THE PHYSIOLOGY OF INDIAN NODULE BACTERIA.

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Chapter I.

Introduction.

It was theoretically a scientific assumption that, under the special conditions of Indian soils and host plants, such adaptable organisms as bacteria might exhibit certain variable peculiarities which could give us a better insight into their nature; perhaps also certain phenomena could be better observed under the laboratory conditions naturally prevailing in our country and acquire the full significance; perhaps even certain features were obscured and certain potentialities completely hidden by more conspicuous phenomena observed in foreign laboratories.

This surmise, which was merely reasonable, proved in fact also correct, and the result of our work is that we are now able to assert certain characteristics about our Indian strains without having merely to rely on analogies; and on the other hand we have added in some places new information about the organisms studied.

In addition, we provide for future workers a reliable techniqu ecovering all the ground of the physiological study of nodule bacteria which was developed after long and careful study of the methods available and which in our country was found to succeed where other methods, apparently successful abroad, had failed.

Summary of the Method.

I. The first step was the isolation of the micro-organism in pure culture. For that, three typical host plants were chosen. It was thought advisable to select three leguminous plants of economic importance in India and indigenous to the Old World. For purposes of more accurate comparison the experiments on the three isolated organisms should be carried on at the same time.

The groups according to the latest work done (Baldwin and Fred, 1929) in this matter are :—

(*a*) *Rhizobium leguminosarum* Frank. The organism causing the formation of nodules upon the roots of *Lathyrus, Pisum, Vicia* and *Lens.*

(*b*) *Rhizobium trifolii* Dangeard. The organism causing the formation of nodules upon the roots of *Trifolium* sp.

(*c*) *Rhizobium phaseoli* Dangeard. Causes the formation of nodules on *Phaseolus vulgaris, Ph. angustifolius* and *Ph. multiflorus.*

(*d*) *Rhizobium meliloti* Dangeard. The organism causing the formation of root nodules upon *Melilotus, Medicago* and *Trigonella.*

(*e*) *Rhizobium japonicum* (Kirchner) Comb. Nov. The organism causing the formation of root nodules on *Soja max.*

The plants chosen were *Cajanus indicus, Dolichos biflorus* and *Psophocarpus tetragonolobus* (Hooker, 1874). The isolation and identification of the micro-organism was made to satisfy all the microscopical and morphological data as well as the typical reactions ; and was always counterproved by the biological positive and negative test, as will be seen later.

Once obtained in pure culture, we proceeded to study the organism in question using all pertinent culture media suggested by the experience obtained during the last twenty years of microbial research.

The physiology of a micro-organism, the growth, the colonial aggrupation, the rate of reproduction,are all dependent on the nature and the variety of the enzymes, and these are tested on carefully selected media of varying composition.

The micro-reactions are sometimes made more evident by the use of indicators. This study occupied the body of our work and the results are given below.

II. A remarkable fact which we encountered in the progress of our work is that in the case of *C. indicus* we isolated, quite consistently, from the root nodules two organisms. The typical symbiont and another one, referred to below, which was not able to produce nodules, when inoculated into a pot in which the plant from which it was isolated was grown under sterile conditions.

The typical symbiont was able to produce nodules not only on the same plant from which it was isolated, *i.e., C. indicus* but also on others on which we tried it, *viz., D. biflorus, Ps. tetragonolobus* and even cowpea (*Vigna catiang*).

III. The organism of *Cajanus indicus* has been placed by Löhnis and Leonard, 1926, and others in the cowpea group, a group which is considered to have an alkaline reaction on litmus milk with the formation

or not of serum zone. But in our experiments we found that the organism of *Cajanus indicus* does not behave as those of the cowpea group. In all the experiments we found that the organism in question does not give an alkaline reaction. On the contrary it is distinctly and consistently acid.

Also in its fermentation reactions we found that it deviates from the reactions of the organisms of the cowpea group as will be mentioned later.

Various attempts were made to grow plants in flasks as recommended by Garman and Didlake ; but, although the plants did grow and develop to a stage in which nodules could be expected, we never observed actual nodule formation, even when modifying in different ways the conditions of the experiment.

IV. As regards pot experiments it was found that the usual method of growing the plants in open pots did not give consistent results.

As contamination cannot always be avoided with this method, it is always to be feared. The technique of protecting the pots with gauze, providing at the same time glass tubes as outlets for the plants and a test tube opened at both ends as an inlet for watering, proved very successful.

Isolation.

In order to isolate the organism from the nodules in the beginning of our work the method generally followed by the other workers was adopted.

A few good-sized and apparently-sound nodules were selected and cut off the roots leaving a few millimetres of the root on both sides in order to make the handling with forceps easier and also to keep the nodules intact, thus preventing the disinfecting fluid from penetrating them. The nodules were then washed several times in distilled water to remove the soil particles adhering to them. They were then sterilised by putting them into the following sterilising liquid :—

Mercury Bichloride	.. ..	1 gm.
Hydrochloric Acid	.. ..	2·5 c.c.
Water	.. ..	500 c.c.

The nodules were well shaken in the above solution for 3–4 minutes. Then they were transferred aseptically to a sterile dish containing sterile distilled water. They were washed 3–4 times, dried on a sterile blotting paper, dipped into 95% alcohol, and then passed quickly through the flame to remove the last traces of alcohol. A fair-sized nodule was transferred to a flamed glass slide and cut open aseptically. The central soft portion of the exposed surface was removed on to a few drops of sterile water with an arrow-headed needle. This was well shaken to make an emulsion.

A little of this emulsion was transferred to a medium tube and plated (Giltner and others).

This in the first place is a long and tedious process; that, however, would not have been considered as an obstacle if good results were obtained to compensate for the efforts, but as a matter of fact time and again it produced negative results.

This difficulty has been experienced by all the workers (Joshi in our country amongst others). Therefore an attempt was made to derive more light from their experience and develop a suitable standard method, at least a standard method which could be considered as satisfactory for our study.

The nodules, after being washed in ordinary tap water, were put into a sterile test tube with a cotton plug. A few c.c. of the above disinfecting fluid were added to the tube containing the nodules. The plug was replaced and the tube was put in an Erlenmeyer vacuum flask and connected to the exhaust pump. The pump was worked to exhaust the air. This step is taken in order that the disinfecting fluid may reach the entire surface of the nodule. The whole procedure does not take more than 2–3 minutes with a good "Genco" pump. The nodules that were at the bottom of the solution would float when the air was exhausted. The flask was disconnected and the tube was taken out of it. The plug and the mouth were flamed, and the mercuric chloride thrown away, care being taken that the nodules did not pass out. Sterile water was added to the tube, it was plugged and the same process of exhausting the air repeated, so that the last traces of mercury bichloride were removed as recommended by Hass and Fred (1919).

The nodules were washed with sterile water 4–5 times. As a measure of control of future operations, before an emulsion was made, we always plated out the last wash; by which we had perfect assurance that no other soil organisms were present. The presence of an occasional air or water organism is of course immaterial. It was found generally that this fourth or fifth wash was sufficient, as the rinsing liquid did not show any growth. The nodules were then broken inside the tube and an emulsion made. A drop of the emulsion was further diluted and plated out. By following the outlined procedure we always obtained good growth of colonies and free from undesirable contamination.

