants (5 to 20 mm. per day). This slow growth rate may be characteristic of trees, or there is also the possibility that the methods and nutrients developed for roots of herbaceous plants may not be entirely satisfactory for woody plants. Bonner (1942b) has also shown an inhibitory effect, reversible by application of p-amino-benzoic acid, resulting from the treatment of tomato roots with sulphanilamide, sulphapyridine, and sulphathiazole.

Robbins (1940a) has demonstrated a specific response to light of the root hairs of excised Datura roots. He also found (1940b) that B(-4 methylthiazolyl-5)-alanine was capable of replacing thiamin-thiazole in the nutrition of excised tomato roots. Moreover, he showed (1942) that pyridoxine (Vitamin $B_6$) was highly specific in its effects on cultures of tomato roots. Bonner (1943) observed that tomato roots responded to nutrition with thiamin plus pyridoxine, but not to thiamin plus glycine, while different strains responded differently to nicotinic acid. Similar results were obtained by Day (1941, 1943). White (1943) using four clones of tomato roots from three different laboratories showed that both glycine and pyridoxine are effective supplements to thiamin, the degree of response varying somewhat among the different clones. Nicotinic acid appeared to improve growth when glycine, pyridoxine and thiamin were all likewise present, but was without beneficial effect when combined with any one or two of these other substances. The best results were obtained when all four organic compounds were included in the medium.

White points out that although these papers still indicate a considerable interest in method, the major emphasis has shifted from how to grow roots, to what can be done with roots when grown. White himself (1938b, 1938c) had already used root cultures successfully in investigations on "root pressure". Henderson and Stauffer (1944) employed excised tomato roots in a study of respiratory processes. McClay (1940) showed that thiamin is synthesised by maize roots, and Reid and Robbins (1942) studied the synthesis of ascorbic acid by excised tomato roots. Bonner (1942c, 1943) demonstrated syntheses of thiamin, nicotinic acid and riboflavin by roots of a number of species and subspecific strains of plants. Dawson (1942) has employed the technique in a particularly interesting way as a final proof of the localisation of synthesis of nicotinic acid and other alkaloids in roots of tobacco. This was originally demonstrated by means of reciprocal grafts. Hart (1943) has used a similar technique in studying the syntheses and interconversions of sugars in the roots of sugar cane. The problem of heterotic vigour has also been investigated by means of excised root tips (van Overbeek, 1944; Whaley and Long, 1944) and the effects of polyploidy on growth rates of excised tomato roots have been investigated by Robbins and Kavanagh (1943).

Since, as we have noted, the main object of the present author was to obtain nodules on cultures of leguminous roots, mention must now be made of the work of Lewis and McCoy (1933) who investigated the possibility of nodule formation on etiolated whole plants, etiolated excised shoots and excised whole roots of black wax bean (Phaseolus sp.) The excised roots of this experiment, however, are not comparable with those which have been discussed in the previous pages which were grown from root tips about 0.5 cm. in length. In this case, after germination had proceeded for four days, the entire root system was excised, cultivated on agar nutrient medium (nitrogen-free), and inoculated with appropriate bacteria. Four nodules formed on 1 out of 60 such roots. With regard to the etiolated whole plants, no nodules appeared on those grown in medium without added carbohydrate, but a small proportion (9 out of 60) of those to which 0.5 per cent. sucrose was added possessed a few nodules. Frequent reference to these results will be made throughout the thesis.

It also seems necessary to consider briefly some of the conditions which are known to affect the nodulation of normal whole plants, and which might be expected to exert similar effects upon nodule formation by excised roots. Very early in the investigations on leguminous plants it was noted that nitrates, and combined nitrogen in general, hindered the development of nodules upon their roots (Rautenberg and Kühn, 1864; Hellriegel and Wilfarth, 1888; Lawes and Gilbert, 1891). Why this should be the case was explained in a variety of ways. Hiltner (1900) thought that the effect was on the plant, i.e., a plant supplied with enough combined nitrogen was able to resist bacterial attack. Others, basing their ideas on Laurent's (1891) statement that as little as one in a thousand concentration of nitrates in sterile plant sap stops the growth of Rhizobia, suggested that the effect of the nitrates was on the bacteria themselves rather than on the plant. Another suggestion was that of Giöbel (1926) who states that the nitrate ion diffuses into the plant more rapidly than does the organic nitrogen compound produced in the nodule, thus causing the products of fixation to accumulate and eventually stopping the process. Mazé (1898) believed that the excretion of carbohydrate from the host plant was essential before bacteria could invade it, and thought that inorganic nitrogen compounds united with the carbohydrate of the plant and reduced its level to a point where excretion was prevented. Several other workers (Hopkins and Fred, 1933; Leonard, 1926; Rüffer, 1932; Allison, 1935) also assume that a certain level of carbohydrate in the host is essential for the development of nodules and the fixation of nitrogen. The so-called carbohydrate/nitrogen hypothesis (Weber, 1930; Wilson,1935) considers simultaneously the effects of both carbohydrate and nitrogen because as Wilson and Fred (1939) state "Various responses of the symbiotic nitrogen fixation system are functions of the supply of both carbohydrate and nitrogen as measured by the relationship between them". Thornton (1936) growing lucerne in agar, showed that nodule bacteria not only cause deformation of root hairs but also increase their number and average length. Combined nitrogen checked this stimulation of growth probably by reducing the carbohydrate/nitrogen relationship below some critical level. This would appear to be borne out by the fact that the addition of glucose to the medium overcame the injurious effects of the combined nitrogen. Hopkins and Fred (1933) added mannitol along with combined nitrogen to red clover plants and found that it nullified the effects produced by the combined nitrogen when added alone. Hopkins, Wilson and Peterson (1932) conclude that the effect of the combined nitrogen is not merely to prevent entrance of the bacteria, because moderate quantities do not suppress the formation of nodules but affect their size and distribution. Thornton and Nicol (1936) showed that nitrates reduced the volume of bacterial tissue within the nodule more than they reduced nodule number or nodule length. Giöbel (1926) indicated that the number of nodules on Soya beans was not markedly reduced in the presence of sodium nitrate, but the dry weights of the nodules were reduced in proportion to the level of combined nitrogen.

Several investigators have studied the effect upon nodule formation of increasing the carbohydrate/nitrogen relationship by addition of carbon compounds to the rooting medium. The present author is particularly concerned with the effect of sucrose since this is the carbohydrate used as an energy source in the culture media for excised legume roots. The aseptic experiments of J.K. Wilson (1931) and Lewis and McCoy (1933) show that sucrose is very beneficial, and in some cases essential to nodule production by etiolated leguminous plants. There would appear to be no previous records of truly aseptic investigations into the effect of sucrose upon the nodulation of normal illuminated plants. However, as we shall see in greater detail in Section II, the results of several non-sterile experiments indicate that, in the main, sucrose either had a beneficial effect or else no effect at all upon nodule formation. (Ritter, 1911; Prucha, 1915; J.K. Wilson,1917). Some indication that sucrose may be injurious to growth and nodulation is suggested by Prucha (1915) and Ludwig and Allison (1935) but under their non-sterile conditions it may be that the effects noted were due to fermentation reactions taking place in the media.

As we have seen, the media used for the cultivation of excised leguminous roots contain small quantities of aneurin and nicotinic acid as 'accessory growth factors'. The author is not aware of any previous work on the effect of these substances upon nodule formation. It has been shown that aneurin greatly increases the growth of nodule bacteria on a synthetic medium (Nilsson, Bjälve and Burström, 1938; West and Wilson, 1939), although nicotinic acid has no such effect. The presence of aneurin is merely essential for the initiation of growth, since West and P.W. Wilson (1938) found that once growth has started, the bacteria can synthesise it for themselves. Bonner and Greene (1938) showed that the aneurin content of pea plants kept in the dark does not increase, whereas in light this content of the leaf rises rapidly, and they concluded therefore, that the aneurin is produced in leaves in light. Whole pea plants growing in light did not respond to addition of aneurin and thus it was decided that they must be capable of synthesising all that is required for their own needs.

Reference to the work of Thornton (1929) will be met with repeatedly in this thesis. It will suffice to say here that, using young lucerne plants, he found that a water-soluble substance which causes bacterial multiplication and eventual invasion of the host is excreted from the roots into the surrounding medium at a time coincident with the opening of the first true leaves.

## METHODS.

In all the following investigations involving the cultivation of excised root tips, sucrose was present in the rooting medium. The maintenance of sterility was therefore of great importance and most of the experimental operations were carried out in an inoculating box, similar to that described by Mackie and McCartney (1946), which could be at least partially sterilised by passing in a jet of steam. On cooling, the steam condensed on any dust particles or bacterial and fungal spores present in the atmosphere and carried them down to the floor of the box enclosed in water droplets. Manipulations could then be carried out in this 'washed' air with much less risk of infection.

The seeds most widely used in the author's experiments were those of Field Pea (Pisum arvense L.), variety 'New Zealand Maple'. Large and well-formed seeds with undamaged testas were selected. They were surface-sterilised inside the inoculating box by shaking with absolute alcohol for 2 minutes, then with 0.1 per cent. mercuric chloride for 6 minutes, and finally were washed in six changes of sterile water. They were left to imbibe overnight in the last washing water and were then transferred aseptically to Petri plates containing a shallow layer of 1.0 per cent. agar, about nine seeds being placed on each plate. These were left at a temperature of 19° C for germination to proceed. The advantage of using agar was that any bacteria or fungi remaining on the testas could be detected by their growth on its surface, and peas from such plates were discarded. Any slight modifications to this procedure which were made when clover and bean roots were employed are described in the appropriate places in the text. When root cultures were to be started, tips 0.5 cm. long were excised from the radicles after they had attained a length of one to two centimetres and were transferred with sterile forceps to flasks of sterile nutrient.

The standard nutrient solution used in the experiments, except where otherwise stated, was as follows:-

| | |
|---|---|
| $Ca(NO_3)_2.4H_2O$ | 242 mg. |
| $MgSO_4.7H_2O$ | 42 mg. |
| $KNO_3$ | 85 mg. |
| $KCl$ | 61 mg. |
| $KH_2PO_4$ | 2 mg. |
| $FeCl_3$ | a trace |

Made up to one litre with glass-distilled water. When a nitrogen-free solution was desired , appropriate amounts of $K_2SO_4$and $CaSO_4.2H_2O$ were substituted for the potassium and calcium nitrates, to maintain the K and Ca concentrations at the same level. Accessory growth substances in the form of 0.1 mg. aneurin hydrochloride (B.D.H.) and 0.5 mg. nicotinic acid (B.D.H.), were added per litre of solution. Stated amounts of analytically pure sucrose were also added as required. The above mineral solution, together with the accessory growth substances and 4 per cent. sucrose, and with ferrous tartrate substituted for the ferric chloride shown above, is that devised by Bonner and Devirian (1939) for the growth of excised root tips of pea. The pH of the solution as used for peas was 4.7 when freshly autoclaved and its osmotic pressure was 4.18 atmospheres. By means of a pipette, 50 cc. of nutrient was placed in each of the required number of clean dry Erlenmeyer flasks of 250 cc. capacity. These were then plugged with cotton wool, covered with parchment paper, and sterilised in the autoclave for 15 minutes at 18 pounds pressure. When cool, they were ready to receive the excised root tips.

Since the cultures had to be kept sterile, the roots could not be removed from the flasks from time to time to note increases in length. A method of measurement was therefore devised whereby the shadow of the root was cast on a piece of thin paper, traced with a soft pencil, and measured by means of a map-measuring wheel. The bench lamp employed as a light source was always kept at the same angle and height above all the flasks in order to secure uniformity of conditions. Although this method may result in minor discrepancies, chiefly in root thickness, due to optical effects in the solution, it has been proved by removing a root from the medium and superimposing it upon its traced image, that such differences as do exist are very small indeed.

The strains of the nodule organism used for the inoculation of roots in the following investigations were cultured on yeast-mannitol agar slopes prepared according to the formula of Fred, Baldwin and McCoy (1932):-

| | | | |
|---|---|---|---|
| Agar | 15 gm. | NaCl | 0.1 gm. |
| Mannitol | 10 gm. | $CaCO_3$ | 3.0 gm. |
| $K_2HPO_4$ | 0.5 gm. | Yeast water | 100 cc. |
| $MgSO_4.7H_2O$ | 0.2 gm. | Distilled water | 900 cc. |

Preparation of inoculum was carried out in the inoculating box by adding a little sterile water to the bacterial culture and making a uniform suspension by manipulation with a sterilised piece of glass tubing. The tubing served as a pipette in the actual inoculation operation, approximately 1 cc. of suspension being added to each flask containing roots. When pea roots were involved, the particular organism employed was the effective HX pea strain originally obtained from Professor A.I. Virtanen. Agar cultures were often inoculated according to the method of Chen and Thornton (1940). After the tubes or flasks of medium had been removed from the autoclave, they were placed in a water bath operating at 42°C. and left until they had assumed this temperature, at which the agar was still liquid, but was not hot enough to kill bacterial cells. Several vessels at a time were transferred from the bath to the inoculating box, approximately 1 cc. of bacterial suspension added to each, and then well shaken.

## EXPERIMENTAL RESULTS.

### Experiment 1. Growth of excised root tips of pea in nutrient solution containing yeast extract, and the temperature best suited to their healthy development.

In reviewing the history of root culture we have seen that before the discovery of the exact components of yeast extract which were essential for the growth of excised roots tips of pea, the extract was added in its entirety to all culture media. In preliminary experiments carried out in this Department by Miss Helen Frazer, this same procedure was adopted and root growth and lateral root development were achieved. It was decided to repeat these experiments in order to become familiar with the technique which she had successfully employed, and also to observe the extent of growth which could be attained under different temperature conditions.

0.1 gm. of yeast was boiled for twenty minutes in 30 - 50 cc. of glass distilled water, left to settle in the refrigerator, and filtered twice through a Buchner funnel. The whole of the filtrate was then used in making up one litre of nutrient, being substituted for the aneurin and nicotinic acid given in the formula of Bonner and Devirian (1939).

One excised root tip of pea was placed in each of eighteen flasks of medium. Twelve of these were placed at a temperature of 15 - 16°C. and the remaining six at 19 - 20°C. Within three days all the roots had increased to twice their original length and were showing a response to gravity. Those at the higher temperature were beginning to turn brown especially at the cut surface. At the end of fifteen days the roots were transferred to freshly prepared solution and were allowed to remain for another two weeks before being removed, examined and measured along a ruler.

### Table 1.

Growth of excised root tips of pea in nutrient solution containing yeast extract at different temperatures. Growth period = 4 weeks.

| Temperature | Av. length of main root (cm.) | Av. no. of laterals | Remarks |
|---|---|---|---|
| 19 - 20°C | 12.3 | 13 | Roots dark brown |
| 15 - 16°C | 14.0 | 14 | Young parts white |

It can be seen from Table 1 that there was little difference in length between roots from the two temperature levels. The difference in appearance, however, was very marked. Roots at the higher level became completely brown at an early stage, the tendency being evident even after the third day. Before the termination of the experiment the older parts of the roots at the lower level had also become brown but the younger regions were still white. It appeared therefore that temperatures averaging about 15 - 16° C. were best suited to the healthy growth of excised root tips of pea.

The seeds used in the above investigation were over a year old. The experiment was repeated using peas from the crop of the current year in order to observe whether seed age had any effect on the growth of excised root tips.

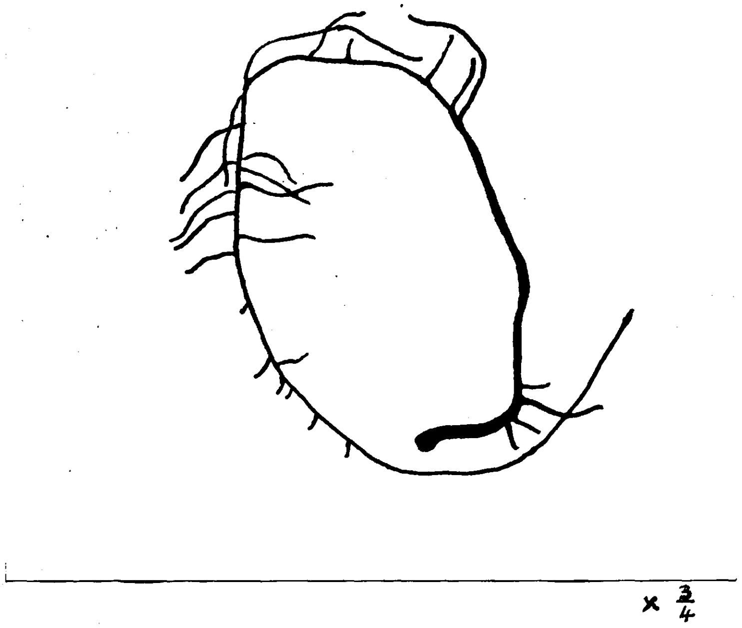


Fig. 1. Tracing of a pea root grown from an excised root tip in nutrient solution containing yeast extract.
Growth period = 4 weeks.

## Table 2.

As for Table 1, but using seed from the current year's crop. Growth period = 4 weeks.

| Temperature | Av. length of main root (cm.) | Av. no. of laterals | Remarks |
|---|---|---|---|
| 19 - 20°C. | 17.0 | 17.6 | Roots brown |
| 15 - 16°C. | 13.0 | 17.4 | Young parts white |

It is evident from Table 2 that in comparison with the roots of the first experiment, an improvement was shown both in the development of the main root and in the production of lateral roots. Tracing of typical root system can be seen in Fig. 1.

There was again a tendency to browning which was more marked in roots grown at the higher temperatures. Specimens from each temperature level were examined for root hairs, which were found to be numerous but very short.

## Experiment 2. Growth of excised root tips of pea in nutrient solution aontaining aneurin and nicotinic acid as "accessory growth factors".

Bonner and Devirian (1939), as noted, found that in addition to the usual mineral salts and a carbohydrate supply of 4 per cent. sucrose, excised root tips of pea required small quantities of aneurin and nicotinic acid before growth would proceed. These substances effectively replaced the yeast extract used by earlier investigators. The formula for their culture medium has already been given in the preceding pages and it was now employed in the preparation of the nutrient for the present experiment.

Following the usual procedure, three root tips were transferred to each flask of solution. Five flasks were placed at a temperature of 15 - 16°C, and six at 19 - 20°C. The growth period extended over 4 weeks.

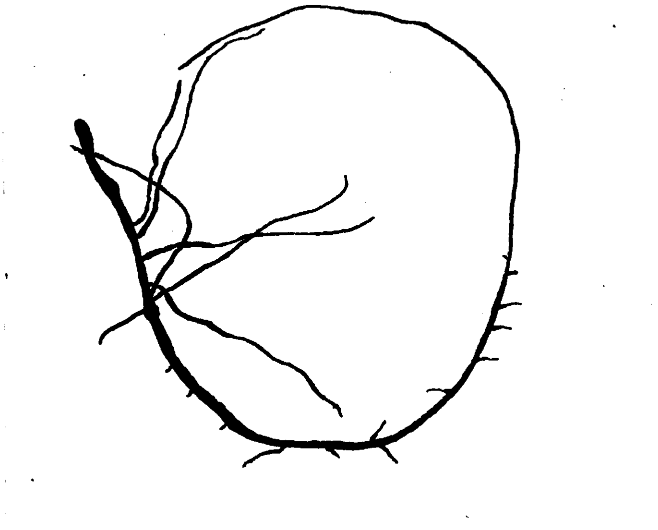
Although the roots at the higher temperature level showed a slight initial advantage, they began to turn brown after about 17 days and their condition at the conclusion of the experiment was much inferior to that of the roots cultured at the lower level.

Table 3.

Growth of excised root tips of pea in nutrient solution containing aneurin and nicotinic acid. Growth period = 4 weeks.

| Temperature | Av. length of main root (cm.) | Av. no. of laterals |
|---|---|---|
| 19 - 20°C | 19.0 | 14.0 |
| 15 - 16°C | 18.0 | 16.6 |

Comparing the results in Tables 2 and 3 it can be seen that main root length was greater in the present test than in the preceding one where yeast extract was employed and although the lateral roots were fewer in number they, too, were longer. All specimens were whiter and sturdier than those grown in nutrient solution containing yeast extract and the root hairs produced were longer and much more numerous. Tracing of a typical root system is shown in Fig. 2., while Fig. 3 shows similar roots at an older stage.



$\times \frac{3}{4}$

Fig. 2. Tracing of a pea root grown from an excised tip in nutrient solution containing aneurin and nicotinic acid. Growth period = 4 weeks.

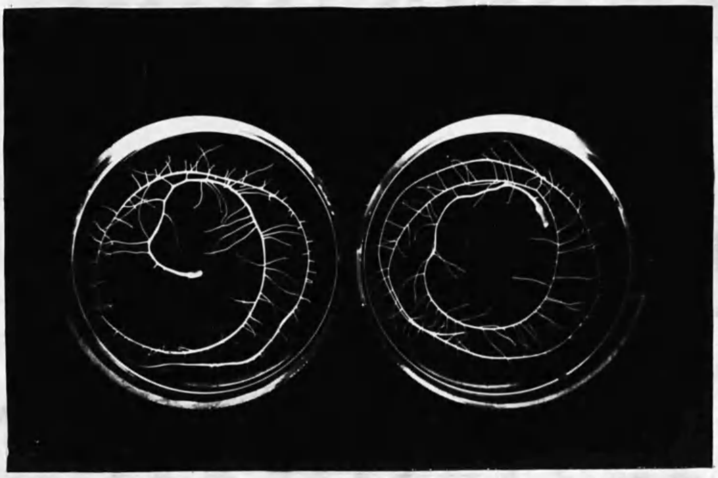


Fig. 3. Excised roots of pea, photographed after seven weeks' growth in nutrient solution containing aneurin and nicotinic acid.

It appears therefore that for the growth of excised root tips of pea the more suitable medium is that containing aneurin and nicotinic acid and the most suitable temperature lies in the region of 15 - 16°C.

Simultaneously with the progress of the above experiments, it was decided to investigate whether greater aeration of the nutrient medium would be accompanied by increased growth of the excised pea roots.

Experiment 3. Growth of excised root tips of pea in sand watered with nutrient solution containing yeast extract.

Ten Erlenmeyer flasks of 250 cc. capacity were used as culture vessels. Into five of them was weighed 250 gm. of fine quartz sand and the remainder received the same quantity of coarse silver sand. The flasks were then sterilised for ninety minutes at 18 - 20 pounds pressure. Fifty cc. of nutrient solution containing yeast extract was added to each and a second sterilisation carried out for fifteen minutes at the same pressure. When thoroughly cool, a groove was made in the surface of the sand with a sterile platinum wire and an excised root tip placed along the groove. All the flasks were left in the dark at a temperature of 15 - 16°C and examined at intervals throughout a 4 - week growth period,

After three days the tips began to turn downwards and numerous fine root hairs could be seen with the naked eye. When two weeks had elapsed, it was noted that the roots in fine sand had difficulty in penetrating downwards and their efforts to do so had resulted in an upward curvature of the main part of the root, thus reducing the number of points at which it could absorb nutrient. The flasks were therefore opened and by means of a sterile scalpel the roots were completely covered over with the moist sand.

Table 4.

Growth of excised root tips of pea in sand watered with nutrient solution containing yeast extract. Growth period = 4 weeks.

| Medium | Av. length of main root (cm.) | Av. no. of laterals |
|---|---|---|
| Fine sand | 8.1 | 2.6 |
| Coarse sand | 4.5 | 2.75 |

From Table 4 it is obvious that the growth of excised root tips of pea in sand culture is far below that attained in purely liquid medium, despite the improved conditions of aeration. Root hairs were very plentiful on all the specimens but the roots in fine sand were longer, whiter and showed a better development of hairs than those in coarse sand.

Experiment 4. Growth of excised pea roots on agar medium.

As noted in the Introduction, Malyschev (1932) favoured the use of an agar substrate for the growth of excised root tips, while White (1933), in comparing his results with those of Malyschev, showed that much superior growth had been obtained in liquid culture. In the experiments of the present author, since nodule formation was the end in view, it was thought that growth on a solid medium might possess certain definite advantages. For example, aeration would be better since the roots would either lie on the agar surface or make air spaces for themselves as they penetrated through it, and moreover, there was the possibility that the root hairs might be longer and more numerous. Therefore it was decided to grow excised root tips of pea on the usual nutrient medium containing aneurin and nicotinic acid solidified by the addition of 1.0 per cent. agar.

Two root tips were 'planted' in each of ten Petri plates containing the solidified medium and allowed to grow for a period of ten weeks. At first growth was rapid, and within three or four days many of the roots extended right across the agar surface and were beginning to turn downwards where they reached the sides of the plates. This initial growth rate was not maintained however, and at the end of the growth period the average length of the main root was only 18.4 cm. and the average number of lateral roots 10.65. Comparison of these figures with those in Table 3 shows that during a much shorter growth period (4 weeks), excised pea roots in liquid culture attained the same length as those above and produced more numerous lateral roots.

### Experiment 5. Effect on the growth of excised root tips of pea of artificial aeration of the liquid medium.

It was possible that in the two preceding experiments the increased aeration might be proving beneficial to root growth but its effects were perhaps nullified by the greater difficulty of absorbing nutrient from sand and agar substrates. Hence in the present test, the roots were grown in liquid medium and the better aeration was provided by an air stream drawn through the solution.

Every precaution was taken to try to obtain sterility of the apparatus and of the air stream. All the tubing and glassware employed were wrapped in sulphite paper and sterilised in the autoclave, and all connections were made inside the inoculating box. The air was drawn by means of a suction pump from a greenhouse adjoining the laboratory, and was passed through a double copper spiral to assume the temperature of the surroundings and was then washed with sodium hydroxide and passed through flasks of cotton wool.

Nine excised root tips were transferred to each of six pressure flasks of one litre capacity containing 550 cc. of nutrient solution (unless statement is made to the contrary it can be assumed that the medium used in succeeding experiments is that which contains aneurin and nicotinic acid). The flasks were placed in a light-proof box in the laboratory for a period of three weeks. Three of them were aerated and the other three left as controls.

Despite all precautions, after ten days, two of the three aerated flasks began to show the presence of fungal and bacterial infection. Therefore, the results for aerated roots given in the following Table are the average values for the nine roots contained in the one non-infected flask.

Table 5.

Effect of aeration on excised root tips of pea growing in liquid medium. Growth period = 3 weeks.

| Treatment | Av. length of main root (cm.) | Av. no. of laterals | Av. dry wt. (mg.) |
|---|---|---|---|
| Aerated | 14.3 | 4.5 | 10.33 |
| Non-aerated | 15.2 | 16.2 | 11.53 |

From Table 5 it can be seen that the control roots made better growth than those which were aerated, and their superiority was evident from the second day of the experiment.

It was concluded, therefore, that aeration of the culture solution was not beneficial to the growth of excised root tips of pea. Assuming that oxygen content is the most significant factor affected by aeration, it appears that the roots grow best in the presumably reduced oxygen tension provided by the non-aerated solution.

Experiment 6. Glycerol and mannitol as substitutes for sucrose in the growth of excised root tips of pea.

In enquiring into the conditions affecting the growth of excised roots of pea it seemed of some interest to determine whether their energy source must always be carbohydrate in nature or whether they were capable of utilising the higher alcohols. As we have seen, Hopkins and Fred (1933) added mannitol along with combined nitrogen to their red clover plants and found that it cancelled out the injurious effects caused by the combined nitrogen when added alone. Thus the clover must have been able to absorb the mannitol and utilise it to increase the carbohydrate/nitrogen ratio within the plant. As noted in "Methods", mannitol is also effective as a source of carbon for the root nodule bacteria. With regard to glycerol, several investigators (Boehm, 1883; Meyer, 1886; É. Laurent, 1887; Pfeffer, 1900) have shown that it could be absorbed and used by leaves, cuttings and floral parts of plants and also by whole rooted plants of Phaseolus vulgaris, corn and radish (Acton, 1889; J. Laurent, 1904; Molliard, 1907).

Two root tips were placed in each of nine flasks containing medium with the normal carbon supply of 4 per cent. sucrose. These acted as controls. Two similar series of flasks contained respectively 4 per cent. glycerol and 4 per cent. mannitol in place of the 4 per cent. sucrose. Thus there were eighteen roots at each treatment. All flasks were set aside for a period of 4

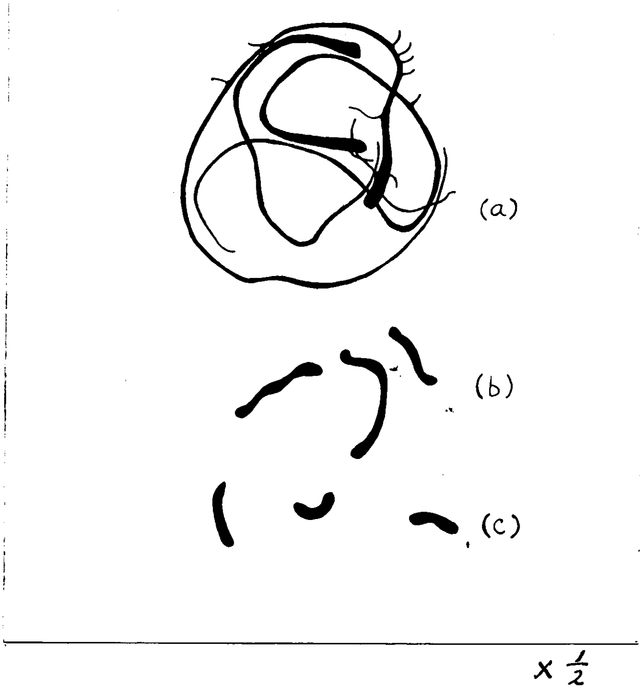


Fig. 4. Tracings of excised roots of pea grown for 4 weeks in nutrient medium containing (a) 4 per cent. sucrose, (b) 4 per cent. glycerol, (c) 4 per cent. mannitol.

weeks.

Table 6.

Glycerol and mannitol as substitutes for sucrose in the growth of excised root tips of pea. Growth period = 4 weeks.

| | 4 per cent. sucrose | 4 per cent. glycerol | 4 per cent. mannitol |
|---|---|---|---|
| Av. length of root (cm.) | 18.2 | 2.9 | 1.2 |

It can be seen clearly from Table 6 and Fig. 4 that neither glycerol nor mannitol in the given concentration, was effective as a substitute for 4 per cent. sucrose in the nutrition of excised root tips of pea.

### Experiment 7. Effect upon the growth of excised roots of pea of transferring them from full nutrient medium to solutions containing only fractions of the original concentration of nitrate.

It is generally accepted, as already noted, that the presence of combined nitrogen in the rooting medium has a depressing effect upon the production of nodules by intact leguminous plants. Possibly the same would hold good for the excised roots of legumes. It seemed advisable, therefore, to investigate the possibility of growing excised roots of pea at nitrate levels lower than that supplied in the medium of Bonner and Devirian (1939). If satisfactory growth occurred, then it might be expected that nodules would form on such roots more readily than on roots growing in the solution with the full complement of nitrate.

Nutrient solutions containing one half and one quarter of the full nitrate concentration were prepared as indicated in "Methods". The osmotic pressuresof the reduced nitrate solutions were determined by the depression of the freezing point method and it was found that no change in osmotic pressure had been involved in the reduction of the nitrate concentration.

The pH values of the solutions were also determined, by an indicator method using the Hellige apparatus, and the following results obtained:-

Full nitrate soln.freshly prepared and autoclaved, pH = 4.7
$\frac{1}{2}$ " " " " " " pH = 4.4-4.6
$\frac{1}{4}$ " " " " " " pH = 4.4 - 4.6
Full nitrate solution in which roots had grown 2 days, pH = 6.2
Full nitrate solution in which roots had grown 28 days, pH = 6.7

Therefore, no appreciable change in pH had been caused by varying the nitrate content of the solution. The freshly prepared medium was acid but within two days a considerable rise had occurred. This is compatible with the observations of Trelease and Trelease (1933, 1935) on whole plants of wheat growing in water culture. They found that in solutions where the ratio of nitrates to ammonium salts was high, pH values gradually increased until a limiting value of 6.5 was reached. Similarly Jacobson (1925) had observed that 97 days' old wheat plants placed in culture solution were capable of changing its pH from 3.9 to 6.3 during the first 12 hours. Presumably the explanation of both these results is that there is preferential absorption of the $NO_3$ ion of the nitrate salts leaving the metallic cations free to combine with free OH groups. The new compounds thus formed were then able to partly neutralise the acidity of the solution.

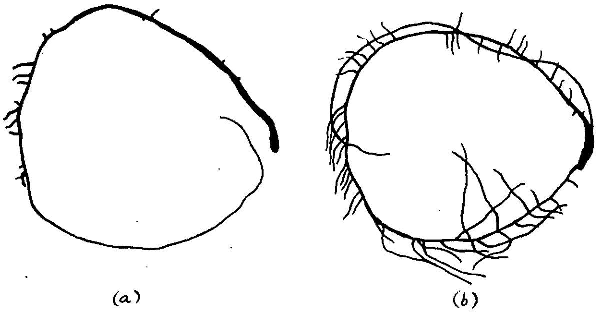
The excised pea roots employed in the present experiment had grown for 4 weeks in full medium and had attained a considerable length. They were traced and measured before being transferred aseptically to flasks containing freshly prepared full nitrate, half nitrate and quarter nitrate solutions, one root to each flask and nine flasks at each concentration. After two weeks all roots were again traced and measured.

Table 7.

Effect upon the growth of excised roots of pea of transferring them from full nutrient solution to media containing only fractions of the original nitrate concentration.

| Treatment | Av. increase in length (cm.) | Av. increase in no. of laterals |
|---|---|---|
| Full nitrate | 6.6 | 15.7 |
| Half nitrate | 11.8 | 21.0 |
| Quarter nitrate | 5.8 | 27.2 |

From Table 7 it is evident that growth occurred at both of the reduced nitrate levels and exceeded that achieved by the full nitrate controls. The greatest



× 1/2

Fig. 5. Tracings of excised roots of pea, (a) before, (b) after, two weeks' growth in nutrient solution containing one quarter of the original concentration of nitrate.

increase in length was found in the half nitrate flasks and the greatest increase in number of laterals in those with quarter nitrate (Fig. 5). Roots at the quarter nitrate concentration were the whitest and therefore the most healthy looking.

In yet another experiment, freshly excised root tips of pea were transferred directly to solutions containing one quarter, one eighth and one sixteenth of the full nitrate value and also into a solution without nitrate. The maximum increase in length noted over a period of 3 weeks was only 1.0 cm., and in the majority of cases, no growth whatever occurred. Thus it is obvious that solutions low in nitrate have a serious inhibiting effect upon the growth of newly excised root tips of pea.

### Experiment 8. Effect of various concentrations of sucrose upon the growth of freshly excised root tips of pea.

In Section II it will be seen that 4 per cent. sucrose had a very marked depressing effect upon the number of nodules formed on whole pea plants, growing either in darkness or in light. The medium employed for the culture of excised root tips of pea contains sucrose at this concentration, and therefore it was thought necessary to determine whether or not they would grow when supplied with sucrose at levels less harmful to nodule production.

Investigations were made of the effects of the sucrose concentrations shown in Table 8. There were five flasks at each concentration and two excised root tips per flask.

Table 8.

Effect of various concentrations of sucrose upon the growth of freshly excised root tips of pea. Number of roots per treatment = 10. Growth period = 4 weeks.

| Concn. of sucrose (per cent.) | 4.0 | 3.0 | 2.0 | 1.0 |
|---|---|---|---|---|
| Av. length of root (cm.) | 24.8 | 16.9 | 8.9 | 3.6 |

It is clear from the results in Table 8 that reduction of the sucrose even to the 3 per cent. level had a decidedly injurious effect on the growth of freshly excised root tips of pea. Moreover, the roots in the medium with 4 per cent. sucrose, besides being longer than the other specimens, were much thicker and showed a better development of lateral roots.

From the foregoing experiments it was now known that well-developed root systems of pea could be cultured on demand from excised tips in numbers sufficient to permit of investigations into their ability to form nodules. This part of the work was therefore commenced forthwith and the following tests carried out.

### Experiment 9. Inoculation with nodule bacteria of excised pea roots growing on agar medium.

We have already discussed the possible advantages of an agar substrate over liquid medium with regard to the process of nodulation, e.g., the aeration would be better and the root hairs might be longer and more numerous. This latter point is particularly important since it is through the root hairs that the organisms gain entry to the tissues of the host.

Six excised root systems of pea growing in Petri plates on the usual nutrient medium solidified with 1.0 per cent. agar, were inoculated by smearing loopfuls of nodule bacteria over such parts as were above the agar surface. The organisms grew well, forming a dense white band of slime down either side of the root and laterals and showing no tendency to spread uniformly over the rest of the agar. No nodules were formed on any of these inoculated specimens and the roots themselves soon ceased to grow.

### Experiment 10. Inoculation with nodule bacteria of excised pea roots supplied with nitrogen-free solution and solutions with low concentrations of nitrate.

The roots employed were those from a previous experiment and some of them had been growing well in solutions with reduced nitrate. In an attempt to provide the medium most suitable for nodule formation, it was decided to replace these solutions with one which was entirely free from nitrogen. Unfortunately, the roots were now too large to permit of transfer from one flask to another without grave risk of infection. Thus the method was adopted of sucking off the old solutions through sterile glass and rubber tubes attached to a sterilised pressure flask fitted to a suction pump. As usual, all operations were performed inside the inoculating box and the tubes were changed at regular intervals. The older brown portions of the roots were cut off with sterile scissors and the nitrogen-free solution, previously autoclaved, was then introduced through the side arm of a large pressure flask to ensure that the necks of the flasks containing the roots were kept quite dry.

Two flasks from each of the previous nitrate concentrations were left uninoculated as controls. The remainder were inoculated with a few drops of a suspension of nodule bacteria from nine-day-old slopes. The roots were examined at intervals throughout a 7-week growth period and the following observations then recorded:-

1. No nodules were formed on any of the inoculated specimens although they were in a solution free from nitrate and possessed numerous root hairs.
2. The bacteria multiplied in the medium and formed white slime strands adhering to the roots.
3. All roots turned brown and this browning was not due to the presence of the bacteria since a similar effect was noted in the uninoculated specimens.

4. Growth ceased in the majority of cases and again this was not the result of bacterial action alone, since the controls also ceased to grow.

It was thought that the changeover to a solution completely free from nitrogen had proved too drastic and therefore a similar experiment was performed in which roots which had attained a good length in full medium were transferred to solutions containing one quarter, one eighth, and one sixteenth of the original nitrate concentration, and were then inoculated. All the roots continued to show some reduced growth but no nodules were obtained during an 8-week growth period. Bacterial multiplication caused the medium to become milky.

### Experiment 11. Inoculation with nodule bacteria of excised pea roots supplied with various reduced concentrations of sucrose.

As already mentioned, it will be shown in Section II that sucrose at the 4 per cent. level is markedly injurious to the nodulation of whole pea plants and we have seen in a previous experiment that excised root tips of pea did not grow well if the sucrose was reduced below this level. In view of these findings, a further experiment was now performed in which excised pea root tips were allowed to grow for three weeks in full medium with 4 per cent. sucrose before being transferred to solutions containing 2 per cent., 1 per cent. and 0.5 per cent. sucrose. They were then inoculated with nodule organisms.

During a growth period of 6 weeks no nodules formed on any of the root systems, no growth occurred at the two lowest levels of sucrose and there was only a very slight increase in length at the 2 per cent. concentration.

### Experiment 12. 'Double - medium' technique for the growth and inoculation of excised root tips of pea.

Two points had been firmly established, firstly, that 4 per cent. sucrose had a very depressing effect on the production of nodules by the roots of whole pea plants (see Section II), and secondly, that this concentration of sucrose was essential for the growth of excised pea root tips. It has also been shown that when nodule bacteria were added to root cultures containing 4 per cent. sucrose, they multiplied rapidly and had an injurious effect upon root growth. If the depressing effect of the sucrose was being exerted externally, and restricting the actual invasion of the root by the bacteria, it was thought that some advantage might be gained from a technique whereby the sucrose supply was separated from the bacterial suspension.

A layer of nitrogen-free medium without sucrose and with 1.0 per cent. agar added, was poured into each of six Soxhlet flasks (500 cc. capacity) and six deep Petri dishes. Before the agar had set, a glass tube (1 in. x 2 in.) containing the complete nutrient solution of Bonner and Devirian (1939) with 4 per cent. sucrose and 1 per cent. agar, was placed inside each flask or dish as shown In Fig. 6. The apparatus was then sterilised and left in the autoclave until quite cool, usually overnight. The agar, of course had solidified by morning and there was no danger of the media being mixed. This was in fact the reason for the addition of agar, viz., to prevent one type of nutrient spilling into the other when the containers were being moved and examined. It has already been shown that pea root tips can grow well on agar medium, though not so vigorously as in purely liquid culture.

One, or sometimes two, excised root tips of pea were transferred to each of the inner cylinders. The outer medium was surface-inoculated with a suspension of nodule bacteria. After the roots had attained sufficient length, their apical regions were lifted over the edge of the cylinders in the hope that they would grow downwards into the surrounding inoculated medium. The lifting operation was carried out with a loop of sterile platinum wire.

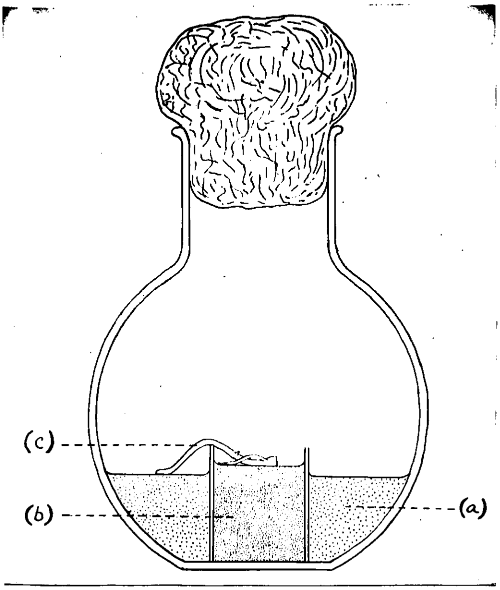


Fig. 6. 'Double-vessel' arrangement for attempted nodulation of excised pea roots. (a) Inoculated mineral-salt agar; (b) nutrient agar containing 4 per cent. sucrose and accessory growth substances; (c) excised pea root.

### Table 9.

'Double - medium' technique for the growth and inoculation of excised root tips of pea. Growth period = 7 weeks.

| Root No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Length of root (cm.) | 7 | 8 | 8 | 9 | 8 | 8 | 7 | 11 | 8 | 5 | 7 | 7 | 6 | 6 |
| No. of laterals | 2 | 1 | 1 | 1 | 1 | 2 | 0 | 2 | 1 | 0 | 0 | 2 | 2 | 1 |
| Growth on outer agar (cm.) | 0 | 0.5 | 0.5 | 1.5 | 0.1 | 2 | 0.5 | 2 | 0 | 0 | 0 | 0.5 | 0.1 | 0 |

In all cases it was found that the inner containers were too high above the level of the outer agar. Thus the roots either never reached the inoculated medium, or, having reached it and having grown for a short distance, the overarched portions then began to dry up and growth soon ceased.

As can be seen from Table 9, in no case did growth compare with that previously achieved on agar medium. All the lateral roots noted above were produced in the inner container where the medium contained nitrates and the full supply of sucrose. The culture vessels had to be opened at least three times during the setting up of experiment and this may account for the fact that two of the Petri dishes and one of the flasks became infected with fungus and had to be discarded.