For the actual isolation and culture of the specific organism various media were tried. From that we gained a fairly good, and certainly interesting knowledge of conditions of growth, at least in this country.

As many times the success of laboratory procedure depends on small points which are likely to be taken for granted in routine work, we give in some detail below, not only the formula but also, where necessary, the way in which we prepared the different culture media.

Instead of using 1·5% agar so commonly recommended by the various authors, we generally use 2% agar as 1·5% agar does not give a good solid medium under our conditions of temperature and in our case also of pH. (This is otherwise the general practice in our Laboratory.) However, as this organism often produces gum it is advisable to work with a somewhat harder medium and therefore the percentage of 2·5 was found to be the best and was generally adopted for critical work. Besides the condition of the medium may also create difficulty in the passage of slant sub-cultures, when it is impossible to do any approximate comparative work by means of the loopfuls unless the slants offer a smooth and firm surface.

Whenever the agar is available in powder form we recommend soaking it in water for half to one hour before heating it. If the agar is heated in water at once, without previously soaking it in water, as mentioned before, clots will invariably be formed and sometimes even charring may take place. After soaking the agar in water it should be heated on an asbestos gauze and boiled for at least half an hour and after that it is made to the original volume by the addition of distilled water. Then the ingredients are added and the resulting preparation is heated on the water-bath to obtain perfect solution. The pH was adjusted with normal sodium hydroxide and the media tubed in 5 c.c. amounts for the slants and 12 c.c. for the plates and autoclaving was carried out at one atmosphere pressure for half an hour. The glass material used was always Pyrex, Jena or another kind of neutral glass. We cannot too strongly recommend the use of special glass when critical results are aimed at in microbial research, specially with nodule bacteria. As stated above the ingredients, specially carbohydrates, were added last, in order to avoid decomposition by prolonged heating. Moreover, the tubes were always placed in the autoclave when the steam was beginning to pass out freely through the tap. Another precaution taken to avoid decomposition of sugars was to remove the tubes from the autoclave as soon as the pressure went down and to transfer them to the refrigerator. By this method we found that the sugars did not dissociate and no turbidity occurred, the media thus remaining quite clear. If the above precaution was not taken it was very difficult to avoid turbidity. The slants were never used immediately. They were at least kept for 2–3 days in the incubator before being used, so as to be sure of their being sterile.

At no time in our procedure were media allowed to remain for more than 20 days. We found that on standing for a long time, even in the refrigerator, the pH of the media used to vary considerably. Moreover, the media lost much of their water and became so dry that no growth could take place when inoculated.

Coming to the particular media the following data will give enough information. A comparative comment on the results will be given when the description of the methods have been dealt with.

Lipman and Fowler's Synthetic Agar.

Dextrose	10·0 gm.
Potassium hydrogen phosphate	0·5 ,,
Magnesium sulphate	0·2 ,,
Potassium nitrate	0·05 ,,
Agar	20 to 25 ,,
Distilled water	1000 c.c.

Winogradski's Medium.

Distilled water	1000 c.c.
Agar	20 to 25 gm.
Sodium asparaginate	1·0 ,,
Magnesium sulphate	0·2 ,,
Glucose	1·0 ,,
Ammonium dihydrogen phosphate ..	1·5 ,,
Calcium chloride	0·1 ,,
Potassium chloride	0·1 ,,
Ferric chloride	Trace

Barlow's Ash Maltose Medium.

Wood ashes	16 gm.
Maltose	10 gm.
Distilled water	1000 c.c.

his medium was prepared as follows:—

Wood ashes were mixed with water and boiled for one minute. Filtered through two sheets of filter paper. The filtrate was mixed with agar and sugar and heated till dissolved. It was filtered through cotton wool, tubed and sterilised.

Wood Ash Extract Sucrose Medium.

Wood ashes	15·0 gm.
Mono-potassium dihydrogen phosphate ..	3·0 ,,
Sucrose	10·0 ,,
Water	1000 c.c.

Greig Smith's Levulose Agar, 1912.

Levulose	20·0	gm.
Asparagin	0·6	,,
Sodium citrate	1·0	,,
Potassium citrate	1·0	,,
Agar	25·0	,,
Distilled water	1000	c.c.

Bean Extract Medium.

In the preparation of bean extract medium, after trying the modifications of Löhnis (1930), we did not follow them as the procedure is a long and tedious one and is not justified by striking results. It was found that the original method of Maze (1897) gave as good results as the modification by Löhnis. Nevertheless, we have to note that we made a change in Maze's method, *viz.*, instead of heating the haricot beans for half an hour at 100° C. we heated them with a large quantity of water on direct flame for 2 to 3 hours by which the maceration is affected and the availability of the starch material considerably influenced. As the volume of water and the weight of beans was not specified, we took 200 gm. of seed for one litre of extract. Generally three litres of water were added and the infusion heated as mentioned before for 2 to 3 hours. The extract was passed through a strainer and made up to a litre. To the extract 2·5% agar was added and the medium prepared in the way described in the beginning. 2% of sucrose was added to this medium. Occasionally calcium carbonate was also added, but very often we prepared this medium without calcium carbonate as it served quite well.

Laurent's Sucrose Medium.

Tap water	1000·0	c.c.
Sucrose	10·0	gm.
Mono-potassium dihydrogen phosphate	1·0	,,
Magnesium sulphate	0·5	,,
Agar	20–25	,,

Soil Extract Mannite Agar.

Soil Extract	1000·0	c.c.
Mannite	20·0	gm.
Di-potassium monohydrogen phosphate	0·5	,,
Agar	20–25·0	,,

Soil extract was prepared by heating one kilogramme of rich garden soil in 1500 c.c. of water for half an hour in the autoclave.

Marmite Mannite Medium (Desai).

Marmite	2·0	gm.
Mannite	20·0	,,
Di-potassium monohydrogen phosphate	0·5	,,
Magnesium sulphate	0·2	,,
Agar	20 to 25·0	,,
Distilled water	1000·0	c.c.

Calcium carbonate added if desired.

Ashby's Mannitol Agar (modified).

Distilled water	1000·0	c.c.
Mannitol	12·0	gm.
Mono-potassium dihydrogen phosphate	0·2	,,
Magnesium sulphate	0·2	,,
Sodium chloride	0·2	,,
Calcium sulphate	0·1	,,
Calcium carbonate	5·0	,,

Yeast Water Mannitol Agar.

Agar	20–25·0	gm.
Mannitol	10·0	,,
Di-potassium monohydrogen phosphate	0·5	,,
Magnesium sulphate	0·2	,,
Sodium chloride	0·1	,,
Calcium carbonate	3·0	,,
Distilled water	900·0	c.c.
Yeast water	100·0	,,

Yeast water was obtained by soaking 10 gms. of yeast in 100 c.c. of water for 2 hours, then autoclaving for forty to sixty minutes and allowing to settle a week before using.

Of all the abovementioned media, bean extract medium gives best growth. The next best are soil extract and marmite mannite medium. In the first case slant growth in the limits of visibility can be observed after twenty-four hours. A growth to the full extent is reached after about seventy-two hours. In the second case growth is apparent only after thirty-six hours, while full growth is attained after no less than eighty-four hours.