It seems probable that refinements of this method may offer the best solution to the problem of eliminating the injurious effect of sucrose while still meeting the requirements of the roots for the 4 per cent. concentration. It should also provide a means of overcoming the reduction in root growth apparently caused by the rapid growth of the bacteria in media containing sucrose. Therefore it is planned to proceed in future with further experiments along these lines.

While the above work on the reduction of sucrose and nitrate as preliminaries to inoculation was proceeding, some consideration was also given to two other factors which might exert an influence on nodule production by excised roots.

### Experiment 13. Effect of extracts from the tops of whole pea plants on the nodulation of excised roots of pea.

Thornton (1929) found that the active, nodule-stimulating substance excreted from the roots of lucerne plants when the first true leaf opens is not formed in this leaf, since removal of the leaves while still closed has no effect upon nodule appearance. Nevertheless, it was decided to test the possibility that some specific substance originating in the shoot might be essential for nodule formation on peas and thus the excised root might be expected to benefit from the addition to the medium of extracts from the tops of whole pea plants. With this idea in mind, the following small-scale preliminary experiment was carried out.

Inoculated pea seeds were planted in sterile sand watered with nitrogen-free mineral solution. When they had attained a height of three to four feet, the four topmost nodes were cut off from each of eight plants, cut into small segments with scissors, and placed in a mortar along with a little clean sand and 50 cc. of glass distilled water. The mixture was pounded for ten minutes, by which time the shoots were reduced to a fine pulp, and the liquid then filtered twice through a Buchner funnel. Five cc. of filtrate was pipetted into each of three test tubes and the tubes plugged with cotton wool and autoclaved for 15 minutes at 15 - 20 pounds pressure. The high temperatures of the autoclave caused coagulation of the protoplasmic proteins which appeared as a green flocculum. Three representative excised roots were selected from the inoculated specimens of a previous experiment and five cc. of sterile top extract added to each.

Observations made over a period of 8 weeks showed that no nodules were formed on any of the root systems. This result might indicate that the top extract contained nothing which was beneficial to nodule production, but it was also possible that the sterilisation in the autoclave might have decomposed any nodule-promoting substance originally present. An unsuccessful attempt to obtain sterility without heating was made with a Berkefeld filter and obviously other such attempts are required in the future.

### Experiment 14. Effect of auxins upon the growth and nodulation of excised pea roots.

Bonner (1940a) stated that he had observed 'no significant secondary thickening' in his numerous root cultures, and concluded that the initiation of this process must depend on factors different from those which govern root growth in length. According to Snow (1933, 1935) and Avery, Burkholder and Creighton (1937), cambial activity is initiated by auxins, and since nodule development resembles cambial activity in being a meristematic process, it was decided to treat excised root tips of pea with auxins and subsequently observe whether they could produce nodules. Therefore, although it was known (Bonner and Koepfli, 1939) that auxins had an inhibiting effect upon the actual growth of excised pea roots, the following experiment was performed in the hope that the treated roots, though stunted, might form nodules more rapidly than those left untreated.

Ten freshly excised root tips of pea were soaked overnight in β-indolyl acetic acid and ten others in α-napthalene acetic acid[*] before being transferred

---

[*] Unfortunately no record of the concentrations used is available.

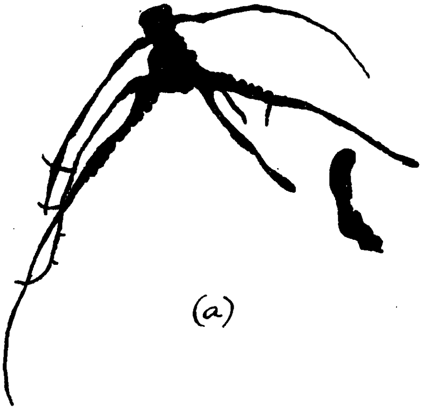
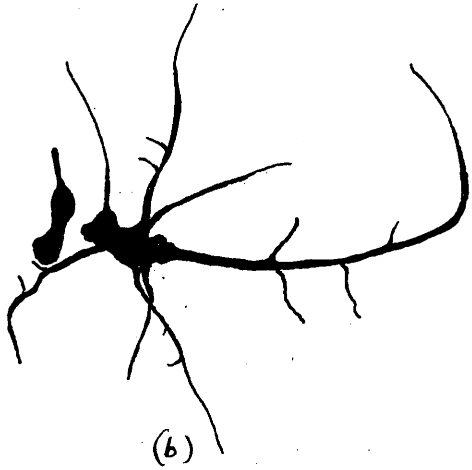


Fig. 7. Tracings of excised pea roots treated with (a) $\alpha$-napthalene acetic acid, (b) $\beta$-indolyl acetic acid, then grown for five weeks in nutrient containing aneurin and nicotinic acid.



to flasks of the usual medium. They were examined at intervals throughout a 5-week growth period and it was found that the main root remained stunted, numerous lateral roots developed and the cells of the cortical parenchyma were stimulated to divide rapidly, giving rise to irregular mounds of spongy tissue. Tracings of representative roots are shown in Fig. 7. One half of the roots at each treatment were inoculated with nodule organisms, but after a further 4 week's growth there was no sign of any nodule formation. The milkiness produced indicated that once again the bacteria had grown well in the medium.

Thus it would appear that treatment of the freshly excised tips with auxins could not overcome the conditions which inhibit the development of nodules on excised roots of pea.

In addition to all the foregoing investigations using excised pea roots, attention has also been paid to the possibilities of cultivating the excised roots of other legumes and eventually securing nodules upon them.

### Experiment 15. Growth of excised root tips of Soya bean (Soja Max Piper), Broad bean (Vicia Faba L.), sweet pea (Lathyrus odorata L.), and sea pea (Lathyrus maritima L).

As we have seen, excised root tips of New Zealand field pea grow very successfully in the culture solution devised by Bonner and Devirian (1939) and tests were also made on the suitability of this solution for the growth of roots tips of two other types of pea and two types of bean.

In a series of experiments employing Soya beans, excised root tips were transferred to solution containing yeast extract, to solution containing aneurin and nicotinic acid and to flasks of fine and coarse sand watered with the latter solution. In all cases some growth was achieved but the tap root remained short and there was a tendency to form a system of small, thin, repeatedly-branching laterals which quickly turned dark brown. It seems obvious, therefore, that the solution does not contain the factors necessary for the healthy growth of root tips of Soya bean.

A similar conclusion was reached as the result of experiments using sweet pea and sea pea where in both cases growth was exceedingly poor.

Some measure of success was obtained with cultures of root tips of a dwarf variety of broad bean known as Beck's Gen. After 6 weeks in the solution containing aneurin and nicotinic acid, the average length of the main root was 11.2 cm. and the average number of laterals was 5.6. These were then transferred to freshly prepared nutrients containing one quarter, one eighth and one sixteenth of the original nitrate concentration, and were inoculated with appropriate nodule bacteria. Observations over a 6-week period showed that although the organisms multiplied well in the medium, and there was a slight increase in root length, no nodules were formed.

## Experiment 16. Growth of excised root tips of clover in liquid medium, and the effect of inoculation with nodule bacteria.

Bonner (1940a) investigated the growth requirements of excised root tips of clover and found them to be essentially the same as those which he had already determined for pea. The same mineral salt mixture was suitable, the same concentrations of aneurin and nicotinic acid were necessary, but 2 per cent. sucrose was substituted for the 4 per cent. employed with peas.

The seeds used in the present experiments were those of New Zealand Montgomery red clover. They were selected under a lens and surface-sterilised by the same method as for peas except that they were treated for 3 minutes with absolute alcohol and only 4 minutes with mercuric chloride. Following the usual procedure, 3 excised root tips 0.5 cm. long were transferred to each of ten Erlenmeyer flasks of 250 cc. capacity containing 50 cc. of medium with 2 per cent. sucrose. Two flasks containing medium with 4 per cent. sucrose were 'planted' as controls.

Growth at first was very slow but geotropic curvatures soon developed and large root caps were much in evidence. After 11 days, when the roots had increased to approximately twice their original length, five of the flasks were inoculated with an effective strain of clover organism (Rothamsted strain 49). They were then examined frequently during a 9-week growth period.

### Table 10.

Growth of excised root tips of clover in liquid medium, and the effect of inoculation with nodule bacteria.

Growth period = 9 weeks.

|  | Root | Length of root (cm.) | No. of laterals | No. of laterals as long as main root | No. of nods. |
|---|---|---|---|---|---|
| UNINOC. | A | 9.0 | 236 | 10 | - |
|  | B | 8.8 | 125 | 3 | - |
|  | C | 4.3 | 10 | 0 | - |
| INOC. | A | 2.1 | 3 | 0 | 0 |
|  | B | 1.0 | 0 | 0 | 0 |
|  | C | 1.7 | 0 | 0 | 0 |

Figure 8 shows the contents of a typical inoculated, and a typical uninoculated flask transferred to Petri dishes for purposes of photography. It is obvious that although the organisms multiplied in the medium and produced milky strands, their presence had a very injurious effect upon the growth of the roots. From examination of Table 10 which contains data on the roots pictured in the Figure, it appears that growth ceased altogether very soon after the inoculum had been added. The uninoculated roots formed numerous branches many being as long as the main root which was thus difficult to distinguish.

No nodules were formed on any of the inoculated specimens. Microscopic examination of the roots showed that starch grains were present in very large numbers in

Fig. 8. Excised roots of clover grown in liquid medium containing 2 per cent. sucrose. Those on right were uninoculated, those on left were inoculated with nodule bacteria which produced milky strands in the medium and inhibited root growth.

the cells of the young lateral roots formed in the uninoculated flasks. They were not so prevalent in the larger roots and were completely absent from those which were inoculated. Thus it is apparent that in the case of uninoculated clover roots some of the 2.0 per cent. sucrose absorbed from the solution is transformed into starch and stored as such in the tissues of the developing laterals.

Although the contents of only two of the flasks were actually photographed and measured, these observations can be taken as typical for each series.

Growth in the control flasks containing 4 per cent. sucrose was no better than that in the flasks at the 2 per cent. level.

In an experiment similar to the above but using Petri dishes as culture vessels, their unsuitability for this purpose was well demonstrated. Within 10 days all those containing solution with 2 per cent. sucrose and all but three of those with 4 per cent. sucrose showed fungal infection. In the majority of cases the fungus had started to grow at the sides of the dish and was spreading inwards.

Experiment 17. Growth of excised root tips of clover on agar medium, and the effect of inoculation with nodule bacteria.

The solution employed was identical with that used in the preceding experiment except that it was now solidified by the addition of 1 per cent. agar. As before, three excised root tips were transferred to each flask of nutrient. Seven flasks were left uninoculated as controls, ten were inoculated while the agar was molten, and another ten were surface inoculated after the agar had 'set'. Observations were made over a period of 12 weeks.

In the inoculated flasks, the bacteria multiplied rapidly giving a distinct opacity to the agar, and on its surface they grew particularly well in the immediate neighbourhood of the root tips, forming dense patches of gum. The roots themselves soon turned dark brown, and ceased to grow when they had elongated to about three or four times their original length. In some cases, one or two very short laterals formed and they also became brown. There were no signs of nodules on any of the roots.

The uninoculated controls grew vigorously, penetrated the agar, and formed a dense mass on the base of the flask. Towards the end of the growth period, their very oldest portions began to turn brown, but the rest of the root system was white and healthy looking. The striking growth differences between typical examples of these roots and those taken from an inoculated flask are shown in Fig. 9. Therefore it is evident that the presence of the nodule bacteria has a marked depressing effect upon the growth of excised root tips of clover on agar medium and we have already seen that the same is true for growth in liquid culture.

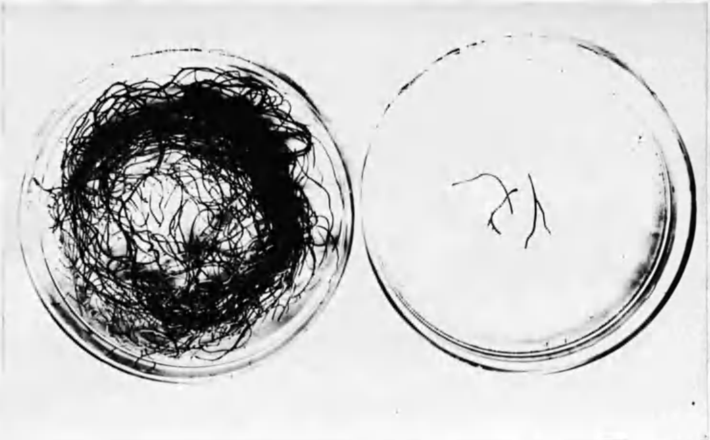


Fig. 9. Roots of clover grown from excised tips on agar medium containing 2 per cent. sucrose. Left, uninoculated roots, right, roots inoculated with nodule bacteria.

Experiment 18. Growth of excised roots of black wax bean, (1) from complete roots excised after 4 days' contact with the cotyledons, (2) from excised root tips 0.5 cm. long.

As we have seen in the Introduction, Lewis and McCoy (1933) secured 4 nodules on one out of 60 roots of black wax bean which were excised in their entirety after a germination period of 4 days. Although such roots are not strictly comparable with those grown from excised root tips in accordance with the more generally accepted sense of the term 'tissue culture', it would always be a step in the right direction if good nodule formation could be secured on roots of this type. In order to gain some idea of any advantages possessed by excised whole roots over excised root tips the following comparison was made of their ability to grow on a given medium.

Lewis and McCoy used as culture vessels 32 oz. bottles containing medium prepared according to the formula of J.K. Wilson (1931) $MgSO_4$, 0.2 gm.; $KH_2PO_4$, 0.2 gm.; NaCl, 1.0 gm.; $CaSO_4$, 0.1 gm.; Agar, 15 gm.; CaCO3, in excess; Water, 1 litre; Sucrose, 0.5 per cent. After sterilisation, sterile $CaCl_2$ was added to give a 1:500 molar concentration. The seeds were sterilised in alcohol and mercuric chloride and allowed to germinate for 2 days on agar plates. They were then transferred to the bottles and left for another 2 days before the tops were severed from the roots. Roots thus cultured, at a temperature of 27°C., often reached a length of 15 cm. or more, produced 30 to 50 secondary roots, and after inoculation with appropriate bacteria, 1 in 60 of them developed nodules.

In the present experiment, Petri plates containing a shallow layer of the Wilson medium were used instead of 32 oz. bottles. For Part (1), the above procedure of Lewis and McCoy was followed and at the time of excision the average length of the bean roots was 8.0 cm. and the average number of laterals was 12. In Part (2) of the experiment, after the seeds had germinated for 2 days on agar, tips 0.5 cm. long were excised from the radicles and transferred to the Petri plates of medium.

Both series of roots were left at a temperature of 25°C. for a period of 9 weeks, by which time it was evident that all growth had ceased.

Table 11.

Growth of excised roots of black wax bean, (1) from complete roots excised after 4 days' contact with the cotyledons, (2) from excised root tips 0.5 cm. long. No. of excised whole roots = 10, No. of excised root tips = 13. Growth period = 9 weeks.

| | Av.length of main root (cm.) | Av. no. of laterals | Av.increase in length (cm.) | Av. increase in laterals. |
|---|---|---|---|---|
| Excised whole roots | 12.3 | 63.7 | 4.3 | 51.7 |
| Excised root tips | 3.5 | 5.3 | 3.0 | 5.3 |

It is clear from Table 11 that the growth achieved by roots excised in their entirety after 4 days of germination greatly exceeds that of excised root tips cultured under the same conditions. In both sets of roots, those parts which touched the agar surface became dark brown in colour while the rest of the root retained its original whiteness. In the case of the excised whole roots, many of the lateral rootlets were 4 to 5 cm. long whereas the laterals which formed on the excised root tips never exceeded 1 cm. in length.

If the absence of nitrogen from the Wilson medium had been the limiting factor for the growth of the excised root tips of the above experiment, it seemed possible that they might thrive in the complete solution of Bonner and Devirian (1939), with accessory substances, to which 0.5 per cent. sucrose had been added. As we have just seen, Lewis and McCoy used this level of sucrose in their work with whole bean roots. If, on the other hand, this low concentration of sucrose had been limiting then good growth might be attained in the solution of Bonner and Devirian with the usual 4 per cent. sucrose employed for the culture of excised root tips of pea. The following experiments were therefore carried out.

Experiment 19. Growth of excised root tips of black wax bean in the medium of Bonner and Devirian with 4 per cent. and 0.5 per cent. sucrose, and the effect of inoculation with nodule bacteria.

Proceeding according to the methods already described, two root tips were placed in each of 10 Erlenmeyer flasks (250 cc. capacity) containing medium with 4 per cent. sucrose, and two to each of 6 similar flasks with 0.5 per cent. sucrose. After 17 days, growth in all cases was found to be extremely poor but nevertheless, half the roots of each series were inoculated with nodule organisms and the flasks set aside for another 8 weeks.

Table 12.

Growth of excised root tips of black wax bean in the medium of Bonner and Devirian with 4 per cent. and 0.5 per cent. sucrose, and the effect of inoculation with nodule bacteria. Growth period = 10 weeks.

| | Uninoculated | | Inoculated | |
|---|---|---|---|---|
| Conc. of sucrose (per cent.) | 0.5 | 4.0 | 0.5 | 4.0 |
| Av. length of main root (cm.) | 5.3 | 3.34 | 3.75 | 2.95 |
| Av. number of laterals | 9.5 | 0.1 | 2.5 | 0.9 |
| No. of nodules | - | - | 0 | 0 |

As can be seen from Table 12, growth was poor at both sucrose levels though the lower concentration proved to be slightly more beneficial, especially with regard to the production of rootlets. None of these laterals exceeded 1 cm. in length. Therefore it seems obvious that the medium does not provide the factors necessary for the culture of excised root tips of black wax bean.

The bacteria, as usual, formed white slimy masses which made the solution appear milky and coated the surface of the roots, but no nodules developed in any of the inoculated flasks. Once again the presence of the nodule organisms had a depressing effect upon root growth. Contrary to previous findings with other species of plants, the inoculated roots were not so brown as those which were left uninoculated.

Experiment 20. Inoculation of excised whole roots of black wax bean with an appropriate strain of nodule organism.

It has been mentioned already that the securing of good nodule formation on excised whole roots might prove helpful in efforts to obtain nodules on roots cultured from excised tips. Since it had been demonstrated by Lewis and McCoy (1933), and confirmed by the author, that excised whole roots of black wax bean could make some growth on a nitrogen-free medium containing a low concentration of sucrose, it seemed that they were suitable material for inoculation with nodule forming bacteria. As noted, Lewis and McCoy employed such roots and observed nodules on only one root out of sixty which were cultured.

The procedure followed was that of Lewis and McCoy already detailed in a previous experiment, but since Petri plates had proved to be too small, 500 cc. Erlenmeyer flasks were now used as culture vessels. Each contained approximately 80 cc. of Wilson medium. At the time of excision the average length of the main roots was 6 cm. and the average number of laterals was 12. In 12 of the flasks, roots were inoculated by dropping on to the agar surface about 1 cc. of a suspension of nodule organism. In the remaining 10 flasks, the method of Chen and Thornton (1940) was adopted, i.e., the agar was inoculated while molten before the roots were planted. All flasks were set aside at a temperature of 20°C. and were examined at frequent intervals throughout an 8 week growth period.

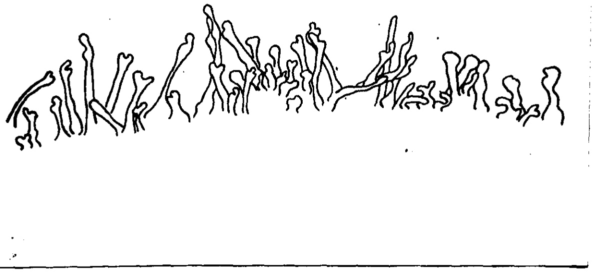
Table 13.

Inoculation of excised whole roots of black wax bean growing on nitrogen-free Wilson medium.
Set 1. Agar surface-inoculated when solid.
Set 2. Agar inoculated when molten. Growth period = 8 weeks.

| | No. of nods. | Av. increase in length (cm.) | Av. increase in no of laterals | Length of longest lateral |
|---|---|---|---|---|
| Set 1. | 0 | 3.5 | 25.8 | 8.3 |
| Set 2. | 0 | 5.8 | 16.0 | 5.0 |

Table 13 shows that no nodules were formed on any of the twenty-two roots cultured. In both sets of flasks the bacteria multiplied rapidly in the immediate neighbourhood of the root and laterals, producing gummy streaks which followed the path of these organs.

Microscopic examination of the root hairs revealed the peculiar deformations shown in Fig. 10. These



× 70

Fig. 10. Camera lucida drawing of deformed root hairs from excised whole roots of black wax bean inoculated with nodule bacteria.

closely resemble the abnormalities observed by McCoy (1932) and shown to be no indication of bacterial attack upon the plant, since the same effects could be produced by cell-free filtrates of nodule organisms belonging to an entirely different cross-inoculation group.

Browning was again noted wherever the roots touched the agar surface, and, in general, growth was not so satisfactory as that obtained in the previous investigation. This may have been due to the lower temperature employed.

It was decided to repeat the experiment, this time adding some nitrate-nitrogen to the medium in an attempt to improve growth and prevent the onset of browning. Calcium nitrate in the concentration used by Bonner (0.242 gm. per litre) was substituted for the calcium chloride of the Wilson formula, but otherwise the procedure was as before. In 4 flasks the agar was surface-inoculated, in 8 it was inoculated while molten and the remaining 7 flasks were left uninoculated as controls.

### Table 14.

As for Table 13. but with calcium nitrate substituted for the calcium chloride of the Wilson formula. Growth period = 8 weeks.

| | No. of nods. | Av. increase in length (cm.) | Av. increase in no of laterals | Length of longest lateral |
|---|---|---|---|---|
| Set 1. | 0 | 3.02 | 20.5 | 3.1 |
| Set 2. | 0 | 4.40 | 16.6 | 7.2 |
| Uninoc. | - | 7.90 | 29.1 | 8.2 |

From Table 14 it can be seen that once again both methods of inoculation did not result in nodule production although, as before, the bacteria formed a distinct zone of gum round the main root and laterals. Again the depressing effect of the presence of the bacteria upon the growth of the roots was in evidence.

The introduction of nitrate into the medium benefited the appearance of the roots during the first 2 or 3 weeks of growth since they remained white with pink-purple tinge which did not appear to be unhealthy. After the third week, however, the dark brown colouration noted before became obvious wherever the roots touched the surface of the agar. The nitrate seems to have had little or no effect upon root growth since increases in length of the main root and laterals were similar to those which occurred in the nitrogen-free medium.

An attempt was now made to secure nodules on excised whole roots of pea similar to those of black wax bean employed above.

Experiment 21. Inoculation of excised whole roots of pea.

One surface-sterilised pea seed was transferred to each of 12 large test tubes (20 cm. x 3 cm.) containing 50 cc. of the complete medium of Bonner and Devirian (1939) with 4 per cent. sucrose, solidified by the addition of 1 per cent. agar. All tubes were inoculated while the agar was molten. After 3 days' growth in the dark, a sterile scalpel was used to sever the tops from the roots, and the tops were removed.

During a growth period of 11 weeks the roots attained an average length of 12.0 cm. and possessed an average of 17.3 lateral roots. One nodule was formed on one root system showing that nodules can develop on excised whole roots of pea growing in the full nutrient devised by Bonner and Devirian. A milky zone, due to bacterial growth, was obvious around the main root and laterals in all tubes.

## DISCUSSION SECTION I.

The results of the experiments described in the preceding pages serve to establish several important facts which will be of value in future attempts to secure nodule formation on excised roots. Firstly, the techniques involved in tissue culture work have been mastered sufficiently to allow of the setting up of tests on a scale large enough to produce analysable results. Roots which had to be discarded due to contamination with fungi or bacteria were few in number but allowance must always be made for a small percentage of losses from this cause.

Verification has been obtained that, for the growth of excised root tips of field pea, the medium of Bonner and Devirian (1939) containing aneurin and nicotinic acid as accessory growth factors is superior to the same medium containing yeast extract. However, although so suitable for field pea, the medium does not provide all the essential substances or conditions for the growth of root tips of Soya bean, black wax bean, sweet pea and sea pea and it allows only a limited growth of a dwarf variety of broad bean.

With regard to aeration of the medium, it is obvious from Table 5, that there is no advantage in attempting to increase artificially the amount of oxygen present. Quantities optimal for the needs of the growing roots must diffuse through the cotton wool plugs of the flasks and dissolve in the solution. It is also evident from Table 4, that the greater aeration prevailing in sand cultures does not compensate for the greater difficulty in absorption. We have also seen that growth on medium solidified with agar is inferior to that in purely liquid culture and this finding is in agreement with the views of White (1933) who demonstrated that his own results were very much superior to those of Malyschev (1932a & b) who had used a similar medium but with agar added.

The majority of investigators (White, Bonner, etc.) have adopted the procedure of excising tips from their original cultures at frequent intervals and transferring them to new flasks or plates of solution. Thus they acquired large 'clones' of roots with identical genetical constitution since their origin could be traced back to the same parent fragment. The aim of the present author was to secure root systems of a size comparable with that of the roots of intact plants and thus suitable for use in attempts to obtain nodules. Therefore no sub-culturing was carried out, the original excised root tip being allowed to attain the maximum development that the given supply of nutrient would support. With this method, roots 10-12 cms. long have developed within two weeks, while after longer periods roots well supplied with lateral rootlets and reaching an average length of 40 cms. have obtained. This growth rate is below that of Bonner and Devirian (1939) who state that excised pea roots in their medium grow at the rate of 70-85 mm. per week. The discrepancy may be due to differences in the temperatures at which the roots were cultured. To maintain in a white, healthy condition the relatively large root masses required for nodule work, it has been found profitable to store them at temperatures averaging about 15-16°C. These are well below the range of 27-30°C. stated by White (1943b) to be about optimal for plant tissue cultures in general. However, as we have seen, growth of excised pea roots at 19-20°C. resulted in browning of the older portions of the roots and showed no compensating benefit to root length or lateral root production. In one extreme case two flasks at the lower temperature level were set aside and neglected for several months. The roots which they contained then measured 59.5 cm., 83.1 cm., 109 cm. and 75.5 cm. and possessed numerous laterals. It is evident therefore, that no renewal of nutrient is necessary during the growth periods required for experiments on nodule formation.

As we have seen, many previous workers (Laurent, 1891; Hiltner, 1900; Giöbel, 1926; Thornton, 1936; and others) emphasise the injurious effects of inorganic nitrogen upon the process of nodule formation, and Wilson, (1940) summarises diagrammatically how invasion of the plant and fixation of nitrogen vary with changes in the carbohydrate/nitrogen relationship within the host. With regard to invasion, the process with which the present author is primarily concerned, it appears that at all levels of carbohydrate, increase in nitrogen decreases nodulation and at all levels of nitrogen, increase in carbohydrate increases nodulation. Therefore, at the time, it seemed profitable to investigate the growth of excised pea roots in solutions containing only fractions of the original supply of nitrogen before inoculating them with nodule bacteria. It is now clear that freshly excised root tips cannot thrive if the nitrate concentration is reduced below that advocated by Bonner and Devirian. Table 7 however, shows that roots which have grown in full solution for a period of 4 weeks and are then transferred to media with reduced nitrate, continue to grow and produce numerous laterals. Unfortunately the experiment was terminated two weeks after the transfer to the lower nitrate levels, and therefore the growth observed may have been due, in the main, to utilisation of nitrogenous material stored within the root tissues during the period in normal medium. Therefore, despite the results in Table 7, it is not possible to say with accuracy that concentrations of nitrate as low as one quarter of the value given in the Bonner formula, are sufficient to support the continued growth of excised roots of pea. When roots which had attained an adequate size in the full medium were transferred to solution completely free from nitrate and then inoculated with nodule bacteria, both controls and inoculated roots ceased to grow, presumably due to lack of nitrogen, and no nodules were formed.

Repetition of the experiment using solutions containing one quarter, one eighth and one sixteenth of the original nitrate concentration again yielded no nodules although this time the controls remained healthy and showed some growth. It seems reasonable to suppose that some factor or factors other than nitrate concentration must be inhibiting nodule development, since it is unlikely that such low concentrations of inorganic nitrogen could prove very injurious.

Experiments about to be described in Section II show that the presence of sucrose in the rooting medium markedly reduces the number of nodules which form on pea plants growing in darkness and in light, 4 per cent. sucrose being the most injurious of all the concentrations tested. In the present Section, Table 8 shows that freshly excised root tips of pea do not grow well if the concentration is reduced below the 4 per cent. level. Reduction even to 3 per cent. caused a fall in root length from 24.8 cm. to 16.9 cm. and there was also an unfavourable effect upon root thickness and number of laterals. Transfer of well-grown roots from full medium to solutions containing 2 per cent., 1 per cent. and 0.5 per cent. sucrose, followed by inoculation with bacteria did not result in nodule formation. Growth was much reduced in the 2 per cent. sucrose medium and ceased altogether at the other levels. As we shall see, these concentrations permit a certain limited amount of nodule production on etiolated whole pea plants. In the case of the excised roots, however, it may be that the reduced sucrose has a harmful effect upon root growth which outweighs any advantage that the reduction may have with regard to nodulation. Unfortunately there were no uninoculated controls to show whether this was the case, and therefore the possibility also exists that the depression of growth observed in the above concentrations of sucrose may have been partly or wholly due to the bacteria and not merely to the initial reduction of the sucrose level. The injurious effect of the presence of nodule organisms on the growth of excised clover roots in both liquid and agar cultures has been well demonstrated, little or no further development having occurred after the inoculation with bacteria. Similar depressing effects on growth were observed in inoculated cultures of the roots of black wax bean. This may have been due to the accumulation of some harmful by-product of bacterial multiplication, or, more probably, to competition for food substances. The rapidly increasing number of organisms might soon reduce the available sucrose below some critical level essential for root growth. This seems to be the most acceptable explanation, though competition for dissolved oxygen may be involved also. It is interesting to note here the difference between the effect of the bacteria on the growth of excised roots and their effect on the roots of etiolated whole plants. In experiments described in Section II, etiolated pea plants produced well developed root systems although they were growing in inoculated agar containing sucrose. The bacteria multiplied rapidly in the medium, particularly round the root and rootlets, but there was no sign of the depression of growth so evident with excised roots. It may be that the additional supply of food within the cotyledons of the etiolated plants favoured the production of more vigorous roots and these were able to compete successfully with the bacteria for the available sucrose.

In view of all the above considerations it is obvious that a high concentration of sucrose is needed by excised pea roots, but that this, as shown by whole plant experiments, is depressing to nodule numbers and also encourages rapid development of the bacteria in the medium, apparently 'swamping' the roots. An attempt was made therefore, to separate the sucrose supply of excised roots from the bacterial inoculum so that the external effects of sucrose upon nodule formation might be eliminated, and yet the root continue to receive its full complement. Table 9 shows that some growth of excised pea roots was achieved using a double vessel technique, but all the lateral roots noted were produced in the sucrose medium. Microscopic examination of the parts of the roots which had grown along the surface of the inoculated agar showed deformations of the root hairs similar to those observed on black wax bean. According to McCoy (1932) these do not necessarily indicate an attempted invasion of the host by the bacteria, since such abnormalities can be occasioned by cell-free filtrates of organisms which would not normally infect pea roots. Modifications and improvements to the method of this experiment are being evolved and it is hoped to test them out in the near future. Similar experiments with excised roots of clover might also prove profitable.

From Table 11 it is clear that the growth increments of 4-day-old roots of black wax bean excised in their entirety far exceed those of excised bean root tips cultured under the same experimental conditions. This was to be expected because the J.K. Wilson medium employed is notrogen-free and therefore growth would be limited by the amount of the nitrogenous reserves present in the original fragment. Moreover, the sucrose content of the medium is relatively low (0.5 per cent.). Thus it would appear that the larger supplies of nitrogen compounds, carbohydrates and other substances translocated from the cotyledons and present in the complete excised root systems, enable them to grow on the Wilson medium, showing a greater elongation of the main root and producing nearly ten times as many laterals as did the excised root tips. It seems probable also that substances which favour or specifically induce nodule formation may be transferred similarly from the cotyledons during thefirst four days of the germination period and in the complete root may be present in sufficient quantity to permit of some restricted nodule production. This may account for the one nodule observed by the author on an excised whole root of pea, and for the nodules noted by Lewis and McCoy (1933) on excised whole roots of black wax bean. Thornton (1929) suggests that a substance which induces nodulation of young lucerne plants and increases the growth of the nodule organism on agar, is excreted from the roots at a time co-incident with the opening of the first true leaves. In Section II we shall see that such an excretion appears to be involved also in the nodulation of whole pea plants grown under normal light conditions. The distinct zones of dense bacterial growth observed round excised whole roots of pea and black wax bean seem to indicate the workings of a similar excretion from these organs. This being the case, the active substance either must have its origin in the root itself, or be translocated from the cotyledons and/or young shoot during the first three days of the germination period. If the latter explanation holds, then this may account for the failure of nodulation on roots cultured from tips 0.5 cm. long since such tips would contain only very small amounts of the required nodule-promoting substance. With this idea in mind it is intended to add to the culture medium for excised root tips, root washings from whole plants and observe their effect upon nodule production. Details of the method of obtaining these washings will be given in connection with above-mentioned experiment in Section II. It has been observed that when small quantities of autoclaved extract from the shoots of normal pea plants are added to the medium in which inoculated excised pea roots are growing, no nodules form but another method of obtaining sterility of the extract will have to be employed before any definite conclusions can be reached about its effect upon the nodulation process. It may well be that the heat of the autoclave decomposes any substances which might stimulate nodule formation.

A preliminary experiment involving the treatment of excised root tips with auxins before planting in the nutrient medium, did not result in nodule production when nodule bacteria were subsequently added. The results merely confirmed the findings of Bonner and Koepfli (1939) that auxins have an inhibiting effect upon the growth of excised pea roots.

## SUMMARY SECTION I.

1. Well-developed root systems of field pea (*Pisum arvense* L.) var. New Zealand Maple, have been obtained from excised tips 0.5 cm. in length grown under sterile conditions in the nutrient solution of Bonner and Devirian (1939).

2. This solution is unsuitable for the growth of excised root tips of sweet pea, sea pea, Soya bean and black wax bean, but allows a certain limited growth of tips of a dwarf variety of broad bean.

3. The temperature best suited to the maintenance of healthy excised root systems of pea is in the region of 15-16°C. At higher temperatures, browning of the older portions of the roots becomes more evident.

4. Aeration of the nutrient solution is not beneficial to root growth.

5. Growth of excised pea roots in liquid medium exceeds that on agar medium or in sand watered with the nutrient solution.

6. Freshly excised root tips of pea cannot grow in solution with reduced concentrations of nitrate. Older excised roots transferred from full medium to solutions low in nitrate can continue to grow for at least two weeks.

7. Freshly excised root tips of pea cannot make

good growth if the sucrose content of the medium is reduced below the 4 per cent. level.

8. Glycerol and mannitol are ineffective as substitutes for sucrose in the growth of excised root tips of pea.

9. Inoculation with nodule bacteria of excised pea roots growing in liquid, sand or agar culture, with or without the presence of nitrates, does not result in nodule formation. Microscopic examination of such roots reveals no deformation of the root hairs which could be associated with bacterial attack.

10. Reduction of the level of sucrose supplied to well-grown excised pea roots just prior to inoculation does not result in nodule formation and the roots themselves cease to grow.

11. Some growth of excised pea root tips, but no sign of nodulation has been achieved using a double-vessel technique involving a separation of the sucrose supply from the bacterial inoculum.

12. Pre-treatment with auxins results in inhibition of the growth of excised pea roots and such roots do not form nodules when inoculated with nodule organisms.

13. Addition to the medium of an extract from the tops of whole pea plants grown in light does not result in nodule production on excised pea roots.

14. Excised root tips of clover grow well in both liquid and agar culture in the medium advocated by

Bonner and Devirian (1939) which contains sucrose at the 2 per cent. level. When nodule bacteria are added they multiply rapidly, but root growth ceases and there is no sign of nodule formation.

15. Four-day-old roots of black wax bean excised in their entirety make much superior growth to that of excised bean root tips when both are cultured on nitrogen-free agar medium supplied with 0.5 per cent. sucrose. Inoculation of the excised whole roots with appropriate bacteria does not result in nodule production.

16. One nodule formed on an inoculated pea root which was excised in its entirety after being in contact with the cotyledons and plumule for a period of three days.

17. Failure of nodule formation on excised roots may be attributed therefore, to several possibilities:

(i) the concentration of sucrose required for root growth may cause the bacteria to multiply so rapidly that they overgrow the roots;

(ii) the absence of additional nutrient substances, normally derived from the cotyledons, may reduce the vigour of the excised roots and their ability to compete with the bacteria for available food;

(iii) some specific substance, normally derived from the cotyledons or shoot and necessary for nodule production, may be absent from the excised roots (this substance may take the form of a root excretion) ;

(lV) there may be some other, as yet unknown, effect of the sucrose present in the medium.

# SECTION II.

The effect of certain factors on the formation of root nodules by whole plants of pea and other leguminous plants. (Embodying material accepted for publication by the Royal Society of Edinburgh.)

## INTRODUCTION.

The experiments described in the present section were undertaken, as we have seen, as the outcome of an investigation into the possibility of securing nodule formation on excised roots of leguminous plants. It soon became clear that additional experiments were necessary, in order to test the effect, on the nodulation of whole plants, of certain conditions under which nodulation of excised roots was being attempted. Etiolated plants have, to a considerable extent, been used in these further investigations, since in their nutritional requirements they more closely resemble excised roots than do plants grown in light. They are incapable of carrying out not only photosynthesis as such, but also all other syntheses which may depend on the incidence of light upon the shoot. Observations on their responses to inoculation with nodule bacteria might be expected to further our knowledge of the place of origin of substances essential to nodule production. Moreover, it seemed reasonable to suppose that their reactions to imposed experimental conditions would bear some resemblance to the behaviour of excised roots under similar conditions, and explanations advanced for the one might prove at least partially applicable to the other. Any great differences in behaviour could be attributed to the cotyledonary reserves to which the etiolated plants have access and some idea of the content and functional scope of these reserves might thus be determined. It also seemed necessary to make some observations on normal plants grown in light in order to elucidate certain points which arose during the etiolation tests, and in this way the work was sometimes extended beyond the immediate interests of root cultures.

The investigations consist of:-

(a) a study of the extent of nodulation on plants grown in continuous darkness,

(b) studies of the effect of sucrose, nitrate and accessory growth substances on the nodulation of etiolated and light-grown plants,

(c) experiments relating to production by whole plants of substances favouring nodule formation.

A brief notice concerning some of these observations has already appeared (McGonagle, 1944), while a further paper has been accepted for publication by the Royal Society of Edinburgh.

The effects of partial or complete exclusion of light upon the processes of nodulation and nitrogen-fixation have attracted the interest of several workers. Vines (1888) showed that when he placed Vicia Faba plants in the shade no nodules were formed. Prazmowski (1890), on the other hand, grew peas in garden soil in the dark and found that nodules were as plentiful upon them as upon his controls, although he reports that such nodules remained much smaller. Thornton (1930) investigated the effect of excluding light upon the nodulation of young lucerne seedlings. Those kept in the dark from the time of germination produced only 3.5 nodules per 20 plants, those darkened after the opening of the first true leaf, 16 nodules per 20 plants, while those kept continuously exposed to light showed 46.5 nodules per 20 plants. Moreover, in the case of the seedlings darkened after the first true leaf had opened, the nodules already initiated ceased to grow and no new ones were formed. In the former the bacteria became parasitic, attacking the cell contents and nuclei of their hosts in order to obtain carbohydrates. J.K. Wilson (1931) obtained only one nodule on 40 vetch plants cultured aseptically on sucrose-free agar medium. When lucerne, red clover and pea were used instead of vetch, he obtained no nodules at all on plants grown without sugar. Lewis and McCoy (1933), in their investigations, grew black wax beans aseptically in the dark on nitrogen-free agar medium and observed no nodules on the 60 control plants to which no carbohydrate was added.

Both J.K. Wilson and Lewis and McCoy (1933) considered that the addition of sucrose to the rooting medium stimulated nodule production on etiolated legumes in aseptic culture. However, as we shall see, the nodules which appeared were still very few and developed on only a small proportion of the plants. Schweizer (1932) found that a strain of Soya bean which possesses no chlorophyll, and normally forms no nodules, would do so when sucrose was supplied. Ritter (1911), Prucha (1915), J.K.Wilson (1917) and Ludwig and Allison (1935), studied the effect of sucrose on nodule formation by leguminous plants growing in natural light. Their work will be referred to in some detail in the Discussion.

Previous findings regarding the depressing effect of combined nitrogen on nodule production have been mentioned in the Introduction to Section I, and mention has also been made of experiments in which addition of carbon compounds along with combined nitrogen served to nullify the injurious effect of the combined nitrogen alone. The work of other investigators on the synthesis of aneurin in illuminated pea plants and its role in initiating the growth of nodule bacteria has also been dealt with in the above section, pp. 39 - 40.

# METHODS

The whole plants employed in the following investigations were generally grown in large test tubes (20 cm. x 3 cm.) plugged with cotton wool, and were rooted either in agar medium or in fine quartz sand. An aseptic technique (as regards the root system and rooting medium) was used in most cases so that the results would be directly applicable to root tissue cultures, and also because the sucrose present in many of the treatments might otherwise have been rapidly decomposed by contaminating microorganisms. In addition the agar medium used in several of the experiments was convenient in that nodule formation and development could be observed easily throughout the growth period. The nutrient solution, except where otherwise stated, was the one whose formula has already been given in Section I, p.42. It was, in most cases, either solidified with 1.0 - 1.5 per cent. agar, or was added at the rate of 1 part by 4 parts of weight of fine quartz sand. For agar cultures, tubes containing 50 cc. of medium were sterilised in the autoclave for 20 minutes at a pressure of 15 lbs. When sand was used, it was given a preliminary sterilisation for 3 hours at 15 lbs. pressure, weighed out into tubes (80 gm. to each), the nutrient solution added, and the tubes autoclaved for 20 minutes at 15 lbs. pressure. Inoculation of agar cultures was carried out according to the already described method of Chen and Thornton (1940).

In the case of sand cultures, inoculation was achieved by dipping the seeds in the bacterial suspension before planting. One germinated seed was transferred aseptically to each tube. (Seeds were surface-sterilised and germinated according to the directions detailed in Section I.) If the tubes were to be placed in the greenhouse, the basal portions, extending to about half an inch above the sand or agar surface, were wrapped in black paper in order to exclude light from the root systems. All greenhouse experiments were conducted during spring or summer months.

As the shoots elongated, they generally managed to grow unaided through the cotton wool plugs, or up between plus and glass, but in a small number of cases where emergence was proving difficult, the tubes were again placed in the inoculating box and the plugs loosened a little. The emergence of the shoots did not have any injurious effects upon the sterility of the rooting medium. Sterility was judged by visual inspection of the tubes and only about 2.5 per cent. had to be discarded because of the presence of contaminating organisms.

Plants which were to be etiolated were grown throughout in a dark room constructed for photographic purposes, giving complete exclusion of light. When being examined, the plants of some of the experiments were exposed to low-intensity white artificial light for periods which amounted to approximately half an hour per week. In the other experiments, red light was used for inspection.

The methods employed for obtaining washings from the roots of lucerne and pea plants are described in the accounts of the experiments concerned.

## EXPERIMENTAL RESULTS.

### Experiment 22. Formation of nodules on etiolated pea plants, and the effect of sucrose, nitrate, and accessory growth substances.