The results with ash maltose medium were not so satisfactory. Better results were obtained on ash medium with sucrose as the carbohydrate. This made us direct our attention to the prominent part which the specific carbohydrate might play in determining and stimulating a favourable

growth. This surmise of ours was justified later on by the fermentation experiments which we carried out at a later stage of our work and which will be described further on.

We also want to remark in this connection that for critical biochemical work, media containing such ingredients as " marmite " and wood ash are not so easily controllable as those containing more definite and isolated compounds. In fact the objection could be raised as well in the case of Yeast extract.

* * *

(A) Two kinds of colonies were found in the plates, specially in Bean extract medium, on which the emulsion from the nodules of *Cajanus indicus* was plated. One type of colonies, as mentioned before, showed abundant gum so that when an attempt was made to take out a little bit of it with the loop regular long threads were formed. The other type of colonies did not show so much gum. Both of them however correspond to the general description of nodule-bacteria, and so special attention was devoted to them. The gum formation is by no means a character on the basis of which an identity can be accepted or rejected. We preserved carefully the stock of both kinds of colonies, namely the one producing greater and the other producing a lesser amount of mucilage ; and in subsequent experiments the " concomitant " was tested along with the others.

We tried this latter organism by different methods and we found that, although the organism behaved in many ways like *Rhizobium radicicola*, it failed to produce nodules on *Cajanus indicus*.

A fuller description of this interesting organism is published elsewhere. We have proposed for it the name of *Bacillus concomitans*.

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The second organism isolated from *C. indicus* was the typical *Rhizobium* and once identified with the preliminary tests, it was set apart for further studies together with the organisms isolated from the two other host plants singled out for our research.

The colonies of the organism of *C. indicus* were round, smooth, watery, entire, translucent, convex.

Streaks are raised translucent, spreading over the whole surface and often flowing down and accumulating to some extent at the bottom.

When forty-eight hours growth smears were prepared a Gram negative straight rod with round ends was revealed. The rods measured from 1·5 to 3·0 microns. Branched forms were never noted in this organism either.

(B) *Isolation of organisms from the root nodules of* D. biflorus *and* Ps. tetragonolobus.—The nodule organisms from *D. biflorus* and *Ps. tetragonolobus* were isolated from the root nodules of the plants grown in our laboratory in the same way as the organism from *C. indicus*. In their isolation no contamination was met with.

After isolation, the organisms were tested for their identity both by their reactions on different media and by the biological test. They were found to be true to type.

The colonies of *D. biflorus* were more watery, transparent, becoming somewhat milky as age advances, not very slimy, glistening, smooth, entire, not as convex as those of *C. indicus* (flattening out with age), and with a tendency to coalesce and extending up to 8 mm. in diameter.

Forty-eight hours mounts from marmite mannite agar showed Gram negative rods varying from more or less cocci-bacilli form to well-shaped rods up to 2·5 microns in length. Swollen vacuolated forms were found when mounts were prepared from old cultures.

Colonies of *Ps. tetragonolobus* organisms were found to be much smaller compared with those of *C. indicus* and *D. biflorus*. They were drop-like, more convex, not very mucilaginous, glistening, opaque, smooth, entire, extending up to 2–3 mm. in diameter.

Smears showed Gram negative rods with round ends. Vacuolated forms were very often seen. Branched forms were not seen, neither in artificial cultures nor in nodules.

Chapter II.

Physiological and Biochemical Reactions in Experimental Media.

If soil inoculation with a given nodule bacterium is to be done, with a view to grow some particular leguminous plant that has not hitherto been grown in a particular area, it is quite essential that the identity and specific affinities of the micro-organisms be established or at least the probabilities of cross-inoculation, when a specific organism is not available, or other leguminous crops have previously been grown there. On the other hand, in order to divide the organisms into various groups and assign them definite reactions towards various media, it is necessary to examine as many of the organisms in that group as possible, if any generalisation is to be attempted.

With regard to the organisms isolated by us very meagre data were at our disposal, so it was thought necessary to make a fresh and systematic study of the cultural reactions and physiological behaviour in general.

Reaction on potato medium.—As already mentioned, Löhnis and Hansen (1921) have proved that Rhizobia organisms do not grow, or grow very scantily on potato slants. So potato slants were prepared by cleaning the potatoes thoroughly and cutting out of them, by means of a cork-borer, cylinders just wide enough to get into test-tubes. These cylinders were cut diagonally so as to give slant surfaces. They were then washed in water and placed into a solution of sodium carbonate (1 in 500) for half an hour. They were then taken out and washed several times in water and transferred to sterilised test-tubes having small pieces of glass rods as supports and a few c.c. of water at the bottom, and were autoclaved for half an hour. In order to avoid overcooking the tubes were placed in the autoclave after the steam had begun to pass out freely.

After a few days the slants prepared as above were inoculated with the organisms in question. The result was that except in the case of *Ps. tetragonolobus* no growth was observed, while in *Ps. tetragonolobus* sometimes very slight growth was noticed.

Carrot slants were also prepared in the same way as potato ones and inoculated with our organisms. No growth was observed in any case.

Milk medium.—Löhnis and Hansen (1921) showed that considering the growth of Rhizobia on milk media they can be divided into two definite groups. Members of one group form a " serum zone " and they correspond to those which are fast growers, whilst those of the other group do not produce " serum zone " and belong to the slow growers. Cowpea (*Vigna catiang*) group organisms are classed as belonging to the latter one. In 1925 Stevens introduced indicators in the milk and was able thereby to note the changes distinctly. He divided the nodule bacteria from their reaction on milk into three groups. (1) Those that produce acidity in milk. (2) Those that produce alkalinity in milk but at the same time give a 'serum zone'. (3) Those which do not produce 'serum zone' but are basic in reaction. According to this classification the Cowpea group organisms are placed in the third group. In testing our micro-organisms we observed the unexpected facts that *C. indicus* and *D. biflorus* commonly placed in the Cowpea group do not behave in this reaction as the members of the third group.

Milk media, both containing indicators and no indicators, were prepared in the following way :—

Fresh pasteurized milk was obtained and placed in a shallow dish in the refrigerator overnight. Next morning the skimmed milk was siphoned out into another vessel leaving the cream in the first one. It was tested to see whether it titrated above—1·7% normal (Fuller's scale). In most

cases there was no need to adjust to normality. It was tubed in sterile tubes, plugged and sterilized in the water-bath on four successive days for twenty minutes, care being taken that the tubes were placed after the water had begun boiling.

When milk containing indicators was required, they were added before tubing. In the case of litmus milk 2% of a standard solution of Kahlbaum's azolitmin was added. Also brom-thymol-blue milk was prepared on several occasions. All these were inoculated with the organisms in question.

Their reaction in the beginning was alkaline in all cases. But *C. indicus* organism reduced the litmus and later on produced acidity. While the "concomitant" from *Cajanus indicus* and the organism from *D. biflorus*, and Goa-bean produced 'serum zone' and remained alkaline throughout. (See Plate V, Fig. 1).

From this it appears, as hinted above, that if the *C. indicus* and *D. biflorus* organisms are to be retained in the Cowpea group and the paramount criterion is the biological test (cross-inoculation) then the aggrupation of organisms obtained by the milk reactions is not substantiated.

In our attempt to prepare milk medium we tried to autoclave the milk, containing indicator or not, by placing the tubes in the autoclave after the steam had begun to come out, allowing then the temperature to rise to 110° C., stopping the gas and when the pressure went down finally transferring the tubes to the refrigerator. It was found that this method of preparing the milk served as well as the ordinary process of sterilising successively for four days. It saved time in addition.

Glycerol phosphate medium.—Glycerol phosphate medium having the formula given below was tried to see what changes in morphology occurred as Percival suggests that bacteriods are formed in this medium. After several attempts it was found that none of the abovementioned stocks would grow. A modification, however, is contemplated.

Distilled water	.. ..	1000·0 c.c.
Magnesium sulphate	.. ..	0·1 gm.
Di-potassium phosphate	.. ..	2·0 „
Glycerol	.. ..	10·0 „

heated to boiling, filtered, tubed, sterilised for three successive days.

Carrot agar.—Carrot agar medium was prepared according to Müller and Stapp (1925). 250 gms. of well washed and finely minced carrot were cooked in the steamer with 500 c.c. of water for half an hour. The filtrate was taken out and made up to 500 c.c. and adjusted to pH 7·0 with a strong

solution of sodium carbonate. 500 c.c. of 3·6% neutral water solution of agar was added to the extract, mixed well, tubed and sterilised at 110° C.

Slants of the above media, when inoculated with all our stocks, gave a fair growth but they became dry much sooner than in the case of other media. They turned the medium brown suggesting tyrosinase reaction. So we prepared a medium to which a given amount of tyrosin was added and the reaction verified.

Tyrosinase reaction.—It was of interest to test in our Indian micro-organisms the presence of an enzyme acting on tyrosin.

After studying the formula used by Stapp, 1923 and used by Almon, we selected Thornton (1922), mannitol nitrate-asparagin medium to which 0·15% of tyrosin was added. It was found that all our cultures produced browning in the medium, but they differed in their grades of darkening. *Ps. tetragonolobus* organism produced the darkest colour and *Cajanus indicus* organism the faintest.

Yeast water mannitol agar is not suitable for this purpose. As has been indicated by Eckhardt, etc. (1931), the natural colour of the yeast water substrate interferes with the colour changes of the tyrosinase reaction.

The formula of tyrosin mannitol nitrates asparagin medium used in our experiments is as follows :—

Water	1000·0	c.c.
Mannitol	1·0	gm.
Asparagin	0·5	,,
Potassium nitrate	0·5	,,
Di-potassium phosphate	1·0	,,
Magnesium sulphate	0·2	,,
Calcium chloride	0·1	,,
Ferric chloride	0·002	,,
Sodium chloride	0·1	,,
Agar	25·0	,,

The presence of gelatinase.—Burill and Hansen, 1917, proved that the nodule organisms are capable of slowly liquefying gelatin, and it was further testified by Stapp (1924) that almost all the nodule organisms belonging to every cross-inoculation group liquefy gelatin slowly. Others dissent radically from this view. Müller and Stapp (1925) emphasised the importance of using only gelatin of the proper reaction. Eckhardt and others (1931) pointed out that the use of Witte's peptone favoured the growth. This was proved to be correct also by our experiments. The kind of gelatin and the kind

of peptone do have a great effect on the growth of organism in gelatin and in its liquefaction.

We carried the final experiments in the following way.

Beef extract peptone gelatin was used according to the following formula :—

Beef extract	..	..	3 gm.
Peptone (Witte's)	..	..	5 ,,
Distilled water	..	..	1000 c.c.
Gelatin	..	..	20–25 %

Adjusted to neutrality.

Contrary to the finding of Müller and Stapp (1925) better results were obtained with Witte's peptone.

In our experiments it was noted that if instead of gelatin stab, gelatin slants were used, liquefaction when it occurs takes place much earlier. The reason for a better growth in gelatin slants than in gelatin stabs may be that in the former a much better oxygen supply is available. Our organisms are strict aerobes, though probably in varying degrees. The reaction of these organisms towards gelatin *plus* oxygen can be made clearer by comparing the normal results with those obtained by the shake culture method.

The result of our observation is that the concomitant bacillus does not seem to liquefy gelatin, while Goa-bean organism liquefies gelatin sooner than the *C. indicus* and *D. biflorus*. They all grow meagrely and that after a long time and get somewhat dirty coloured, indicating tyrosinase reaction. We therefore consider that by our choice of the slant method we can observe gelatin liquefaction in our bacteria and are inclined to give a more favourable report on the subject of liquefaction than that given by Walker. A comparative study of other strains with our slant method is contemplated.

With our simple slant method complete liquefaction takes place in about a fortnight, while the method reported by other authors requires months (*cf.* for instance Eckhardt, Baldwin and Fred, 1931). In our laboratory the stab method gave liquefaction after one month and slant within a fortnight.

It is to be noted that the amount of growth and the liquefaction are not interdependent. In our cultures we obtained growth at the third or fourth day, and beginning of liquefaction after ten days as a rule. But in any case we have to point out that gelatin is not the ideal medium for the growth of our organisms and even in case of good liquefaction the growth may be called slow and in addition scanty.

Dye bacteriostasis.—Stevens (1925), Wright (1925) and various other authors have studied the relation of the growth of nodule organism to crystal violet and have shown that various groups will tolerate different concentrations of the reagent. In our experiments we tried two kinds of media, solid and liquid, and curiously enough with different results. The liquid medium is that suggested by Stevens. In our experiments we used a sucrose peptone solution containing 2·0 gms. of monopotassium phosphate, 0·1 gm. of magnesium sulphate, 20·0 gms. of sucrose, 10 gms. of peptone per litre, sterilising on three successive days. It was noted that Goa-bean organism grows better ; *D. biflorus* organism grows, if at all, very little ; while the *Cajanus* organism and its concomitant did not grow at all. On the contrary all the organisms grew quite well in Leonard's solid medium (1931) containing crystal violet in 1 : 80,000 concentration and produced colonies evenly coloured.

The formula of Leonard's medium is :—

Soil extract	.. ..	1000·0 c.c.
Di-potassium-phosphate	.. ..	1·0 gm.
Sodium nitrate	.. ..	1·0 ,,
Glycerol	.. ..	10·0 ,,
Agar	.. ..	10–15 ,,

(but we used 20 gms. of agar for the reasons mentioned at the beginning of the first chapter).

Soil extract was prepared by heating 1000 gms. of garden soil with one litre of tap water in the autoclave for thirty minutes. A small amount of calcium carbonate was added and the whole filtered through a double filter paper till the filtrate came through it quite clear.

The phenomenon has also been observed in the case of Congo-red.

We use the following formula suggested by Fred, Baldwin and McCoy (1932) :

Mannitol	.. ..	10·0 gm.
Potassium nitrate	.. ..	0·5 ,,
Di-potassium phosphate	.. ..	0·5 ,,
Magnesium sulphate	..	0·2 ,,
Sodium chloride	.. ..	0·2 ,,
Ferric chloride	.. ..	Trace.
Manganese sulphate	.. ..	Trace.
Water	.. ..	1000·0 c.c.
Agar	.. ..	25·0 gms.