As we have seen in the Introduction, the etiolated plant bears a closer resemblance to the excised root than does a plant grown under normal light conditions and therefore useful information might be gained from a study of conditions affecting the nodulation of such plants.

In the experiment to which Table 15 refers, plants were grown from seed in aseptic tube culture in continuous darkness for a period of six weeks at a temperature which was mainly in the region of 13°C. The rooting medium was the usual mineral-salt mixture (with accessory substances) solidified with agar. In some tubes the nitrogen-free formula was used, in others nitrate was supplied in the amounts shown in the original formula. Four per cent. sucrose was added to a proportion of the tubes. Twelve tubes, each with one plant, were set up for each treatment, all the tubes being inoculated with the nodule organism. The plants were examined at intervals under red light.

### Table 15.

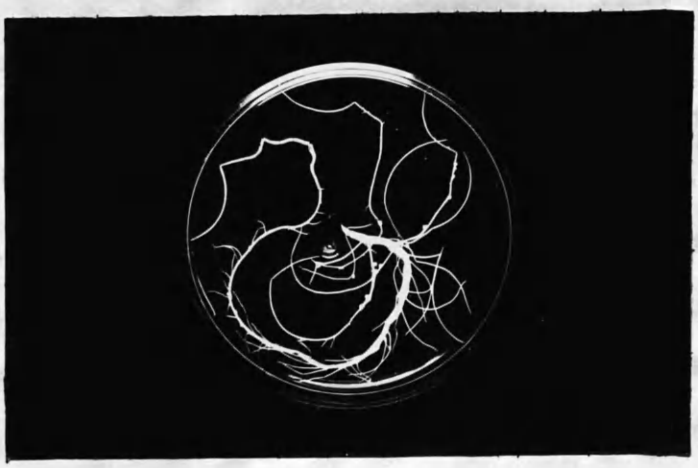
Formation of nodules on etiolated peas, and the effect of sucrose and nitrate. Plants grown on mineral-salt agar for period of 6 weeks. Data are averages of 12 plants for each treatment.

| Treatment | | No. of nodules per plant | | Length (cm.) | | Dry wt. (mg.) | | |
|---|---|---|---|---|---|---|---|---|
| 4% Sucrose | Nitrate | Av. | Range | Shoot | Main Root | Shoot | Root* | Total |
| - | - | 6.0 | o(1p) - 13 | 40 | 25 | 92 | 23 | 115 |
| + | - | 2.3 | 0(5p) - 9 | 28 | 18 | 126 | 40 | 166 |
| - | + | 1.8 | 0(6p) - 6 | 43 | 24 | 100 | 28 | 128 |
| + | + | 0.6 | 0(8p) - 4 | 38 | 17 | 138 | 51 | 189 |

p = plant

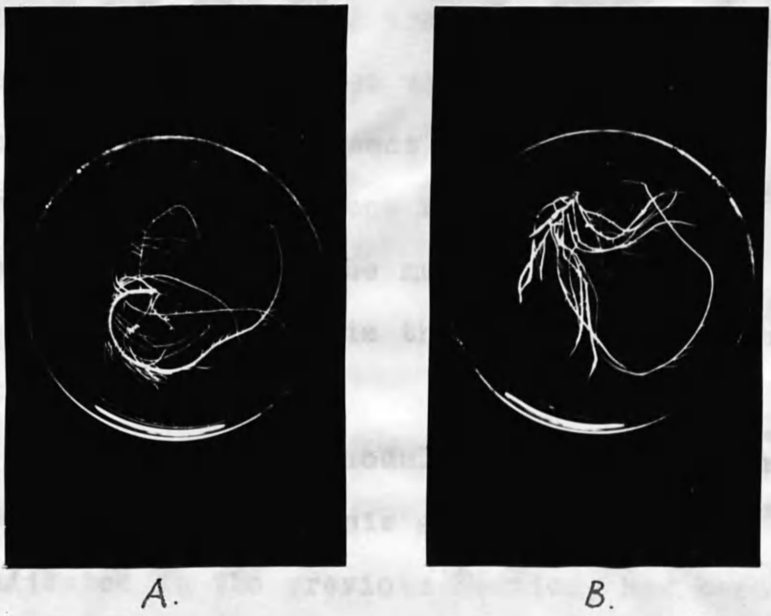
* inclusive of nodules.

Figs. 11A. and 11B. Two root systems from etiolated pea plants grown in nitrogen-free mineral-salt agar, showing the presence of nodules.



It will be observed from Table 15 that despite growth in continuous darkness, appreciable numbers of nodules formed. Thus of the twelve plants grown without sucrose or nitrate, eleven developed nodules the average number per plant being 6.0, i.e., a total of 72 nodules on the twelve plants. In this and also in subsequent experiments considerable variation occurred in the number of nodules formed on individual plants under similar conditions, an indication of the extent of this variation being given in the column headed 'Range'. The nodules, which were of hemispherical shape, were mostly less than 1 mm. in diameter though a few attained a diameter of 2-3 mm. These plants without sucrose were the first to show nodule development. (Figs. 11A and 11B).

In the presence of 4 per cent. sucrose the number of nodules was clearly reduced. Thus of twelve plants, only seven showed nodules, the average being reduced to 2.3 per plant, i.e., 27 nodules on the twelve plants. Information on the effect of sucrose on the size of nodules will be given in connection with the next experiment. The presence of nitrate was also effective in reducing the number of nodules, and when both sucrose and nitrate were present together, nodule numbers were still further reduced. (Figs. 12A and 12B). Some of the nodules in all four treatments had a pink colour resembling that which under normal conditions is due to the presence of haemoglobin.



Figs. 12A and 12B Roots from etiolated pea plants grown in inoculated agar medium containing 4 per cent. sucrose. A. medium nitrogen-free. B. medium with nitrate, roots much thicker.

Shoots and roots of plants supplied with sucrose alone were considerably shorter than in the absence of sucrose (Figs. 13A and 13B). Plant dry weights were increased by the addition of both sucrose and nitrate. Surrounding the main root and the rootlets, particularly in the case of plants supplied with sucrose, there was a well-defined milky or opalescent zone (Fig. 14). When some of the agar from this zone was examined under the microscope, it was found to be much more thickly populated with nodule bacteria than agar taken from other parts of the tube.

The effect on the nodulation of etiolated peas of adding aneurin and nicotinic acid to the medium in the amounts indicated in the previous Section, has been tested in two similar experiments under aseptic conditions. Details of technique were as for the previous investigation, the medium being prepared according to the nitrogen-free formula. The experiment was planned so as to provide evidence on the effect of the accessory growth substances, both in the presence and absence of 4 per cent. sucrose. The plants of the first experiment were examined under low intensity artificial white light, while in the second experiment red light was used. Results are summarised in Table 16.

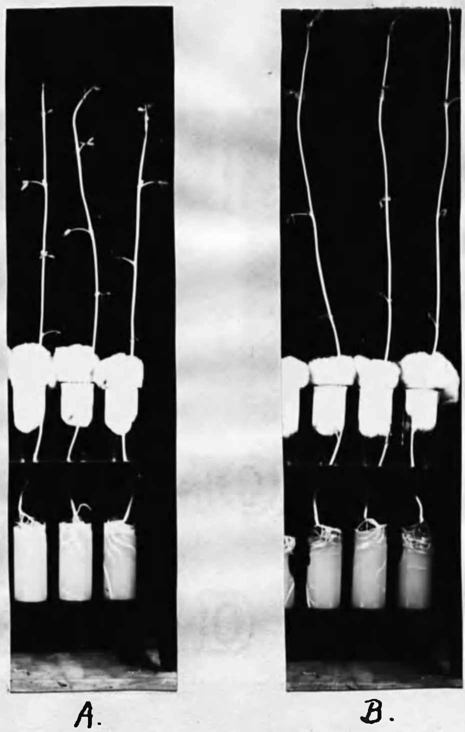


Fig. 13. Etiolated peas growing in nutrient agar. Those on right sucrose-free, those on left supplied with 4 per cent. sucrose. Shoots are shorter and sturdier when sucrose present.

Fig. 14. Milky zone of dense bacterial growth surrounding the main root and rootlets of an etiolated pea plant.

## Table 16.

Formation of nodules on etiolated peas, and the effect of sucrose and accessory growth substances (aneurin and nicotinic acid). Plants grown on mineral-salt agar for a period of 8 weeks. Data are averages of 6 (Expt. 1) or 12 (Expt. 2) plants per treatment.

| Treatment | | No. of nodules per plant, Expt.1. | | No. of nodules per plant, Expt.2. | |
|---|---|---|---|---|---|
| 4% Sucrose | A.G.S.* | Av. | Range | Av. | Range |
| - | - | 7.6 | 0(1p) - 14 | 8.8 | 4(1p) - 20 |
| + | - | 1.8 | 0(3p) - 6 | 1.9 | 0(6p) - 8 |
| - | + | 9.5 | 3(1p) - 26 | 6.3 | 0(1p) - 22 |
| + | + | 4.8 | 0(1p) - 10 | 1.7 | 0(5p) - 6 |

* A.G.S. = accessory growth substances.

It will be seen from the Table that plants grown without the addition of sucrose or accessory substances formed appreciable numbers of nodules, the data for these plants being very similar to those recorded in the previous experiment. The presence of sucrose decreased the average number of nodules per plant, and also decreased the maximum number of nodules on any one plant by more than half. In this experiment observations were made on the size of nodules formed. The percentage of 'large' nodules ( 1 mm. or more in diameter) on plants supplied with sucrose was 52.4, while on plants to which no sucrose had been added it was only 19.4. Thus although sucrose reduces the number of nodules, such nodules as are formed tend to be larger. Once again, nodules appeared on the sucrose-free plants before they were detected on those supplied with sucrose.

The addition of accessory growth substances appears to have had some effect on the average number of nodules per plant. Application of the t test shows however, that the differences noted fall within the bounds of experimental error. The same test shows that the differences obtained for the effect of sucrose on nodule numbers are statistically significant at the 1 per cent. level.

The milky bacterial zone round the roots and the pink colour in the nodules were again in evidence in all four treatments.

Experiment 23. Effect of sucrose concentrations lower than 4 per cent. upon the formation of nodules by etiolated peas.

It has been shown that sucrose at the 4 per cent. level has a marked depressing effect upon the numbers of nodules formed on etiolated peas. As we have seen, Wilson (1931) and Lewis and McCoy (1933) reported that the presence of 0.5 per cent., 1.0 per cent. or 2.0 per cent. sucrose was beneficial, if not essential, to the production of nodules on etiolated plants. It was decided, therefore, to determine the effect of such lower concentrations on the nodulation of etiolated peas and observe whether the results agreed with those of the above investigators.

Plants were grown from seed in continuous darkness under aseptic conditions, in tubes of inoculated, solidified, nitrogen-free medium (without accessory substances). Sucrose was added in the concentrations shown in Table. Red light was employed for all observations.

Table 17.

Effect of various sucrose concentrations upon nodule formation by etiolated peas. Number of plants. per treatment = 12. Growth period = 9 weeks.

| Concn. of sucrose. | No.of nods./ plant. | Range | Dry wt. nods.mg./ plant | Dry wt. per nod. (mg.) | Dry wt. /plant (mg.) | Length (cm.) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Shoot | Root |
| 0 | 13.3 | 0(1p)* - 32 | 0.33 | 0.025 | 131 | 25.4 | 45.9 |
| 0.5% | 6.73 | 1(1p) - 15 | 0.818 | 0.122 | 152 | 20.0 | 51.6 |
| 1.0% | 9.75 | 3(2p) - 18 | 1.660 | 0.171 | 151 | 22.2 | 47.3 |
| 2.0% | 4.45 | 0(2p) - 12 | 0.636 | 0.143 | 162 | 24.3 | 46.3 |
| 3.0% | 1.83 | 0(4p) - 6 | 0.160 | 0.091 | 197 | 29.9 | 55.5 |
| 4.0% | 0.27 | 0(9p) - 1 | - | - | 215 | 30.3 | 49.9 |

* p = plant.

It is evident from the Table that the addition of sucrose, even in concentrations as low as 0.5 per cent., restricts the number of nodules formed on etiolated plants.

Once again the presence of sucrose proved beneficial to plant dry weights and to dry weights of such nodules as were formed at the lower levels. Nodules on plants supplied with 4 per cent. sucrose were too few in number for their dry weight to be determined with any degree of accuracy. In contrast to previous findings, shoot lengths of the sucrose-supplied plants exceeded those of the controls.

This, however, was not evident in the early stages of the experiment when, as usual, shoots of plants without sucrose were first to appear above the cotton wool plugs. Eleven of the twelve plants without sucrose formed nodules, the maximum number being 32, and the average number 13.3 per plant. These values are higher than any so far obtained by the author for etiolated plants.

Experiment 24. Spectroscopic examination of a nodule from an etiolated plant to determine the nature of the pink pigment.

One of the larger pink nodules from an etiolated pea plant grown in the preceding experiment was examined spectroscopically as follows: A fairly thick section of nodule tissue was mounted on a small square mirror, a drop of pyridine added, then a few crystals of sodium hyposulphite. The mirror was fixed at an angle to the illumination from a strong electric bulb while a student's hand spectroscope, held about half an inch to one inch above the mirror's surface, was manoeuvred until an absorption band could be clearly observed in the spectrum. For purposes of comparison, a pink nodule from a clover plant grown under normal light conditions was also examined and a similarly placed band noted. In both cases the band appeared towards the yellow end of the green area and therefore corresponded with that at 557 mu obtained by Keilin and Wang (1945) for the haemoglobin, treated with sodium hyposulphite, which

Fig. 15. Arrangement for growing pea plants in sand culture with root system and medium under aseptic conditions.

they extracted from Soya bean nodules. Thus it is evident that the pink colouration observed in the nodules of completely etiolated peas during the author's experiments, is due to the presence of haemoglobin, the pigment which regularly occurs in the effective, nitrogen-fixing nodules of legumes growing in light.

### Experiment 25. Effect of sucrose upon the nodulation of pea plants grown in light.

Having noted the marked depression in nodule numbers caused by the addition of 4 per cent. sucrose to etiolated peas, it was decided to investigate the effect of this and of lower concentrations upon the nodulation of pea plants whose shoots were exposed to normal light.

Two experiments were carried out, the experimental procedure being the same in both cases. Plants were grown in the greenhouse for eight weeks in tubes of sand supplied with the nitrogen-free form of the usual mineral solution containing the concentration of sucrose under examination (See Fig. 15).

Table 18.

Effect of sucrose on nodulation of pea plants grown in natural light. Plants grown by aseptic technique for a period of 8 weeks in sand supplied with nitrogen-free mineral salt solution. Data are averages of 6 plants per treatment.

| | | | | | | |
|---|---|---|---|---|---|---|
| Concn. of sucrose per cent. | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 |
| Av.number of nodules per plant | 16.3 | 12.5 | 6.2 | 4.0 | 3.2 | 1.2 |

## Table 19.

Effect of sucrose on growth and nodulation of pea plants in natural light. Plants grown by aseptic technique for period of 8 weeks in sand supplied with nitrogen-free mineral salt solution. Data are averages of 12 plants per treatment.

| Concn. of sucrose | Av.no. nods. per plant | Dry wt., mg. per plant | | | | Length (cm.) | |
|---|---|---|---|---|---|---|---|
| | | Nodules | Shoot | Root | Total | Shoot | Root |
| 0 | 44 | 3.0 | 170 | 66 | 239 | 47 | 11.1 |
| 0.5% | 26 | 4.3 | 177 | 87 | 268.3 | 41 | 12.2 |
| 1.0% | 26 | 5.4 | 235 | 80 | 320.4 | 47 | 12.7 |
| 2.0% | 14 | 4.5 | 222 | 84 | 310.5 | 39 | 12.9 |
| 4.0% | 10 | 4.4 | 243 | 93 | 340.4 | 38 | 12.2 |

It is obvious from both Tables that as the concentration of sucrose increased, the number of nodules decreased, (see Figs. 16A and 16B) and that a concentration as low as 0.5 per cent. had a marked depressing effect. On the other hand, the size of the nodules steadily increased with increasing amounts of sucrose, as can be seen from the fact that although the number of nodules per plant was decreasing, the dry weight of nodules per plant remained

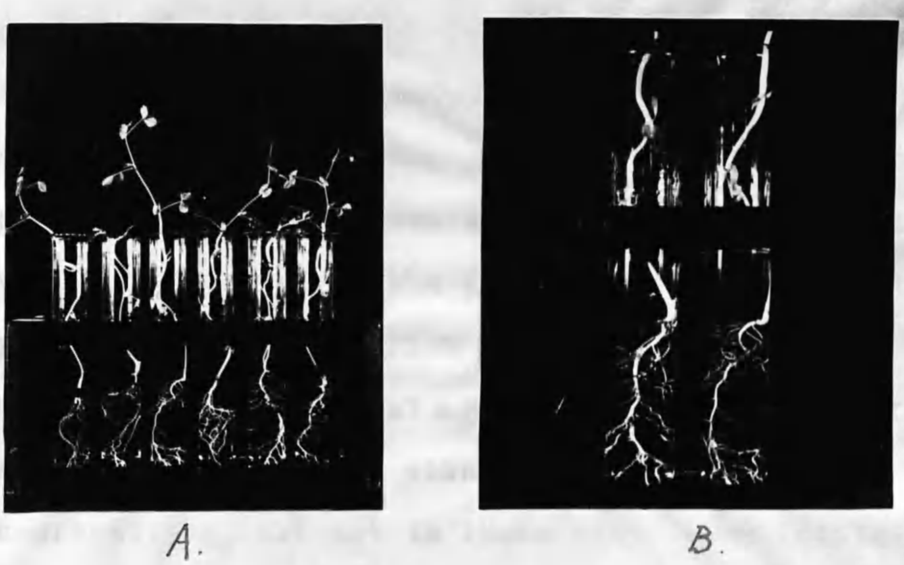


Fig. 16. Peas from sand culture experiment.
A. Three plants on right, with 0.1 per cent. sucrose, have nodules.
B. Details of two root systems. Right, with 4 per cent. sucrose, left, with 0.1 per cent. sucrose.

fairly constant at a value above that of the control, Further data on this point are given in the Discussion. Shoot and root dry weights also benefited from the addition of sucrose, but shoot length was reduced. Red pigmentation was evident in the leaves of all except the control plants, and was particularly marked when sucrose was at the 4 per cent. level. At the end of the growth period, sugar tests were carried out on extracts from the sand of the second experiment. These showed that sugar was still present in the tubes originally supplied with 2 per cent. and 4 per cent. concentrations, but not in those with lower initial concentrations. Quantitative estimations of residual sugar in tubes initially at the 4 per cent. level showed that plant and bacteria had together used 0.3 gm. of the 0.8 gm. of sucrose which was present in each tube at the beginning of the experiment.

### Experiment 26. Effect on nodulation of cultivation of the HX strain of pea nodule organism on media with various concentrations of sucrose.

Prucha (1915) found that pea nodule bacteria made good growth on inorganic-salt agar containing 10 per cent. and 20 per cent. sucrose and some slight growth occurred even when the sucrose level was raised as high as 40 per cent. After 10 weeks, one agar slope with 10 per cent. sucrose had three million organisms, while a slope with 20 per cent. sucrose had one million. The infective powers of these bacteria were undiminished. In the author's experiments it has been demonstrated repeatedly that the presence of sucrose in the rooting medium decreases the number of nodules formed on pea plants grown in darkness and in light. Therefore, it was thought advisable, despite the above findings of Prucha, to test whether the harmful effects of the sucrose were being exerted directly upon the nodule bacteria.

As already stated, the organisms used throughout the present experiments were cultured on the yeast-extract agar of Fred, Baldwin and McCoy (1932) which contains 1.0 per cent. mannitol as a source of carbon. Comparison was now made between growth of the HX strain on media containing 1.0 per cent., 2.0 per cent., 3.0 per cent. or 4.0 per cent. sucrose and that on media containing the same concentrations of mannitol. Cultures were grown on slopes in test tubes (2 cm. x 15 cm.), there being 6 slopes at each treatment. Care was taken that approximately the same amount of inoculum was used in every case and a single stroke with the platinum wire was made down the centre of each slope. After 7 days at 19°C., the organisms had grown well in all the tubes, and therefore it was evident that sucrose in concentrations up to, and including, 4 per cent., did not interfere with bacterial multiplication. The actual amount of growth, as judged by the breadth of the area of gum produced, varied slightly in the various treatments as can be seen in Fig. 17. To

Fig. 17. The HX strain of pea nodule organism growing on agar slopes containing,,left, one to four per cent. mannitol, and right, one to four per cent. sucrose. Growth has occurred in all cases.

obtain this photograph, a method of securing differentiation between the gum region and the rest of the agar had to be devised. In collaboration with Mr. Leitch of this department the following technique was worked out and found to be successful. Harrison and Barlow's gentian stain for bacterial gum was prepared according to the modified formula given on p. 69 of Fred, Baldwin and McCoy (1932). A little was poured into each tube, quickly poured out again and the tube washed out several times with water. A saturated solution of picric acid in 95 per cent. alcohol, was now poured in, immediately poured out and the tube again washed repeatedly. The bacteria and gum were gradually removed by the numerous washings, leaving a pale yellow area surrounded by agar which was stained deep purple. Slopes treated in this way were then suitable for photographic purposes.

Thus it is seen that the substitution of the above amounts of sucrose for the mannitol usually present in the culture medium did not have any marked injurious effect on the growth of the HX strain of nodule bacteria. It now remained to discover whether the ability of the organism to infect the appropriate host had been impaired. Moore (1905) stated that growth on medium rich in nitrogen reduces infectivity of nodule organisms, while Lewis and Nicholson (1905) decided that culture on bouillon with 2 - 5 per cent. sucrose gave the best infectivity of all the media which they tested. Prucha (1915) on the other hand, concluded that the type of medium, even if it is nitrogenous, has not much influence on the infective powers of the organism.