Congo-red was added just before tubing 1 in 400 aqueous solution of the dye.

All the cultures of our strains produced whitish colonies without the absorption of the dye. This is an important test which differentiates our organisms from *B. tumefacians* which absorbs the dye.

We also found this medium preferable to others as the growth upon it is not too abundant and the colour absorption reactions are more delicate and distinct.

Hydrogen-ion reactions.—Fred and Davenport (1918) worked extensively on the reactions of Hydrogen-ion concentrations and limits of growth of the different cross-inoculation groups. We proceeded to study our organisms in this respect and tried to determine the range of pH of the cultures isolated by us. For this purpose Bean extract medium was selected as our previous work had shown it to be the most favourable medium for their growth.[1]

Slants were prepared varying from pH 3·0 to 9·6 and inoculated with our strains. It was found that *B. concomitani, C. indicus* and Goa-bean organisms had their lowest limit 4·8 and their highest limit more than 9·6 (above the limits of the apparatus), while *D. biflorus* organism had 5·2 as its lowest limit and the highest, as the others, more than 9·6. All showed their optimum growth at 6·6 to 7·8 pH.[2]

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[1] In the beginning, we prepared media tubed and autoclaved them and then adjusted the pH with normal acid or alkali using sterile pipettes and other apparatus needed. But once we found that the control also was contaminated perhaps by chance. In our procedure, everything that was used was sterile and manipulations were done aseptically excepting the acid or the alkali used. Perhaps the contamination was pure chance. Strange as it looked, even to ourselves, we decided in our later attempts to sterilize the acid and alkali as well before using them. McClung (1932), we have found later, used also sterile reagents. The fact is that never since did we experience contamination.

[2] In this connection, we may mention that it occurred to us that the acidity of the juice of tamarind plant, to mention a common Indian plant, might be in the way of the symbiotic micro-organism and prevent the connection required for nodule formation. To investigate this point, we grew some tamarind plants in the ordinary garden soil previously used for the growth of nodules of *Cajanus, Dolichos* and Goa-bean. When the seedlings were about three weeks old, they were removed carefully, without disturbing their roots very much. The soil sticking to the roots was collected and its pH was determined, the pH of the sap of the root was also taken. The result was that the pH of the soil, before sowing the seeds, was 6.5 and that of the soil sticking to the roots was 5.2 and the extract of the root has 6.3. It thus appears that the acidity of the soil surrounding the rootlets does not increase so as to prevent the growth of the nodule organism (minimum pH 4.8). It was considered that the sap may contain some substance which would prevent the attack of the organism. Consequently, a media was prepared out of the extract of the rootlets of *Tamarindus indicus*, and adjusted to 6.3, its original pH, and inoculated with the organisms. It was found that the four strains organisms grew quite well. It is yet to be investigated whether a particular organism would adapt itself to form nodules on *Tamarindus indicus* roots, or, perhaps, its rootlets secrete a particular substance which prevents the action of these bacteria.

For the adjustment the colorimetric method was used by means of Hellige Comparator on the following scale of indicators : Bromphenol blue 3·4·6, Methyl red 4·4–6·0, Bromthymol blue 6·0–7·6, Cresol red 7·2–8·8, Thymol blue 8·0–9·6, Thymol blue 1·2–2·8, Cresol purple 5·2–6·6, Phenol red 6·8–8·4, as recommended by Clark and Lubs.

Fermentation and utilization of various carbohydrates.—It is reported by different authors that the relative value of different carbon compounds varies in the case of the growth of different nodule organisms. Differences in their reaction on sugars have been noted and also their preference for particular sugars. Temple (1916) reported that sucrose and glucose give better growth than lactose. Joshi (1920) found out, working with the organisms of Cowpea, pea, sunnhemp, etc., that mannitol stands best for the growth of the nodule organisms. Müller and Stapp (1925) showed that the organisms belonging to different cross-inoculation groups behave differently towards carbon compounds. McCoy, Baldwin and Fred (1931) have studied the growth and fermentation characteristics of *Rhizobium lupini* on a nitrate medium containing different carbohydrates and have found that there was an obvious disparity of results when different sugars were taken. Besides the quantitative growth, the fermentation reactions are of great interest. The organisms belonging to various cross-inoculation groups have been studied by Fred and Baldwin (1927), who observed that the organisms of Cowpea, Soy-bean and Lupine produce alkalinity in nearly all the sugars tested by them, while those of Alfalfa, Pea, Bean, and Red Clover show mostly acidic reaction. However, variations in their reactions were observed in the case of different strains of the same organism. It is accepted as a common rule that the organisms belonging to the fast-growing group produce acidity, especially in galactose, and this is considered to be one of the best criteria of differentiating the organisms of the two groups.

It was our point to enquire into the constancy of these findings in the case of the organisms under research.

In our experiments we used the medium recommended by Baldwin and Fred (1927) having the following formula :—

Agar	.. ..	25·0 gm.
Di-potassium phosphate	.. ..	0·5 ,,
Potassium nitrate	.. ..	0·5 ,,
Magnesium sulphate	.. ..	0·2 ,,
Sodium chloride	.. ..	0·2 ,,
Calcium carbonate	.. ..	3·0 ,,
Sugar or any organic carbon compound	..	10·0 ,,
Distilled water	.. ..	1000·0 c.c.

5 c.c. of 0·5% alcoholic solution of brom-thymol-blue added per litre to indicate the changes in the acidity.

As usual to avoid hydrolysation of the sugars these were added last in the preparation of the medium. Tubes were placed in the autoclave in small batches after the steam began to flow freely. They were sterilised at one atmosphere pressure and rapidly cooled by transferring them to the refrigerator as soon as the pressure went down. Sugars belonging to various series, *i.e.*, pentoses, hexoses, disaccharides and trisaccharides and mannitol, were used and the growth of the organisms on them and their reaction were studied. The results of these studies are given in Tables 1 and 2.

TABLE 1.

Growth of the Organisms on a Nitrate Medium containing various Carbon Compounds.

Sugars	*Cajanus indicus* organism	*Dolichos biflorus* organism	*Psophocarpus tetragonolobus* organism	*C. indicus* concomitant organism
Rhamnose ..	++	++	++	++
Arabinose ..	++	++	++	++
Xylose ..	++	++	+	++
Fructose ..	++	++	++	++
Glucose ..	++	++	++	+++
Galactose ..	++++	++++	++	+++
Mannose ..	+++	++	+	++
Sucrose ..	++++	++++	++++	++++
Lactose ..	+++	++	+	++
Maltose ..	+++	+++	++	+++
Raffinose ..	++++	+++	+++	+++
Dextrin ..	+	+	+	+
Mannitol ..	++++	+++	+++	++++

+ Scanty growth ; ++ Moderate growth ; +++ Good growth ; ++++ Profuse growth.

TABLE 2.

Fermentation Characteristics of the Organisms on a Nitrate medium containing various Carbon Compounds.

Sugars	*Cajanus indicus* organism	*Dolichos biflorus* organism	*Psophocarpus tetragonolobus* organism	*C. indicus* concomitant organism
Rhamnose ..	−−	++	++	−−
Arabinose ..	−−	++	++	−−−
Xylose ..	−−	+++	+	−
Fructose ..	+	+++	++	−
Glucose ..	−−	+++	+	−−−
Galactose ..	−−−	+++	+++	−−−
Mannose ..	−−	++	++	−−
Sucrose ..	++	+++	+++	−−
Lactose ..	−−	++	++	+
Maltose ..	++	+++	+++	+
Raffinose ..	++	+++	++	+
Dextrin ..	+++	+++	+++	+++
Mannitol ..	−−	++	++	..

\+ Slightly alkaline ; ++ Moderately alkaline ; +++ Strongly alkaline.
− Slightly acid ; −− Moderately acid ; −−− Strongly acid.

From the examinations of the tables it will be clear that sucrose and mannitol give best growth. Raffinose stands next. Poor growth was observed on pentoses and dextrin.

From the fermentation characters it is clear that the *D. biflorus* and *Ps. tetragonolobus* organisms produce alkaline change in all the carbohydrate compounds used, agreeing in this respect with the slow-growers, while *Cajanus indicus* organisms and its concomitant both have acid and alkaline reaction depending on the sugars. Judging from their reaction on galactose they behave as fast-growers. It is interesting to note that all the organisms at the start show an alkaline reaction in all the sugars,

while in the end the reactions are as indicated in the tables. The results of two weeks' observations are reported in these tables.

Chapter III.

To establish conclusively the identity of the organism by the biological test we decided to grow plants in flasks and test-tubes containing medium as advocated by Garman and Didlake. This is very handy and convenient method of growing plants in sterile conditions. It does not require too much attention to keep the experiment going and maintain sterile conditions. (See Plate VI.)

For this purpose we first selected the medium for growing seedlings, recommended by Thornton, having the following formula :—

Di-potassium phosphate	..	0·5	gm.
Magnesium sulphate	..	0·2	,,
Sodium chloride	..	0·1	,,
Calcium phosphates	..	2·0	,,
Ferric chloride	..	0·01	,,
Agar	..	10·0	,,
Water	..	1	litre.

This medium was put in 50 c.c. and 150 c.c. amounts respectively into big Pyrex test-tubes of 100 c.c. and Erlenmeyer flasks of 250 c.c. They were plugged and sterilised at one atmosphere pressure. Seeds of *Cajanus indicus*, *Dolichos biflorus* and Goa-bean, Lucerne, Cowpea and Soy-bean were sterilised in the same way as the nodules by subjecting them to the action of 0·2% mercuric chloride solution and exhausting the air to insure complete contact of the disinfecting solution with the whole surface of the seeds. Fifty seeds of each of the two plants *C. indicus* and *D. biflorus* were put in a tube ($1\frac{3}{4}$ " by 7") and washed together. In the case of *Ps. tetragonolobus* on account of the size we used about 15 (fifteen) seeds.

Sterilisation of seeds was continued (3 to 5 minutes) for a longer time than in the case of nodules, as it was found that they were not injured in any way by this prolonged action. This was manifested by the fact that all the seeds germinated and produced fair growth as was also proved by Fellers (1919). After sterilisation of the seeds they were washed in several (at least six) changes of sterile water. The last wash as suggested by us in the case of nodule washing was plated out to make sure of the sterile condition of the seeds. They were embedded aseptically in the surface of the agar, two seeds in the case of every test-tube and four seeds in every flask, taking care that the seeds were placed near the walls so that the growth of the roots and the formation of nodules could be conveniently seen. As

soon as the seeds germinated, they were inoculated with a loopful of the culture to be tested by spreading the inoculum near the seedlings, leaving some flasks with their seedlings alone as control. The seeds were not inoculated with the organisms before sowing, as is a common practice, paying attention to the remark of Hiltner and Stormer (1930 *a*) that direct inoculation of large seeds may not lead to nodule formation, or to the contention of Haas and Fred (1919) that the mercuric bichloride remaining in the seed coat has an injurious effect on bacteria, although this is not likely to occur in our method. They were allowed to grow till they wilted. Growth was quite good in all cases. After a period of three weeks from their germination a few plants were taken every week and examined for nodules. In all cases, whether inoculated with the organisms or not, we found that no nodules had been formed.

This experiment was repeated several times with the above-mentioned medium but we always obtained negative results. It was thought proper to try some other medium and repeat the experiment. Ashby's modified medium recommended by Fellers (1919) was chosen having the formula given below :—

Mannite	.. ..	10·0 gm.
Monopotassium phosphate	.. ..	0·2 ,,
Magnesium sulphate	.. ..	0·2 ,,
Calcium sulphate	.. ..	0·1 ,,
Calcium carbonate	.. ..	1·0 ,,
Distilled water	.. ..	1000·0 c.c.
Agar	.. ..	10·0 gm.

In this case too, as before, negative results were obtained. Then we proceeded to use Bean extract medium as we had found all along our work that it gave the best growth for nodule bacteria. Again we obtained negative results.

In our above-mentioned experiments we had the flask and tubes exposed to the light. Although we have sufficient evidence to be rather sceptical about the destructive action of light on nodule bacteria, we thought to exclude the possibility of that objection being raised against our experiments. Consequently another experiment was started with the above-mentioned three media and the flasks covered with black papers. This time too we found the plants and the roots quite well developed but no nodules were formed on the inoculated plants.

At this stage we are not in a position to say why the method of Garman and Didlake, 1914, which is so handy and convenient is not successful in

India. Joshi (1920) also seems to have tried the method and abandoned it. He writes, "for cross-inoculation the plants which are to be inoculated may be grown in (1) nutrient solution contained in flasks or test-tubes, (2) in agar contained in flasks or in test-tubes, which is advocated by Garman, or (3) in sand in pots.

" The first two methods though convenient were not found very successful in this laboratory on a previous occasion and were therefore thought unsuitable for cross-inoculation."

We did not continue our work in this direction, as the method was not absolutely necessary for the study of the physiological reactions of the organisms. We visualise the possibility of adjusting the conditions so as to make this method of culturing plants and inoculating them with nodule organisms a successful procedure. It is surmised that by changing the conditions of the procedure and perhaps also the condition of physical texture of the medium, this method can be made successful. We hope to devote in future some attention to this work.

The other method of growing the plants in sterile soil or sand was adopted. We attempted these experiments with ordinary flower-pots.

The flower-pots, of about eight inches diameter, were first sterilised in the autoclave at 120°C. for an hour on two consecutive days. The soil was first charred and then the pots were filled with it and autoclaved for one hour in order to accomplish complete sterilisation as mentioned by Brown and Stallings (1921). Seeds were sterilised by the method mentioned before. They were then transferred to the pots with sterile forceps and sown into soil and watered with ordinary tap water. At the close of the experiment we found the results were not consistent. So we changed the procedure. The chances are that soil might not be properly sterilised. It is very difficult to sterilise properly the ordinary soil even if the process is carried out for hours on end. We also adopted sterile water for watering the plants. The water coming from our Stoke's still (1933 model) is found to be quite suitable.