Pea plants growing in light in sand watered with nitrogen-free mineral salt solution were inoculated with suspensions of bacteria prepared from slopes containing the above amounts of sucrose. Plants inoculated from slopes with 1 per cent. mannitol served as controls. (See following Table.) After a six-week growth period nodule numbers were found to be very poor in all cases, the reason for this not being apparent.

| | Sucrose slopes (per cent.) | | | | Mannitol slopes |
|---|---|---|---|---|---|
| Source of inoculum | 1 | 2 | 3 | 4 | 1 per cent. |
| No. of nods. per 6 plants | 3 | 4 | 5 | 12 | 10 |

The experiment was repeated using only inoculum from slopes containing 4 per cent. sucrose and 1 per cent. mannitol. This time there were 12 plants per treatment.

No. of nods./plant inoculated from 1 per cent. mannitol = 35.6

No. of nods./plant inoculated from 4 per cent. sucrose = 43.0

It is evident therefore, that growth on medium containing sucrose at the 4 per cent. level has no permanent detrimental effect on the infective powers of the HX strain of pea nodule bacteria.

## Experiment 27. Effect of the accessory growth substances, aneurin and nicotinic acid upon the growth of etiolated pea plants.

It has been shown in two previous experiments that the presence of these accessory substances has no significant effect upon the number of nodules formed on etiolated pea plants, but its effect on the growth of such plants was not particularly noted. As mentioned before, Bonner and Greene (1938) concluded that aneurin was produced in illuminated leaves and since pea plants growing in light did not benefit from additions of aneurin to the medium, it appeared that they could synthesise sufficient for their needs. Etiolated plants would be incapable of this synthesis and thus it seemed possible that supplying aneurin to their roots would result in increased growth.

The effect of the accessory growth substances was tested both in the presence and absence of 4 per cent. sucrose. Peas were grown in the usual way in aseptic tube culture in nitrogen-free mineral-salt agar to which the accessory substances and sucrose had been added as shown in Table 20. After 8 weeks' growth in darkness, all plants were harvested and their dry weights determined.

## Table 20.

Effect of aneurin and nicotinic acid upon the growth of etiolated pea plants. Growth period = 8 weeks.

Number of plants per treatment =. 12.

| Treatment | | Dry weight, mg. per plant | | |
|---|---|---|---|---|
| 4% sucrose | A.G.S. | Shoot | Root | Total |
| - | - | 91 | 22 | 113 |
| - | + | 88 | 25 | 113 |
| + | - | 118 | 43 | 161 |
| + | + | 119 | 59 | 178 |

From the Table it is clear that the presence of aneurin and nicotinic acid in the rooting medium has little or no effect on the growth of etiolated peas as expressed in terms of plant dry weight. Therefore, if these two substances are essential for the growth of such plants, sufficient quantities of them must be contained in the seed reserves.

Once again it is evident that the addition of sucrose resulted in an increase in plant dry weight, the effect being most marked in the case of the roots. This agrees with the observations of Knudson (1917) who noted an average increase of 149 mg. in the root dry weights of light-grown Canada Field Peas when 2 per cent. sucrose was added, and thought it was possibly due to the fact that these plants were completely enclosed in large tubes. Transpiration was thus restricted, and there would be no current to remove the sugars from the zone of absorption. The peas of the present experiment had the greater part of their shoots exposed to the atmosphere, and this may account for the fact that dry weight increases for roots supplied with 4 per cent. sucrose were only of the order of 20 - 30 mg.

### Experiment 28. Formation of nodules on etiolated plants of black wax bean (Phaseolus sp.), and the effect of two concentrations of sucrose.

As already noted in preceding pages, a variety of black wax bean was used by Lewis and McCoy (1933) in their investigation into the production of nodules on etiolated plants. There was no nodulation of their sucrose-free controls, but nodules did develop on 14 out of 80 of the plants supplied with 0.5 per cent. sucrose. The medium employed was that of Wilson (1931) and the beans were grown at an average temperature of 27° C. The present investigation was undertaken to see if nodules could be obtained on similar plants growing in the medium of Bonner and Devirian with and without sucrose.

Selected seeds were sterilised by shaking for 2 minutes in absolute alcohol and 4 minutes in 0.1 per cent. mercuric chloride, followed by washing in 6 changes of sterile water, and were immediately transferred to agar plates for germination to proceed. Previous experience showed that overnight soaking was harmful to such seeds.

Plants were cultured by aseptic technique for a period of 6 weeks in tubes of nitrogen-free medium (without accessory substances) solidified with 1 per cent. agar. Twelve tubes were supplied with 0.5 per cent. sucrose, six with 1 per cent. sucrose and six were left sucrose-free. The medium in all cases was inoculated with a suspension of an organism known to form effective nodules on plants of the Phaseolus group. Temperatures throughout the growth period never exceeded 21° C.

Table 21.

Formation of nodules on etiolated plants of black wax bean and the effect of 0.5 per cent. and 1 per cent. sucrose. All plants inoculated. Growth period = 6 weeks. Number of plants at 0 per cent. and 1 per cent. sucrose = 6. Number of plants at 0.5 per cent. sucrose = 12.

| Concn. of sucrose | No. of nods./ treatment | Range | Length cm./ plant |  | Dry wt., mg./ plant |  |
|---|---|---|---|---|---|---|
|  |  |  | Shoot | Root | Shoot* | Root |
| 0 | 4 | 0(5p) - 4 | 30.4 | 15.7 | 131 | 24.5 |
| 0.5% | 4 | 0(8p) - 2 | 22.1 | 16.8 | 174 | 51.3 |
| 1.0% | 0 | 0(6p) - 0 | 16.6 | 11.2 | 186 | 40.0 |

* including cotyledons.

From the Table it is evident that nodules can form on etiolated plants of black wax bean without the addition of sucrose to rooting medium. Such nodules, however, were smaller than those on plants to which 0.5 per cent. sucrose had been added. Two of the latter larger nodules were pink in colour, presumably due to the presence of haemoglobin, and therefore etiolated beans, as well as etiolated peas, must be capable of synthesising this pigment. No nodules were formed on plants supplied with sucrose at the 1 per cent. level.

In the majority of tubes growth was not satisfactory perhaps because of the unsuitably low temperature which ranged from 11°C. to 21°C. In the 1 per cent. sucrose series, only one shoot had emerged beyond the cotton wool plug, and in many cases the cotyledons were still enclosed within the testa. Plants supplied with sucrose had browner roots, fewer lateral roots and shorter shoots than those left sucrose-free, but as usual the presence of sucrose increased root and shoot dry weights.

Milky zones of bacterial growth were obvious near to the agar surface where laterals were most plentiful, and were particularly noticeable in the tubes supplied with sucrose.

It is planned to repeat this experiment under temperature conditions more suitable to the growth of beans.

## Experiment 29. Nodule formation on etiolated clover, and the effect of various concentrations of sucrose.

As we have noted earlier J.K. Wilson (1931) secured no nodules on etiolated clover plants which were cultured either with or without added carbohydrate. Since clover has been used in the work with excised roots (Section I) it was decided to test whether Wilson's experience could be confirmed.

In the present experiment, plants of Montgomery red clover were grown from seed in complete darkness on aseptic agar slopes in three-quarter inch test tubes. The medium was that of Chen and Thornton (1940) and had the following composition:- $K_2HPO_4$, 1.0 gm.; $MgSO_4.7H_2O$, 0.2 gm.; $CaH_4(PO_4)_2.2H_2O$, 0.5 gm.; NaCl, 0.1 gm.; $FeCl_3$, 0.01 gm.; Agar, 20.0 gm.; Tap water, 1 litre. To a proportion of the tubes sucrose was added in the amounts shown in the Table.

Seeds were selected under a lens, and any which were damaged or infected were discarded. Surface-sterilisation was effected by shaking for 3 minutes with absolute alcohol, 4 minutes with 0.1 per cent. mercuric chloride, and then washing in 6 changes of sterile water. The washings were extended over 20 to 30 minutes and the seeds shaken from time to time during that period. Using a sterile platinum wire, 4 or 5 seeds were planted per slope. When germination had occurred, each tube was inoculated with a few drops of a suspension of an effective strain of clover bacteria (Rothamsted strain 49). Inoculation was carried out in red light. There were 12 tubes at each treatment.

## Table 22.

Formation of nodules on etiolated clover plants, and the effect of various concentrations of sucrose. Growth period = 8 weeks.

| | Un-inoc. | Inoculated | | | | | |
|---|---|---|---|---|---|---|---|
| Concn.of sucrose | 0% | 0% | 0.5% | 1.0% | 2.0% | 3.0% | 4.0% |
| No.of nods. /12 plants | - | 0 | 0 | 0 | 0 | 3 | 0 |
| Dry weight mg./plant | 1.22 | 1.12 | 1.81 | 2.08 | 2.43 | 2.86 | 3.05 |

It can be seen from the Table that only three nodules were formed, one on each of three plants in the series supplied with 3 per cent. sucrose. Evidence of the beneficial effect upon plant growth of the presence of carbohydrate in the medium is provided by the steady increase in plant dry weight with increase in the concentration of sucrose added. Plants without sucrose had no lateral roots, and, in the majority of cases, the cotyledons had not emerged from the testas. Those supplied with sucrose showed the formation of several short laterals, the cotyledons were expanded, and a small first true leaf had appeared. All plants, however, were very poorly developed. This was probably due to the smallness of their cotyledonary reserves coupled with the lack of nitrogen in the medium. It is intended to repeat the experiment, this time adding some nitrate.

Experiment 30. Effect of root washings upon the nodulation of lucerne seedlings.

Thornton (1929) records that observations on thousands of lucerne seedlings have established that the appearance of nodules coincides with the opening of the first true leaf. This might be interpreted as nothing more than a developmental coincidence, but against this is experimental evidence obtained by Thornton that at the stage in growth mentioned above, a substance is excreted by the roots which favours the growth of the nodule bacteria and infection of the root. Thus root washings prepared from such seedlings stimulated nodule development on younger seedlings and also increased the growth of nodule bacteria in pure culture. Root washings from seedlings not yet showing expansion of first true leaves had no such effects.

If an excretion from the roots preceeds, and induces nodulation in all normal leguminous plants, including pea, it is possible that it may not occur with excised roots and this may explain their failure to form nodules. Therefore it might prove beneficial to add to the culture medium for excised roots, root washings from whole plants. As a necessary preliminary to such a procedure, the experiment of Thornton was repeated in order to become familiar with the technique employed.

Three shallow glass troughs filled with coarse sand were watered with equal volumes of a Rothamsted nutrient solution (Thornton, 1929) and sown with lucerne seed. When the majority of the seedlings in one of the troughs were in the cotyledon stage, they were carefully removed from the sand and the sand packed into a long glass tube approximating to the size of Thornton's, i.e., 2 inches in diameter and 3 feet 6 inches long. Then, following the given directions, it was "clamped in a vertical position and fitted at the lower extremity with a perforated rubbed bung and a glass delivery tube. The solution was extracted with the pressure of a head of distilled water, about one litre of the solution being thus obtained (Extract A). When the seedlings in the second trough had well-expanded first leaves the solution from this culture was extracted in a similar manner (Extract B)." In the case of the third trough, when the first leaves had expanded, the whole of its contents, plants and sand, were packed into the tube and extracted (Extract C). This Extract C was prepared since Thornton's account does not make it perfectly clear whether sand alone or sand plus plants was placed in the extraction tube in his experiment. All three extracts were sterilised in the autoclave.

In order to test the effect of the extracts on nodule formation, twenty-four glazed pots, six for each treatment, were filled with coarse sand, watered with the Rothamsted solution to give 15 per cent. moisture, and sown with lucerne seed. Before the seedlings had emerged, the surface of the sand in 6 pots was uniformly watered with 25 cc. of a suspension of lucerne nodule bacteria made up in distilled water. Six pots were similarly watered with suspensions made up in Extracts A, B and C respectively. Owing to cold weather, it was over 14 days before any of the seedlings showed first true leaves open, but when this had occurred, 20 plants with leaves closed and 20 with leaves open, were examined from 3 pots at each treatment and nodule numbers recorded.

Table 23.

Effect of root washings upon the nodulation of lucerne seedlings. Growth period= 16 days.

| | Plants with leaves closed | | Plants with leaves open | |
|---|---|---|---|---|
| | No.of nods. /60 plants | No.of plants with nods. | No.of nods. /60 plants | No.of plant with nods. |
| Control | 16.0 | 12 | 60.0 | 33 |
| Plants supplied with Extract A. | 10.0 | 7 | 51.0 | 31 |
| Plants supplied with Extract B. | 30.0 | 22 | 85.0 | 38 |
| Plants supplied with Extract C. | 44.0 | 25 | 90.0 | 41 |

The Table shows that both the number of nodules and the number of plants bearing nodules increased when Extract B or Extract C were added. Application of the $\chi^2$

test indicates that the increase in the number of plants with leaves closed which bear nodules when supplied with Extract B is statistically significant only at the 10 per cent. level, but the increase when Extract C was added is sifnificant at the 2 per cent. level. Probably, therefore, Thornton's stimulatory extract was prepared in the manner here described for Extract C, plants as well as sand being packed into the extraction tube. No doubt removal of the plants involves a considerable loss of the active excreted substance since much of it would be coating the root surfaces and contained in the sand which adhered to them.

The presence of Extract A, i.e., washings from the roots of plants with leaves still closed had no statistically significant effect either upon the nodulation of other plants of the same age, or upon the nodulation of older plants whose first true leaves had opened. Similarly the effects of Extract B and Extract C upon plants which had their own leaves expanded were not statistically significant.

We may conclude therefore, that washings from the roots of plants with expanded first true leaves, obtained in the manner of the present Extract C, stimulate the nodulation of younger plants whose leaves are still closed. This is in agreement with the results of Thornton, although the differences noted here as being due to the root excretion are not so striking as those which he recorded.

The experiment was repeated using peas, extracts being made from the sand in which plants with leaves closed (Extract A) and plants with one or two leaves open (Extract B) had been growing. (In both cases the plants were removed before the extraction). The extracts were then autoclaved and, as before, were used for making up the inoculum added to pots of sand containing germinating peas. After 17 days, 15 plants at each treatment were examined for nodules.

Table 24.

Effect of root washings upon the nodulation of young plants of pea. Data are averages for 15 plants per treatment. Growth period = 17 days.

| | Av.no of nods./plant | Range. |
|---|---|---|
| Control | 12.6 | 0(1p) - 34 |
| Plants treated with Extract A | 18.6 | 0(1p) - 65 |
| Plants treated with Extract B | 23.3 | 0(2p) - 51 |

When the *t* test is applied to these results it is found that the increase in nodule numbers on plants supplied with Extract A is not statistically significant whereas the increase noted on plants treated with Extract B is significant at the 5 per cent. level. Presumably there would have been a higher level of significance to the result if the plants had not been removed from the sand prior to making the extract. It appears therefore, that, as with lucerne, a substance which stimulates nodule formation is excreted from the roots of pea plants when their first leaves are opening.

It should be interesting to test the specificity of these two excretions, since Ludwig and Allison (1935) have found that nodule formation on young plants of Soya beans and lucerne is stimulated as much by the presence of corn and wheat plants as by the presence of legumes of the same species.

Experiment 31. Effect on nodule formation on whole pea plants of various concentrations of nitrate, with incidental observations on the effect of exposure of the roots to light.

In view of the results of previous investigators (p.36 ), it seemed necessary to determine whether the nitrate concentrations employed with excised roots of pea have any inhibiting effect upon the nodulation of normal pea plants growing under conditions of water culture. At the time when these trials were being planned, consideration was being given to the preference of some investigators (Robbins and Maneval, 1924; White 1932b) for excised root culture being conducted in light. It was therefore decided to test the effect upon nodulation of exposure of the root systems of whole pea plants to light. It may be noted that the widely held belief that exposure to light has injurious effects upon nodule forming bacteria has been substantiated in the past by the experimental results of only one investigator (Nobles, 1919). Other workers (Otis, 1898; Simon, 1907; Burrill and Hansen, 1917; Albrecht, 1921; Erdman and Wilkins, 1928; Albrecht and Turk, 1930) all agree that 'the bacteria in soils are singularly resistant to light'. Prucha (1915) observed only a slight decrease in nodule numbers on roots which were directly exposed to light. It was decided therefore, to combine an observation on light effect with the above-mentioned investigation into the effect of nitrate.

Nitrogen-free solution was prepared, and also solution containing the full concentration of nitrate advocated by Bonner and Devirian (327 mg. per litre), as well as solutions with one quarter, one eighth and one sixteenth of this concentration. Erlenmeyer flasks of 500 cc. capacity were used as culture vessels and each contained 400 cc. of medium. There were six flasks per treatment. The usual 4 per cent. sucrose was omitted from the medium and the technique employed was not aseptic.

Surface-sterilised seeds were planted in a trough of sterile sand watered with sterile nitrogen-free solution. When the young plants were from ten to twelve days' old, they were each transferred to a flask of nutrient, the plants being supported in cotton wool plugs in such a way that the root system was almost completely submerged. The remains of the testa and cotyledons were kept above the solution level. All flasks were inoculated with 1 cc. of a suspension of nodule bacteria. Four flasks at each treatment were darkened by wrapping with thick brown paper and placed in boxes of sawdust to prevent light from entering through their unwrapped bases. In the remaining flasks the roots were left exposed to the light. The solution was maintained at a constant level by the addition of distilled water, and nodule counts were made after a three-week growth period.

Table 25.

Effect on nodule formation on whole pea plants of various concentrations of nitrate, with incidental observations on the effect of exposure of the roots to light. Growth period = 3 weeks.

| Concn. of $NO_3$ mg./litre | Average number of nodules per plant: Darkened flasks | Average number of nodules per plant: Undarkened flasks |
|---|---|---|
| 0 | 22 | 0 |
| 20.43 | 14 | 0 |
| 40.87 | 75 | 0.5 |
| 81.75 | 101 | 0 |
| 327.0 | over 100 | - |

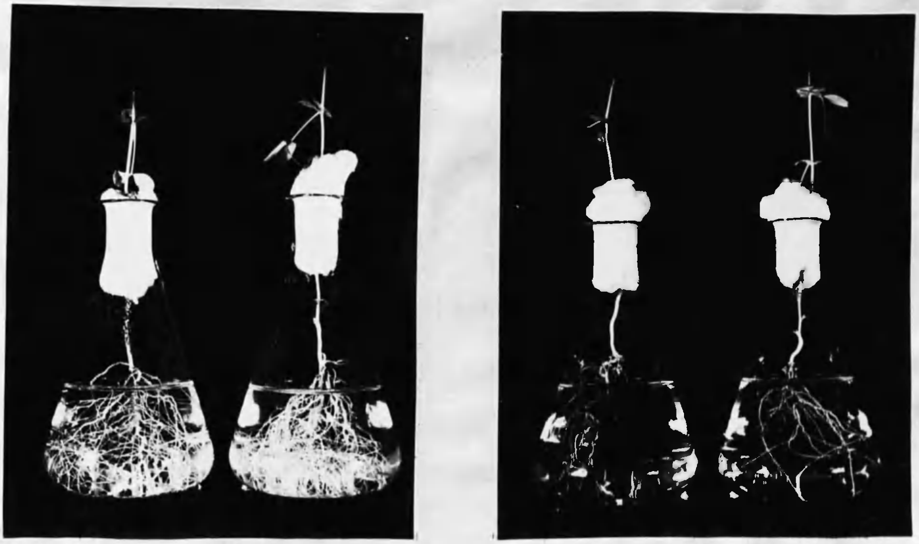
Table 25 shows that nodules developed on darkened roots at all levels of nitrate. As nitrate increased nodules became more numerous but were of much reduced size. In the presence of nitrate the nodules were round and white and widely scattered over the entire submerged root system. In only one case were pink nodules formed.

Fig. 18. Roots of inoculated pea plants growing in mineral solution with $\frac{1}{4}$ normal nitrate. Darkened roots on left show numerous nodules, undarkened ones on right have no nodules.

Two of them were found on uppermost roots near the cotyledons which were at times above the level of the solution. In the nitrogen-free medium the nodules were pink, elongated in shape and clustered near the cotyledons on adventitious roots, or on laterals near to the tap root.

Therefore, nodules can be formed in culture solution containing concentrations of nitrate up to, and including, the full value given in the formula of Bonner and Devirian, but they differ in colour, shape, size and distribution from those formed in nitrogen-free solution (Fig. 19A and Fig. 19B). The results with the undarkened flasks suggest that the incidence of light on the root systems inhibits nodule formation. Typical darkened and undarkened roots are shown in Fig. 18.

As an additional test of the effect of light, one sterile germinated pea was grown in each of eighteen large test tubes, nine containing 50 cc. nitrogen-free solution solidified with 1 per cent. agar, and nine containing 80 gm. sterile fine quartz sand watered with enough of the nitrogen-free solution to give 15 per cent. moisture. The agar medium was inoculated while molten, and the peas to be grown in sand were dipped in a suspension of nodule organisms before planting. All the tubes were wrapped with black paper below the sand or agar level so that in the tubes with sand, light was completely excluded from the root systems, but in the remainder, it could enter through the top surface of the agar and penetrate for some



A.

B.

Fig. 19. Roots of inoculated pea plants growing in water culture, A. with full concentration of nitrogen. Numerous small round nodules are scattered over the entire root system; B. in nitrogen-free solution. The nodules are larger, more elongated, and clustered round the seed or very near to the main root.

distance downwards. After 6 weeks of growth the following results were obtained:-

| Treatment | Av. no. of nods. per plant | Distribution |
|---|---|---|
| Sand | 13 | Over entire root system |
| Agar | 5 | At base of tube. |

Of course it is possible that other experimental conditions, quite apart from the absence of light, may favour the production of nodules in sand culture. However, in view of the results of the preceding experiment, it may be justifiable to assume that the reduction in nodule numbers, and change in their distribution, shown in the Table, is at least partly due to the light which penetrated the agar surface. This may explain the poor nodulation frequently observed by the author in experiments involving the growth of inoculated peas in an agar medium in the greenhouse. With clover plants, on the other hand, no difficulty has been encountered in securing numerous nodules on plants growing under summer greenhouse conditions on an agar medium, and with roots only partially shaded by the rack in which the tubes were placed.

## DISCUSSION SECTION II.

### 1. Possibility of securing nodule formation on etiolated plants.

It has been clearly shown that pea plants grown from seed in complete darkness on medium free from carbohydrate can produce fairly numerous nodules, although their numbers are much below those for plants grown in light under otherwise comparable conditions. The size of the nodules is also much less than that of light-grown plants. Etiolated clover, on the other hand, formed no nodules without the addition of carbohydrate to the medium, and only 4 nodules appeared on one out of six etiolated plants of black wax bean to which no sucrose was added. As we have seen, the results of the majority of previous investigators indicate that growth in darkness markedly reduces nodule production. The author's results, therefore, serve to confirm the findings of these workers with regard to the depressing effect of etiolation, but the nodule numbers recorded here for pea greatly exceed those previously noted for plants grown in darkness, with the possible exception of the findings of Prazmowski (see page 99.). Out of 72 etiolated pea plants grown in the present experiments in agar medium without sucrose, 62 formed nodules, the maximum number per plant being 32, and the over-all average 7.62 per plant. Thornton (1930) found that only 3.5 nodules were formed on 20 lucerne plants kept in continuous darkness, in comparison with 46.5 on the 20 control plants. J.K. Wilson (1931) noted only one nodule on 40 etiolated vetch plants to which no carbohydrate had been added. When peas and clover were used instead of vetch, no nodules at all were mentioned for plants without carbohydrate. Lewis and McCoy (1933), using etiolated black wax beans, observed no nodules on 60 control plants to which no sucrose was added. The relatively large number of nodules observed in the author's experiments with peas may have been due to the particular plant employed, but at any rate it is evident that under the given conditions, considerable numbers of nodules can be formed on peas grown in continuous darkness on mineral-salt agar. It would appear therefore,,that adequate amounts of such substances as are essential for nodule production (additional to those provided by the rooting medium) must be derived from the cotyledonary reserves. The reserves of these substances in small-seeded legumes may not be sufficient to permit of the nodulation of etiolated plants, and this might explain the results obtained by J.K. Wilson with vetch and clover, Thornton with lucerne, and the author with clover. However, this explanation does not hold good for the failure to secure good nodule formation on dark-grown plants of black wax bean, since here the quantity of material stored in the seed is as great, if not greater, than in peas. Therefore, in all the cases mentioned, we must consider the possibility that, apart altogether from the seed reserves, the experimental conditions may be the main factors in determining the occurence and extent of nodulation.

## 2. Effect of sucrose on nodule formation by etiolated and light-grown plants.

In passing to consider the experiments involving the addition of sucrose to the rooting medium, it is necessary to specify that 'addition' implies that analytically pure sucrose was originally added to the medium before autoclaving. After autoclaving, however, the presence of reducing sugars was detected, indicating that hydrolysis of some of the sucrose had occurred in the autoclave. The osmotic pressure of the autoclaved culture solution also led to the same conclusion since its value was 4.18 atmospheres, while the sucrose (if remaining as sucrose) and the mineral salts would account for approximately 3 atmospheres only. In any event it seems probable that the roots themselves would effect a similar conversion, since Burström (1941) observed marked inversion of sucrose in media in which roots of various species of plants had been growing aseptically.

Of the 72 etiolated pea plants supplied with 4 per cent. sucrose, only 35 developed nodules, the maximum number per plant being 10, and the over-all average 1.95 per plant. It is clear therefore that sucrose at a concentration of 4 per cent. has a very marked depressing effect upon the number of nodules which form on etiolated peas, although the size of the nodules developing in the presence of sucrose tends, as noted, to be greater. Table 17 shows that concentrations of sucrose as low as 0.5 per cent. cause a marked decrease in nodule numbers on etiolated peas. The average number of nodules per plant is reduced from 13.33 to 6.73 when 0.5 per cent. sucrose is added, and with 4 per cent. sucrose there were only 0.27 nodules per plant. The number of nodules which formed on etiolated plants of black wax bean was very small in every case, but the addition of 0.5 per cent. sucrose produced a decrease in nodule numbers per plant from 0.66 to 0.33 and plants supplied with 1.0 per cent. sucrose possessed no nodules at all. J.K. Wilson (1931) and Lewis and McCoy (1933) found that the presence of sugars at concentrations lower than 4 per cent. <u>increased</u> the number of nodules forming on etiolated plants, although the responses which they obtained were irregular and confined to a minority of the plants. In the present author's experiments the only evidence of sucrose producing a beneficial effect upon nodulation is provided in the test with etiolated clover where nodules only formed on plants which were supplied with sucrose at the 3 per cent. level (one nodule on each of three plants).

Tables 18 and 19 provide evidence that the addition of 4 per cent. sucrose to pea plants grown in natural light results in a marked reduction in number of nodules, as in the case of etiolated plants. The lower concentrations of sucrose also reduced nodule numbers, although as noted, in these tubes all the sugar had disappeared from the medium by the end of the experiment. The data given in Table 19 show that, although nodule numbers were decreasing, the dry weight of nodules per plant was greater in the presence of sucrose and from these results the following figures may be calculated for the average dry weight of individual nodules:-

| Concentration of sucrose (per cent.) | 0 | 0.5 | 1.0 | 2.0 | 4.0 |
|---|---|---|---|---|---|
| Dry weight per nodule (mg.) | 0.068 | 0.165 | 0.206 | 0.324 | 0.44 |

Thus it is clear that increase in dry weight of nodules accompanied increase in concentration of added sucrose.

As already noted in Section I, there seem to be no references in the literature to truly aseptic experiments involving the addition of sucrose to illuminated legumes in order to study its effect upon nodule production. It is difficult therefore, to make a strict comparison between earlier experiments and the present series of tests because under non-sterile conditions the sucrose originally present would probably undergo more rapid decomposition than in the author's experiments. Ritter (1911) reported that the addition of sucrose was without effect on the nodulation of lupins, and Prucha (1915) concluded that at 2 gm. or less per 300 gm. soil it had no effect on nodule formation on Canada Field Pea. Four, eight and sixteen gm. per 300 gm. soil progressively reduced plant growth, and no nodules formed in the two highest concentrations. Since the conditions were not sterile, it was thought that the decreased growth with increase of sucrose might possibly be due to fermentation reactions taking place in the soil. J.K. Wilson (1917) found that the addition of 2.7 per cent. sucrose to soil cultures of Soya beans considerably increased the number and size of the nodules formed. Ludwig and Allison (1935) noted that the presence of sucrose in the solution supplied to Soya beans in sand culture during the summer months, had a favourable effect on nodule numbers at the beginning of the experiment, but at the end the controls had more nodules than the plants supplied with sucrose. Their final result is therefore in agreement with the findings of the author, but their earlier observations are not, since in the present experiments it was noted that the largest numbers of nodules appeared from the very first on plants to which no sucrose had been added. P.W. Wilson (1940), although he did not record nodule numbers, observed that the size of nodules on red clover plants was greater when carbohydrate was present in the rooting medium.

The significance to root cultures of the above results is obvious and has been commented on in the previous Section.

It now has to be considered why the addition of sucrose to the rooting medium led, in the author's experiments, to a reduction in the number of nodules both on plants grown in darkness and in light. The effect of the sucrose can be envisaged as being exerted externally in the rooting medium, or internally after the entrance of the sugar into the tissues of the plant. In the former case a reduction in the number of penetrations of root hairs by the bacteria would presumably be entailed, while an internal effect might involve a restriction in the proportion of such penetrations that result in the formation of nodules.

It ought to be possible to determine by direct examination which of these alternatives actually obtains (McCoy, 1932), and it is hoped to arrange for this in future experiments. Meanwhile, a few relevant observations may be offered. So far as a limitation of the number of penetrations of root hairs is concerned, this might be occasioned through a reduction in the rate of multiplication of bacteria in the presence of relatively large amounts of sucrose in the medium. That this is not the case has been shown by examination of the growth of the pea nodule organism in pure culture on the usual medium, but with sucrose in concentrations ranging from 0.5 to 4 per cent. substituted for the 1 per cent. mannitol usually supplied. As we have seen, the bacterial colonies grew at a normal rate and showed no signs of inhibition. Pea plants inoculated from 4 per cent. sucrose slopes showed similar nodulation to those inoculated from slopes of normal medium, so that there is no evidence of any reduction in the infective powers of the organism after cultivation on medium with the above amounts of sucrose.

The external effect might alternatively arise through some reduction in the stimulus to enter root hairs. There is some evidence that nodule bacteria present in the soil are specifically attracted to the roots of leguminous plants, where they multiply rapidly in the so-called 'rhizosphere' (P.W. Wilson, 1940). This multiplication, and the eventual invasion of the plant, as already noted, are attributed by Thornton (1929) to the effect of a water-soluble substance excreted by the roots at a time co-incident with the opening of the first true leaves. In the present experiments, since the nutrient medium containing sucrose provided a more suitable substrate for bacterial growth than did the sucrose-free medium, it is conceivable that under these conditions the stimulus which normally brings about the entry of the bacteria into the roots was reduced in intensity. This conception may also explain the failure of nodulation on excised pea roots, which, as we now know, must be cultured in medium containing 4 per cent. sucrose.

The need to bear in mind that the sucrose effect may be exerted internally is shown by the results of previous workers, who observed that the nodulation of legumes can be affected by treatment which increases the relative carbohydrate content of the plant without involving the actual addition of carbohydrate to the rooting medium. P.W. Wilson and Wagner (1935) supplied clover plants with carbon dioxide until they gave evidence of a marked excess of carbohydrate, and then inoculated them with nodule organisms. The total number of nodules formed on the plants amounted to 37 and 167 in different experiments. The corresponding totals for plants in which a lower carbohydrate/nitrogen ratio was induced by supplying ammonium nitrate, were 128 and 275. In the present tests, evidence of a high carbohydrate content in the pea plants grown in light and supplied with sucrose was provided by the development of red pigment in their leaves, the colour being most obvious in plants to which 4 per cent. sucrose was added. Etiolated plants showed no sign of red pigment production. That such colouration is associated with abundant supply of carbohydrate has been noted by Molliard (1907), Knudson (1917), and other workers. Meyer and Anderson (1939) state that "the presence of a relatively high concentration of simple sugars in the cells seems to be universally correlated with anthocyanin formation". Thus it might be that in the illuminated plants, the concentration of carbohydrate (derived from the cotyledons and from photosynthesis) was already at such a level that the addition of even 0.5 per cent. sucrose rendered it excessively high and thereby caused a reduction in nodule numbers. It is difficult to imagine, however, that an excessive level of carbohydrate was the reason for the reduced numbers of nodules on completely etiolated plants. No doubt the possibility does exist in the case of those supplied with sucrose at the 4 per cent. level, since, as we have seen, the cotyledons already possess sufficient carbohydrate to permit of some appreciable nodule formation. However, it seems improbable that the sucrose absorbed from medium containing only a 0.5 per cent. concentration could be sufficient to create an unfavourably high carbohydrate/nitrogen ratio in plants not carrying out photosynthesis. This appears to support the already discussed view that the sucrose effect may be an external one.

The decided increase in the dry weights of both light and dark plants indicates that they are capable of absorbing and utilising the added carbohydrate. This observation is in accord with the results of the aseptic experiments of Molliard (1907) and Georgi, Orcutt and P.W. Wilson (1933) and the non-sterile experiments of Golding (1899, 1900), Knudson (1917) and Knudson and Lindström (1919). They all noted that the addition of sucrose to the rooting medium caused an increase in the dry weights of various plants which were grown in light.

### 3. Effect of nitrate on nodule formation by etiolated and light-grown plants.

The results presented show that the addition of nitrate to the rooting medium had a depressing effect on the nodule numbers of etiolated plants. When 4 per cent. sucrose and nitrate were present together they exerted a cumulative depressing effect. This result differs from that obtained (with light-grown plants) by other investigators, who have found that when legumes are growing in the presence of combined nitrogen, the addition of carbohydrate stimulates nodule formation, and similarly when carbohydrate is excessive, addition of combined nitrogen proves beneficial. (Hopkins and Fred, 1933; Allison and Ludwig, 1934; P.W. Wilson and Wagner, 1935; Thornton, 1936).

Present experiments have also shown that concentrations of nitrate up to, and including, the full value given in the formula of Bonner and Devirian (1939) cause an increase in the number of nodules which form on whole plants of pea grown in the light under conditions of water culture. This is somewhat surprising since no record of any similar increase due to combined nitrogen can be found in the literature. The observed reduction in nodule size is in agreement with the already-mentioned findings of Giöbel (1926), Thornton and Nicol (1936) and Hopkins, P.W. Wilson and Peterson (1932), who also noticed a change in nodule distribution like that described by the author. As we have seen, changes in the colour and shape of the nodules also occurred in the present tests.

Therefore, it would appear that although the full concentration of nitrate present in the usual medium increases the number of nodules formed on whole pea plants grown in light, it has a depressing effect upon the number of nodules which appear on etiolated peas. It is difficult to reconcile these two findings, and as far as excised roots are concerned, we can only assume that since they are more closely akin to etiolated than to light-grown plants, the nitrate in their case too, may be exerting a depressing effect. Again, if the analogy to etiolated plants holds good, the presence of the sucrose, along with nitrate, in the medium may prove even more injurious to nodule formation since we have noted that these two substances exert a cumulative depressing effect upon the nodulation of peas grown in darkness.

### 4. Effect of the accessory growth substances, aneurin and nicotinic acid on the growth and nodulation of etiolated plants.

The presence of aneurin and nicotinic acid had no significant effect upon the nodulation of etiolated pea plants, or upon their growth. As we have seen, aneurin is a necessary constituent of the medium which allows good growth of excised pea roots and therefore it seems possible that the roots of intact plants may normally receive their supply from the cotyledons or some plant of the shoot system. Bonner and Greene (1938), as noted previously, concluded that aneurin is produced in green leaves in light, and therefore it was thought that darkened plants might show improved growth when it was added to the rooting medium. The fact that no improvement was observed seems to indicate that adequate amounts for the development of the etiolated plant must be contained as reserves within the seed. Similarly, since we have seen that aneurin is required for the initiation of bacterial growth (West and P.W. Wilson, 1938) and since no increase in nodule numbers follows its addition to the medium, it appears that quantities which promote the growth which results in best nodulation must be obtained from the plant, perhaps as an excretion from the roots (West, 1939). Alternatively, sufficient aneurin may be added in the inoculum, made up as described, from slopes of agar containing yeast extract.

### 5. Effect of root excretions.

Repetition of the 'root washing' experiment carried out by Thornton (1929) with lucerne has confirmed his finding that a substance which favours the formation of nodules on very young plants is excreted from the roots when the first true leaves are opening. Moreover, as the result of a similar experiment with peas , we have seen that such a substance is also excreted by the roots of pea plants, when their first leaves are unfolding. Thornton has shown that the excretion from lucerne roots increased the growth of the nodule organism on agar medium and if the same be true for the substance excreted from pea roots, then this may be the explanation of the milky bacterial zone observed in the author's experiments with etiolated pea plants.

West (1939) found that young roots of flax and tobacco excrete significant amounts of aneurin and biotin and such excretion may be a common feature among higher plants. If so, then this additional amount of aneurin over and above that often included in the medium of the present experiment, may account for the increased bacterial growth around the roots. We can only conclude that whether the stimulating agent be the aneurin of West, or the unnamed water-soluble compound of Thornton, it must either be present in the cotyledonary reserves or else it can be synthesised by etiolated pea plants without the addition of carbohydrate to the medium. Alternatively, the rapid multiplication of the bacteria in the zone immediately surrounding the roots may be due merely to the accumulation in this region of extra organic matter derived from sloughed-off epidermal cells and root hairs, as suggested by Ludwig and Allison (1935).

The possible significance of root excretions in the nodulation of excised roots has been mentioned already on p.92.

6. Haemoglobin.

As has been mentioned, nodules which formed on etiolated peas both with and without the presence of sucrose in the rooting medium, exhibited a definite pink colour. Spectroscopic examination established that this is due to the presence of the haemoglobin found in the nodules of plants grown under normal conditions (Keilin and Wang, 1945; Virtanen, 1945). Haemoglobin was also present in some of the nodules formed on etiolated black wax beans. The presence of the pigment in nodules on etiolated plants is of some interest, since it is a matter of common observation that when leguminous plants which have been growing under normal conditions are transferred to darkness, within a few days the red colour of the nodule tissues is replaced by brown or green colourations. It is now generally accepted that haemoglobin probably has some function in the mechanism of nitrogen-fixation and therefore it is planned to investigate whether the pink nodules of etiolated plants are capable of carrying out this process.

## SUMMARY SECTION II.

1. Considerable numbers of nodules developed on completely etiolated plants of pea grown aseptically on a rooting medium of agar with nutrient salts. The nodules observed on such plants grown continuously in complete darkness were, however, much inferior in number and size to those formed on plants grown in light under otherwise comparable conditions. Nevertheless, the extent of nodulation was considerably superior to that reported by the majority of previous investigators for various leguminous plants including pea. It thus appears that the etiolated pea can supply the materials required for the formation of a limited number of nodules.

2. A small number of nodules formed on etiolated plants of black wax bean grown on mineral-salt agar, but no nodules were observed on etiolated red clover plants cultured on a similar medium.

3. The presence of sucrose in the rooting medium decreased the number of nodules formed on pea plants grown in continuous darkness, and also on those grown under normal light conditions, but the size and dry weight of nodules were increased; the dry weight of the plants themselves was also increased. The sucrose in some way restricted the invasion of the plant by the nodule bacteria

or reduced the number of nodules initiated, but its presence proved beneficial to the development of such nodules as were initiated.

4. With etiolated black wax beans, the depressing effect of sucrose on nodule numbers and beneficial effect on plant dry weight and nodule size were again evident. Three nodules were observed on etiolated plants of red clover grown on medium to which 3 per cent. sucrose had been added.

5. The presence of combined nitrogen in the rooting medium decreased the number of nodules formed on etiolated pea plants. When sucrose and combined nitrogen were present together, there was still further reduction in nodule numbers.

6. Nitrate concentrations ranging from 20.43 to 327.0 mg. per litre, increased the number of nodules formed on whole pea plants growing in light under conditions of water culture, but reduced their size, and affected their colour, shape and distribution.

7. The addition of aneurin and nicotinic acid to the rooting medium had no significant effect upon the growth or nodulation of etiolated peas.

8. A dense zone of bacterial growth surrounded the main root and rootlets of etiolated pea plants growing

in medium with and without sucrose. The increased bacterial multiplication may have been due to the excretion of some stimulating substance by the roots.

9. The conclusion of a previous investigator that a substance which stimulates nodule formation, and increases the growth of the nodule organism on agar, is excreted from the roots of lucerne plants at a time co-incidental with the opening of their first true leaves, is supported by experiments reported in the thesis. There is also some evidence that with normal whole pea plants a substance which favours the formation of nodules on young plants is excreted from the roots when the first leaves are opening.

10. Nodules formed on etiolated pea plants grown on media with and without sucrose, had the normal pink colour due to the presence of haemoglobin. Apparently a limited formation of this pigment is possible in plants grown in the absence of light.

11. The incidence of light upon the root systems of inoculated whole plants of pea has a marked depressing effect upon nodulation.

12. The bearing of the above results on attempts to secure the nodulation of excised roots is indicated.

## REFERENCES TO LITERATURE.

ACTON, E.H. 1889. The assimilation of carbon by green plants from certain organic compounds. Proc. Roy. Soc. 47: 150 - 175.

ADDICOTT, F.T. 1939. Vitamin $B_1$ in relation to meristematic activity of isolated pea roots. Bot. Gaz. 100: 836 - 843.

______________ 1941. Effects of root growth hormones on the meristem of excised pea roots. Bot. Gaz. 102: 576 - 581.

______________ and BONNER, J. 1938. Nicotinic acid and the growth of isolated pea roots, Science 88: 577 - 578.

______________ and DEVIRIAN, P.S. 1939. A second growth factor for excised pea roots: nicotinic acid. Am. Jour. Bot. 26: 667 - 671.

ALBRECHT, W.A. 1921. Studies on the longevity of B. radicicola in the soil. Missouri Agr. Expt. Sta., Bul. 189: 54 - 56.

______________ 1922. Viable legume bacteria in sun-dried soil. Jour. Am. Soc. Agron. 14: 49 - 51.

______________ and TURK, L.M. 1930. Legume bacteria with reference to light and longevity. Missouri Agr. Expt. Sta., Research Bul. 132: 19 pp.

ALLISON, F.E. 1935. Carbohydrate supply as a primary factor in legume symbiosis. Soil. Sci. 39: 123 - 143.

______________, and LUDWIG, C.A. 1934. The cause of decreased nodule formation on legumes supplied with abundant combined nitrogen. Soil Sci. 37: 431 - 443.

AVERY, G.S., BURKHOLDER, P.R., and CREIGHTON, H.B. 1937. Production and distribution of growth hormone in shoots of Aesculus malus, and its probable role in stimulating cambial activity. Am. Jour. Bot. 24: 51 - 58.

BOEHM, J. 1883. Uber starkebildung aus zucker. Bot. ztg. 41: 33 - 38, 49 - 54.

BONNER, J. 1937. Vitamin $B_1$, a growth factor for higher plants. Science 85: 183 - 184.

_____________ 1938. Thiamin (vitamin $B_1$ ) and the growth of roots: the relation of chemical structure to physiological activity. Am. Jour. Bot. 25: 543 - 549.

_____________ 1940a. On the growth factor requirements of isolated roots. Am. Jour. Bot. 27: 692 - 701.

_____________ 1940b. Specificity of nicotinic acid as a growth factor for isolated pea roots. Plant Physiol. 15: 553 - 557.