The second set of experiments were arranged as suggested by Koch and Butler (1918). Earthen pots were filled with burnt and sterilised sand to which the following inorganic salts were added: 12·5 gm. of calcium carbonate, 10·0 gm. calcium phosphate, 5·0 gm. potassium sulphate and 1·3 gm. magnesium sulphate per 25 kilogrammes of sand. The pots were saturated with water, covered with paper and sterilised in the autoclave for two hours at one atmosphere pressure on two successive days.

After sterilisation the sterilised seeds were sown and watered with sterile water. When the seedlings began to appear the pots were inoculated

by means of a sterile pipette with the organisms to be tested, using a heavy emulsion of them. It was found that plants died in about three weeks before we could expect nodule production.

As the method of Koch and Butler does not seem to be without objection and the inconsistency of results may seriously upset the plans of delicate experiments we decided to proceed independently and devise a method of growing the plants without fear of contaminations which could be relied upon to work under our conditions and with our Indian plants. An important point here is not only to set the experiment going under sterile conditions but to maintain these conditions all through without undue complication either in the apparatus or in the manipulation. Our procedure was as follows :—

The sand was washed with ordinary tap water several times till it showed very slight indication of presence of nitrate (Ring test). It was then heated in flat metal dishes ("lagan") in the hot oven for twenty-four hours continuously at 120°C. to eliminate any nitrogen nutrient compounds and at the same time to sterilise it as thoroughly as possible. The sand was well stirred every few hours so that the heat would reach every portion of it. The pots were sterilised at 120°C. for half an hour on two days. They were then filled with burnt sand. Four small portions of glass tubes open at both ends, having a bore wide enough to let the seeds pass through were fixed in the sand in each pot at equal distance from the centre. A big mouthed test-tube with the bottom cut was thrust into the centre of the pots. All these were plugged with cotton wool. Before fixing the tube in the sand the pot was covered with two or three folds of cotton cloth which was tied round under the rim of the pot with a string. This cotton cloth was perforated just enough to allow the tubes to protrude. If the holes happen by accident to leave too much space around the protruding tube the gap may be plugged with cotton wool. The whole pot so prepared was sterilised at 120°C. for half an hour.

The seeds sterilised by the method described before were placed into the sand through the small tubes under aseptic conditions. They were then watered one by one with sterile water through the small tubes and these plugged again. Watering with sterile water through the individual tube was continued for about five to seven days till the seeds germinated. The watering was then effected through the special big tube placed in the centre. They were then inoculated with a heavy emulsion of the organisms to be tested. When the seedlings grew long enough to come out of the tube, the plugs were removed, the seedlings were allowed to come out freely and sterile cotton was wrapped round their stems. All these manipulations

were done as much as possible under aseptic conditions (see Plate VII, Nos. 5 and 6). After inoculation the plants were also watered with sterile water through the test-tube fixed in the centre of the pot, plugging the tube every time after watering. Plants were supplied every week through the watering tube with Crone's modified solution as used by Bryon having the formula as follows :—

Potassium chloride	.. ..	100·0 gm.
Calcium sulphate	.. ..	25·0 ,,
Calcium phosphate	.. ..	25·0 ,,
Iron phosphate	.. ..	25·0 ,,

For the solution one and half grammes of this mixture were added to one litre of distilled water. In addition to this 0·75 gm. of sodium carbonate was added to it. The mixture was well shaken and allowed to settle overnight, then it was filtered and autoclaved before using.

Plants were allowed to grow in this way and then gradually removed a few every other week to examine nodule formation. By this method we got quite consistently successful results. Consequently in all the experiments that followed we adopted this procedure and it is satisfactory to mention that we never met with any complication whatsoever.

In this manner we first tried to test the identity of the organism isolated from *Cajanus indicus, Dolichos biflorus* and *Psophocarpus tetragonolobus* by growing the seeds of the plants in sterile pots by the method explained above and inoculating them with their own respective cultures. (See Plates VII–X.)

It was found that the concomitant, isolated from the root nodules of *Cajanus indicus* was not able to produce nodules on the roots of the same plant, *i.e., Cajanus indicus,* grown in sterile conditions and inoculated with the organism in question. This experiment was repeated many times with the same negative result.

Hence we tried to inoculate the same organism into the sand in which *Dolichos biflorus* and Goa-bean seeds were sown in sterile conditions. In this experiment we also got negative results. Whilst the other organism isolated from the root nodules of *Cajanus indicus,* which did not possess so much mucilage, when inoculated into the sand in which plants of *Cajanus indicus* were grown, did produce nodules on the roots, the control showing negative results. Similarly the organisms isolated from *D. biflorus* root nodules and Goa-bean root nodules could produce nodules on the respective plants proving the identity of both the organisms. This experiment was counter-proved several times.

Another experiment on the same lines as above was started to find out the probabilities of trans-inoculation in these organisms. It was noticed that all these organisms could cross-inoculate every other plant. The concomitant as stated before did not produce nodules in any of them. The result is given in Table 3.

A further experiment was done with the true *Cajanus indicus* organism to investigate whether it could produce nodules on the roots of Lucerne, Cowpea and Soy-bean plants grown in sterile conditions. It was noted that *Cajanus indicus* organism could not produce nodules on either Soy-bean or Lucerne plants. It produced nodules on the Cowpea, which is in keeping with the cross-inoculation classification commonly accepted.

TABLE 3.

Results of Trans-Inoculations of Cajanus indicus, Dolichos biflorus *and* Psophocarpus tetragonolobus *Plants with the Four Nodule Bacteria isolated from the Same Three Plants.*

Organisms	*Cajanus indicus*	*Dolichos biflorus*	*Psophocarpus tetragonolobus*
C. indicus ..	+	+	+
D. biflorus ..	+	+	+
Ps. tetragonolobus ..	+	+	+
Concomitant bac. ..	—	—	—

TABLE 4.

Results of Trans-Inoculation of Cajanus indicus *Organism on Cowpea, Lucerne and Soy-bean Plants.*

Organism	Cowpea	Lucerne	Soy-bean
Cajanus indicus ..	+	—	—

Conclusions.

1. The physiological reactions of the three types of Indian nodule bacteria (*C. indicus, D. biflorus* and *Ps. tetragonolobus*) have been studied and ascertained, as is indicated in the tables.

2. In the isolation of the nodule organism from *Cajanus indicus* roots we found with certain characters of constancy a contaminant or rather a concomitant, which has not the characteristics of *B. radiobacter*, the only organism likely to be met with.

3. The *Cajanus indicus* root nodule organism is placed by various authors Löhnis and Leonard, in the Cowpea group, the members of which have got an alkaline reaction on milk medium and also on nearly all sugars. The organism of *C. indicus* isolated by us differs in this respect from the members of the Cowpea group in having acid reaction on litmus milk, and mixed reactions on sugars, acidic and alkaline.

4. The use of gelatin stabs, commonly followed in the study of gelatin reactions,may account for much of the irregularity of reaction and disagreement observed in the conclusions of different authors. Our procedure of the gelatin slants gives clear results, sometimes in half the time, and great advantage will be derived from its general adoption.

5. Garman and Didlake's method proved successful as regards the culturing of host plants, but not as regards the nodule formation. Pending the study of a modification of the same, we devised an easy and reliable method of pot cultures which can be depended upon to give clear results in our exacting conditions of temperature and moisture.