_____________ 1942a. Culture of isolated roots of Acacia melanoxylon. Bull. Torrey Bot. Club 69: 130 - 133.

_____________ 1942b. A reversible growth inhibition of isolated tomato roots. Proc. Nat. Acad. Sci. 28: 321 - 328.

_____________ 1942c. Riboflavin in isolated roots. Bot. Gaz. 103: 581 - 585.

_____________ 1943. Further experiments on the nutrition of isolated tomato roots. Bull. Torrey Bot. Club 70: 184 - 189.

______________, and ADDICOTT, F.T. 1937. Cultivation in vitro of excised pea roots. Bot. Gaz. 99: 144 - 170.

______________, and BUCHMAN, E.R. 1938. Syntheses carried out in vitro by isolated pea roots. I. Proc. Nat. Acad. Sci., Wash. 27: 431 - 438.

______________, and DEVIRIAN, P.S. 1939. Growth factor requirements of four species of isolated roots. Am. Jour. Bot. 26: 661 - 665.

______________, and GREENE, J. 1938. Vitamin $B_1$ and the growth of green plants. Bot. Gaz. 100: 226 - 237.

______________, and KOEPFLI, J.B. 1939. The inhibition of root growth by auxins. Am. Jour. Bot. 26: 557 - 566.

BOYSEN - JENSEN, P. 1933. Über der Nachweis von Wuchsstoff in Wurzeln. Planta 19: 345 - 350.

BURRILL, T.J., and HANSEN, R. 1917. Is symbiosis possible between legume bacteria and non-legume plants? Ill. Agr. Expt. Sta., Bul. 202: 115 - 181.

BURSTRÖM, H. 1941. Studies on the carbohydrate nutrition of roots. Lantbrukshögsk., Ann. 9: 264 - 284.

CHAMBERS, W.H. 1923. Cultures of plant cells. Proc. Soc. Expt. Biol. and Med. 21: 71 - 72.

CHEN, H.K., and THORNTON, H.G. 1940. The structure of 'ineffective' nodules and its influence on nitrogen fixation. Proc. Roy. Soc., B, 129: 208 - 209.

DAY, D. 1941. Vitamin $B_6$ and growth of excised tomato roots in agar culture. Science 94: 468 - 469.

_______________ 1943. Growth of excised tomato roots in agar with thiamine plus pyridoxine, nicotinamide or glycine. Am. Jour. Bot. 30: 150 - 156.

DAWSON, R.F. 1942. Nicotine synthesis in excised tobacco roots. Am. Jour. Bot. 29: 813 - 815.

ERDMAN, L.W., and WILKINS, F.S. 1928. Soybean inoculation studies. Iowa Agr. Expt. Sta., Res. Bul. 114: 1 - 56.

FIELDLER, H. 1936. Entwicklungs - und reizphysiologische Untersuchungen an Kulturen isolierten Wurzelspitzen. Ztschr. Bot. 30: 385 - 436.

FRED, E.B., BALDWIN, I.L., and McCOY, E. 1932. Root nodule bacteria and leguminous plants. Madison, Wisconsin.

GALLIGAR, G.C. 1934. Growth studies on excised root tips. Diss., Univ. Illinois.

_______________ 1939. Growth behaviour of one-millimeter excised root tips. Plant Physiol. 14: 163 - 169.

GEORGI, C.E., ORCUTT, F.S., and WILSON, P.W. 1933. Further studies on the relation between the carbon assimilation and nitrogen fixation in leguminous plants. Soil Sci. 36: 375 - 382.

GIÖBEL, G. 1926. The relation of soil nitrogen to nodule development and fixation of nitrogen by certain legumes. N.J. Expt. Sta., Bul. 436: 125 pp.

GOLDING, J. 1899. Sugar as an agent in nitrogen fixation and an aid to the growth of plants. Jour. Soc. Chem. Ind.

18: 564 - 566.

______________ 1900. Sugar as an aid to the growth of plants. Jour Soc. Chem. Ind. 19: 324 - 325.

HABERLANDT, G. 1902. Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien. math. - naturw. - kl. 111: 69 - 92.

HARRISON, R.G. 1907. Observations on the living developing nerve fiber. Proc. Soc. Expt. Biol. and Med. 4: 140 - 143.

HART , C.E. 1943. The synthesis of sucrose in the sugar cane plant. II. The effects of several inorganic and organic compounds upon the interconversion of glucose and fructose and the formation of sucrose in detached organs of the sugar cane plant. Hawaiian Planters Rec. 47: 155 - 170.

HELLRIEGEL, H., and WILFARTH, H. 1888. Untersuchungen über die Stickstoffnahrung der Gramineen und Leguminosen. Beilageheft zu der Ztschr. Ver. Rübenzucker - Industrie Deutschen Reichs. 234 pp.

HENDERSON, J.,and STAUFFER, J.F. 1944. The influence of some respiratory inhibitors and intermediates on growth and respiration of excised tomato roots. Am. Jour. Bot. 31: 528 - 535.

HILTNER, L. 1900. Über die Ursachen welche die Grösse, Zahl, Stellung und Wirkung der Wurzelknollchen der Leguminosen bedingen. Arb. k. Gesundh., Biol. Abt. 1: 177 - 222.

HOPKINS, E.W., and FRED, E.B. 1933. Influence of various nitrogenous compounds and mannitol on nodule formation by clover. Plant Physiol. 8: 141 - 155.

______________, WILSON, P.W. and PETERSON, W.H. 1932. Influence of potassium nitrate on nodule formation and nitrogen fixation by clover. Plant Physiol. 7: 597 - 611.

JACOBSON, H.G.M. 1925. Changes in hydrogen-ion concentration in nutrient solutions. I, In a culture with wheat. II, In cultures with rice, Jour. Am. Soc. Agron. 17: 577 - 583; 583 - 586.

KEILIN, D., and WANG, Y.L. 1945. Haemoglobin in the root nodules of leguminous plants. Nature 155: 227 - 229.

KNUDSON, L. 1917. Influence of certain carbohydrates on green plants. Cornell Univ. Agr. Expt. Sta., Memoir 9: 747 - 809.

______________ 1919. Viability of detached root cap cells. Am. Jour. Bot. 6: 309 - 310.

______________, and LINDSTRÖM, E.W. 1919. Influence of sugars on the growth of albino plants. Am. Jour. Bot. 6: 401 - 406.

KOTTE, W. 1922a. Wurzelmeristem in Gewebekultur. Ber. d. bot. Ges. 40: 269 - 272.

______________ 1922b. Kulturversuche mit isolierten Wurzelspitzen. Beitr. allg. Bot. 2: 413 - 434.

LAURENT, É. 1887. Recherches experimentales sur la formation d'amidon dans les plantes aux dépens de solutions organiques. Soc. Roy. Bot. Belgique., Bul. 26: 243 - 270.

_____________ 1891. Recherches sur les nodosités radicales des légumineuses. Ann. Inst. Pasteur 5: 105 - 139.

LAURENT, J. 1904. Recherches sur la nutrition carbonée des plantes vertes à l'aide de matières organiques. Rev. gén. bot. 16: 14 - 48, 68 - 80, 96 - 119, 120 - 128, 155 - 166, 188 - 202, 231 - 241.

LAWES, J.B., and GILBERT, J.H. 1891. The sources of the nitrogen in our leguminous crops. Jour. Roy. Agr. Soc. 2: 657 - 702.

LEONARD, L.T. 1926. A preliminary note on the relation of photosynthetic carbohydrate to nodule formation on soybeans. Jour. Am. Soc. Agron. 18: 1012 - 1016.

LEWIS, K.H., and McCOY, E. 1933. Root nodule formation on the garden bean, studies by a technique of tissue culture. Bot. Gaz. 95: 316 - 329.

LEWIS, L.L., and NICHOLSON, J.F. 1905. Soil inoculation. Tubercle-forming bacteria of the legumes. Okla. Agr.Expt. Sta., Bul. 68: 1 - 30.

LUDWIG, C.A., and ALLISON, F.E. 1935. Some factors affecting nodule formation on seedlings of leguminous plants. Jour. Am. Soc. Agron. 27: 895 - 902.

MACKIE, T.J., and McCARTNEY, J.E. 1946. Handbook of Practical Bacteriology. Edinburgh.

MALYSCHEV, N. 1932a. Das Wachstum des isolierten Wurzelmeristems auf sterilen Nährböden. Biol. Ztrbl. 52: 257 - 265.

______________ 1932b. The growth of isolated meristems of roots. Preslia 11: 59 - 61.

MAYER, G.G. 1929. Der Einfluss verschiedener Nährstoffzuführung auf das Längenwachstum isolierter Wurzeln. Diss., Giessen.

MAZÉ, M. 1898. Les microbes des nodosités des légumineuses. Ann. Inst. Pasteur. 12: 128 - 155.

McCLAY, J.E. 1940. Synthesis of thiamin by excised roots of maize. Proc. Nat. Acad. Sci.26: 581 - 587.

McCOY, E. 1932. Infection by Bact. Radicicola in relation to the microbiology of the host's cell walls. Proc. Roy. Soc., B. 110: 514 - 533.

Mc GONAGLE, M.P. 1944. Cultures of excised leguminous roots. Nature 153: 158.

MEYER, A. 1886. Bildung der stärkekörner in den laubblättern aus zukerarten, mannit und glycerin. Bot. Ztg. 44: 81 - 88, 105 - 113, 129 - 137, 145 - 151.

MEYER, B.S., and ANDERSON, D.B. 1939. Plant Physiology. New York.

MOLLIARD, M. 1907. Action morphogénique de substances organiques sur les végétaux supérieurs. Rev. Gén. Bot. 19: 241 - 291, 329 - 349, 357 - 391.

______________ 1921. Sur le développement des plantules fragmentées. Compt. Rendu Soc. Biol., Paris, 84: 770 - 772.

MOORE, G.T. 1905. Soil inoculation for legumes; with reports upon the successful use of artificial cultures by practical farmers. U. S. Dept. Agr. Bur. Plant Indus., Bul. 71: 1 - 72.

NAGAO, M. 1936. Studies on the growth hormones of plants. I. The production of growth substances in root tips. Sci. rep. Tôhoku univ. (Biol.) 10: 721 - 731.

NILSSON, R., BJÄLVE, G., and BURSTRÖM, D. 1938. Über Zuwachsfaktoren bei *Bact. Radicicola* I. Ann. d. landwirtsch. Hochschule Swedens. 5: 291 - 322.

NOBLES, C. 1919. Spring inoculation of legumes. Mich. Agr. Expt. Sta., Quart. Bul. 1: 100.

OTIS, D.H. 1898. Root tubercles and their production by inoculation. The Industrialist, Manhattan, Kans. 24: 363 - 378.

van OVERBEEK, J. 1939a. Is auxin produced in roots? Proc. Nat. Acad. Sci., Wash., 25: 245 - 248.

______________ 1939b. Evidence for auxin production in isolated roots growing in vitro. Bot. Gaz. 101: 450 - 456.

______________, and BONNER, J. 1938. Auxin in isolated roots growing in vitro. Proc. Nat. Acad. Sci., Wash., 24: 260 - 264

______________, et al. 1944. Factors affecting the growth of Datura embryos in vitro. Am. Jour. Bot. 31: 219 - 224.

PRAZMOWSKI, A. 1890. Die Wurzelknollchen der Erbse. Landw. Vers. Sta. 37: 161 - 238.

PFEFFER, W. 1900. The physiology of plants. 1: 325 - 326. (Translated by A.J. Ewart).

PRUCHA, M.J. 1915. Physiological studies of Bacillus Radicicola of Canada field pea. Cornell Univ. Agr. Expt. Sta., Memoir 5: 1 - 83.

RAUTENBERG, F., and KÜHN, G. 1864. xviii. Vegetationsversuche im Sommer 1863. Jour. Landw. 12: 107 - 140.

RECHINGER, C. 1893. Untersuchungen über die Grenzen der Teilbarkeit im Pflanzenreich. Abhandl. Zoöl. - bot. - Ges., Wien., 43: 310 - 334.

REID, M.E., and ROBBINS, W.J. 1942. Synthesis of ascorbic acid in excised tomato roots, Science 95: 632 - 633.

RITTER, G. 1911. Beitrage zur N-ernahrung der Leguminosen. Centbl. Bakt. 2 Abt. 29: 650 - 668.

ROBBINS, W.J. 1922a. Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. 73: 376 - 390.

____________ 1922b. Effect of autolized yeast and peptone on growth of excised corn root tips in the dark. Bot. Gaz. 74: 59 - 79.

____________ 1939. Thiamin and plant growth. Science 89: 303 - 307.

____________ 1940a. Light and the growth of excised roots of Datura. Bull. Torrey Bot. Club 67: 762 - 764.

____________ 1940b. Response of excised tomato roots to B(- 4 methylthiazolyl - 5) - alanine. Plant Physiol. 15: 547 - 552.

______________ 1941. Growth of excised roots and heterosis in tomato. Am. Jour. Bot. 28: 216 - 225.

______________ 1942. Specificity of pyridoxine for excised tomato roots. Am. Jour. Bot. 29: 241 - 244.

______________, and BARTLEY, M.A. 1937a. Vitamin $B_1$ and the growth of excised tomato roots. Science 85: 246 - 247.

______________, and ______________ 1937b. Thiazole and the growth of excised tomato roots. Proc. Nat. Acad. Sci. Wash., 23: 385 - 388.

______________, and ______________ 1937c. Use of dextrose by excised tomato roots. Science 86: 290 - 291.

______________, and KAVANAGH, V. 1943. Growth of excised roots of ployploid tomatoes. Am. Jour. Bot. 30: 602 - 605.

______________, and MANEVAL, W.E. 1923. Further experiments on growth of excised root tips under sterile conditions. Bot. Gaz. 76: 274 - 287.

______________, and ______________ 1924. Effect of light on growth of excised root tips under sterile conditions. Bot. Gaz. 78: 424 - 432.

______________, and SCHMIDT, M.B. 1938. Growth of excised roots of the tomato. Bot. Gaz. 99: 671 - 728.

______________, and ______________ 1939a. Vitamin $B_6$, a growth substance for excised tomato roots. Proc. Nat. Acad. Sci. Wash., 25: 1 - 3.

______________, and ______________ 1939b. Further experiments on excised tomato roots. Am. Jour. Bot. 26: 149 - 159.

______________, and WHITE, V.B. 1936. Limited growth and abnormalities in excised corn root tips. Bot. Gaz. 98: 209 - 242.

______________, and ______________ 1937. Effect of extracts from the corn plant on growth of excised root tips. Bot. Gaz. 98: 520 - 534.

______________, WHITE, V.B., McCLARY, J.E., and BARTLEY, M.A. 1936. The importance of ash elements in the cultivation of excised root tips. Proc. Nat. Acad. Sci., Wash., 22: 636 - 639.

RÜFFER, E. 1932. Forschungen zum Kohlenhydratumsatz bei knollchentragenden. und knollchenfreien Sojabohnen. Ztschr. Pflanzenernähr., Dungung u. Bodenk. 24A: 129 - 167.

SCHWEITZER, J. 1932. Über das Varhalten der Bacterienknollchen bei einigen chlorophyllfreien Leguminosen. Verhandl. Naturf. Gesell., Basel, 113: 376 - 377.

SIMON, J. 1907. Die Widerstandsfähigkeit der Wurzelbakterien der Leguminosen und ihre Bedeutung für die Bodenimpfung. Jahresber. Ver. Angew. Bot. 5: 132 - 160.

SNOW, R. 1933. The nature of cambial stimulus. New Phytol. 32: 288 - 296.

______________ 1935. Activation of cambial growth by pure hormones. New Phytol. 34: 347 - 360.

THORNTON , H.G. 1929. The role of the young lucerne plant in determining the infection of the root by nodule-forming bacteria. Proc. Roy. Soc., B, 104: 481 - 492.

______________ 1930. The influence of the host plant in inducing parasitism in lucerne and clover nodules. Proc. Roy. Soc., B, 106: 110 - 122.

______________ 1936. The action of sodium nitrate upon the infection of lucerne root hairs by nodule bacteria. Proc. Roy. Soc., B, 119: 474 - 492.

______________, and NICOL, H. 1936. Reduction of nodule numbers and growth, produced by the addition of sodium nitrate to lucerne in sand culture. Jour. Agr. Sci. 24: 540 - 543.

TRELEASE, S.F., and TRELEASE, H.M. 1933. Physiologically balanced culture solutions with stable hydrogen-ion concentration. Science 78: 438 -439.

TRELEASE, S.F., and TRELEASE, H.M. 1935. Changes in hydrogen-ion concentration of culture solutions containing nitrate and ammonium nitrogen. Am. Jour. Bot. 22: 520 - 542.

USPENSKI, E.E. and USPENSKAJA, W.J. 1925. Reinkultur und ungeschlechtliche Fortpflanzung des Volvox minor und Volvox globator in einer synthetischen Nährlosüng. Zeitschr. Bot. 17: 273 - 308.

VINES, S.H. 1888. On the relation between the formation of tubercles on the roots of leguminosae and the presence of nitrogen in the soil. Ann. Bot. 2: 386 - 389.

VIRTANEN, A.I. 1945. Symbiotic nitrogen fixation. Nature 155: 747 - 748.

_______________, and LAINE, T. 1946. Red, brown and green pigments in leguminous root nodules. Nature 157: 25 - 26.

WEBER, E. 1930. Saltpeterdüngung als Beeintrachtigung der Stickstoffsammlung durch Leguminosen. Zentbl. Bakt. 2 Abt. 82: 353 - 379.

WEST, P.M. 1939. Excretion of thiamin and biotin by the roots of higher plants. Nature 144: 1050 - 1051.

_______________, and WILSON, P.W. 1938. Synthesis of growth factors by <u>Rh</u>. <u>trifolii</u>. Nature 142: 397 - 398.

_______________, and _______________ 1939. Growth factor requirements of the root nodule bacteria. Jour. Bact.37: 161 - 185.

WHALEY, W.G., and LONG, A.L. 1944. The behaviour of excised roots of heterotic hybrids and their inbred parents in culture. Bull. Torrey Bot. Club 71: 267 - 275.

WHITE, P.R. 1932a. Plant tissue cultures. A preliminary report of results obtained in the culturing of certain plant meristems. Arch. exp. Zellforsch. 12: 602 - 620.

_______________ 1932b. Influence of some environmental conditions on the growth of excised root tips of wheat seedlings in liquid media. Plant Physiol. 7: 613 - 628.

_______________ 1933a. Concentrations of inorganic ions as related to growth of excised root tips of wheat seedlings. Plant Physiol. 8: 489 - 508.

______________ 1933b. The .SH radical and some other sources of sulphur as affecting the growth of isolated root tips of wheat seedlings. Protoplasma 19: 132 - 135.

______________ 1933c. Liquid media as substrata for the culturing of isolated root tips. Biol. Ztrbl. 53: 359 - 364.

______________ 1934a. Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9: 585 - 600.

______________ 1934b. Multiplication of the viruses of tobacco and aucuba mosaic in growing excised tomato roots. Phytopath. 24: 1003 - 1011.

______________ 1937a. Seasonal fluctuations in growth rates of excised tomato root tips. Plant Physiol. 12: 183 - 190.

______________ 1937b. Survival of isolated tomato roots at suboptimal and supraoptimal temperatures. Plant Physiol. 12: 771 - 776.

______________ 1937c. Separation from yeast of materials essential for growth of excised tomato roots. Plant. Physiol. 12: 777 - 791.

______________ 1937d. Amino acids in the nutrition of excised tomato roots. Plant Physiol. 12: 793 - 802.

______________ 1937e. Vitamin $B_1$ in the nutrition of excised tomato roots. Plant Physiol. 12: 803 - 811.

______________ 1938a. Accessory salts in the nutrition of excised tomato roots. Plant Physiol. 13: 391 - 398.

______________ 1938b. "Root - pressure", an unappreciated force in sap movement. Am. Jour. Bot. 25: 223 - 227.

______________ 1938c. Root - pressure as a factor in the rise of sap. Nature 141: 581 - 583.

______________ 1938d. Cultivation of excised roots of dicotyledonous plants. Am. Jour. Bot. 25: 348 - 356.

______________ 1939. Glycine in the nutrition of excised tomato roots. Plant Physiol. 14: 527 - 538.

______________ 1940. Vitamin $B_6$, nicotinic acid, pyridine, glycine and thiamin in the nutrition of excised tomato roots. Am. Jour. Bot. 27: 811 - 821.

______________ 1943a. Further evidence on the significance of glycine, pyridoxine, and nicotinic acid in the nutrition of excised tomato roots. Am. Jour. Bot. 30: 33 - 36.

______________ 1943b. A Handbook of Plant Tissue Culture. Cattell Press, Lancaster, Pennsylvania.

______________ 1946. Plant tissue cultures. II. Bot. Rev. 12: 521.

WINKLER, H. 1902. Besprechung der Arbeit G. Haberlandt's "Culturversuche mit isolierten Pflanzenzellen". Bot. Ztng. 60 (2): 262 - 264.

WILSON, J.K. 1917. Physiological studies of *Bacillus radicicola* of Soybean (*Soya Max Piper*) and of factors influencing nodule production. Cornell Univ. Agr. Expt. Sta., Bul. 386: 369 - 413.

______________ 1931. Nodule production on etiolated vetch seedlings. Phytopath. 21: 1083 - 1085.

WILSON, P.W. 1935. The carbohydrate - nitrogen relation in symbiotic nitrogen fixation. Wis. Agr. Expt. Sta., Res. Bul. 129, 40 pp.

______________ 1940. The Biochemistry of Symbiotic Nitrogen Fixation. Madison, Wisconsin.

______________, and FRED, E.B. 1939. The carbohydrate - nitrogen relation in legume symbiosis. Jour. Am. Soc. Agron. 31: 497 - 502.

______________, and WAGNER, F.C. 1935. Combined nitrogen and the nitrogen fixation process in leguminous plants. Wis. Acad. Sci. Trans. 30: 43 - 50.