BIBLIOGRAPHY.

(We are indebted to Fred, Baldwin and McCoy (1932) for the best guide in the bibliography of the subject of nodule bacteria.)

Almond, L. .. .. "Root nodule bacteria and legume plants by Fred, E. B., Baldwin, I. L., and McCoy, E.," 1932, 95.

Ashby, S. F. .. .. "Some observations on the assimilation of atmospheric nitrogen by a free living soil organism—*Azotobacter Chroococum* of Beijerinc.," *Jour. Agr. Sc.* (England), 1907, **2**, 35–51.

Bailey, H. D. .. .. "Practical flagella and capsule stain for bacteria," *Sci.*, 1930, **72**, 95–96.

Baldwin, I. L., and Fred, E. B. "Nomenclature of the root nodule bacteria of the leguminosæ," *Jour. Bact.*, 1929, **17**, 141.

Baldwin, I. L., and Fred, E. B. "The fermentation characters of the root nodule bacteria of the leguminosæ," *Soil Sci.*, 1927, **24**, 217–230.

Bergey, D. H. .. .. *Bergey's Manual of Determinative Bacteriology*, Bailliere, Tindall & Cox, London, 1930.

Boerner, F. I. .. .. See Kolmer, J. A., and Boerner.

Brown, P. E., and Stallings, J. H. "Inoculated legumes as nitrogenous fertilizers," *Soil Sci.*, 1921, **12**, 365–407.

Buchanan, R. E., and Fulmer, E. I. *Physiology and Biochemistry of Bacteria*, Bailliere, Tindall & Cox, London, 1928–1930, 3 vols.

Burril, . . . and Hansen, . . . "Is symbiosis possible between legume bacteria and non-legume plants," *Illinois Agr. Expt. Sta.*, 1917, **202**, 115–181.

Clark, ... "Suggestions concerning legume inoculations," *Mich. Agr. Expt. Sta.*, 1905, **231**, 223–230.

Corbet, A. Steven .. .. *Biological Processes in Tropical Soils*, W. Heffer & Sons, Cambridge, 1935, 93.

Eckhardt, M. M. Baldwin, I. L., and Fred, E. B. "Studies of the root nodule organism of *Lupinus*," *Jour. Bact.*, 1931, **21**, 273–285.

Fellers, C. R. .. .. "The longevity of *B. radicicola* on legume seeds," *Soil Sci.*, 1919, **7**, 217–232.

Fred, E. B., Baldwin, I. L., and McCoy, E. *Root Nodule Bacteria and Legume Plants*, 1932.

Fred, E. B., and Davenport, A. "Influence of reaction on nitrogen assimilating bacteria," *Jour. Agr. Research* (U.S.), 1918, **14**, 317–336.

Fulmer, E. I. .. .. See Buchanan, R. E.

Garman, H., and Didlake, Mary "Six different species of nodule bacteria," *Kentucky Agr. Expt. Sta. Bull.*, 1914, **184**.

Greig Smith, B. .. .. "The determination of Rhizobia in the soil," *Centbl. Bakt.* (etc.), 2 Abt., 1912, **34**, 227–229.

Haas, A. R. C., and Fred, E. B. "The effect of soy-bean germination upon the growth of its nodule-forming bacteria," *Soil. Sci.*, 1919, **7**, 237–245.

Harrison, F. C., and Barlow, B. "The nodule organism of the Leguminosæ—its isolation, cultivation, identification and commercial application," *Centbl. Bakt.* (etc.), 1907, 2 Abt., **19**, 264–272, 426–441.

Hiltner, L., and Stormer, K. .. "Neu Untersuchungen über die Wurzelknöllchen der Leguminosen und deren Erreger," *Arb. K. Gsndhteamt. Biol. Abt.*, 1930, **3**, 151–307.

Hooker, C. B. .. .. *The Flora of British India*, 1879, **2**, 210–217.

Joshi, N. V. .. .. "Studies on the root nodule organism of the leguminous plants," *Memoirs Dept. Agr. India Bact. Ser.*, 1920, **1**, No. 9.

Kellerman, K. F. .. .. "The reaction of crown gall to legume inoculation," *U.S. Dept. Agr. Bur. Plant Indus.*, 1911, Circ. **76**, 6.

Koch, G. P., and Buller, J. R. "Cross inoculation of legumes," *Soil Sci.*, 1918, **6**, 396–403.

Kolmer, J. A., and Boerner, . . *Approved Laboratory Technic*, Appleton & Co., New York, London, 1921.

Laurent, E. .. .. "Recherches sur les nodosités des Legumineuses," *Ann. Inst. Pasteur*, 1891, **5**, 105–139.

Leonard, L. T. .. .. "*Bacillus radiobacter* in reference to commercial legume inoculants," *Jour. Bact.*, 1931, **21**, 35–36.

Levine, N., and Schoenlein .. *A Compilation of Culture Media for the Cultivation of Micro-organisms*, Bailliere, Tindall & Cox, 1930.

Lipman, C. B., and Fowler, L. W. "Isolation of *Bacillus radicicola* from soil," *Science*, (N. S.), 1915, **41**, 256–259.

Löhnis, M. P. .. .. "Can *Bacterium radicicola* assimilate nitrogen in the absence of the host plant?", *Soil Sci.*, 1930, **29**, 37–57.

Löhnis, F., and Hansen, R. .. "Nodule bacteria of leguminous plants," *Jour. Agr. Research* (U.S.), 1921, **20**, 543–556.

Löhnis, F., and Leonard, L. T. "Inoculation of legumes and non-legumes with nitrogen-fixing and other bacteria," *U.S. Dept. Agr. Farmers' Bull.*, 1926, **1496**, 28.

Maneval, W. C. .. .. "The staining of flagella of bacteria with special reference to mordants," *Jour. Bact.*, 1931, **21**, 313– .

Maze, M. .. .. "Fixation de l'azote libre par le bacille des nodosités des légumineuses," *Ann. Inst. Pasteur.*, 1897, **11**, 44–45.

McClung, L. S. .. .. "A note on reaction changes during sterilisation of vegetable extract culture media," *Jour. Bact.*, 1932, **24**, 452– .

Müller, A., und Stapp, C. .. "Beiträge sur Biologie der Leguminoser Knöllchenbakterien mit besonderer Berücksichtigung ihrer Artverschiedenheit.," *Arb. Biol. Reichsants. Land u. Forstw.*, 1925, **14**, 455–464.

Russell, Sir E. J. .. .. *The Micro-Organisms of the Soil*, Longmans, Green & Co., London, 1932.

Schönberg, ... "Untersuchungen über das Verhalten von *Bact. radicicola*, Beij., gegenüber vershiedenen Kohlenhydraten und in Milch.," *Centralbt. Bact.*, 1929, **79**, II Abt., 205–221.

Schoenlein, H. W. .. .. See Levine, N., and Schoenlein.

Shunk, J. V. .. .. "Notes on the Flagellation of the Nodule Bacteria," *Jour. Bact.*, 1921, **12**, 239– .

Stapp, C. .. .. "Beitrage Sum Studium der Bakterientyrosinase," *Biochem. Ztschr.*, 1923, **141**, 42–69.

Stevens, J. W. .. .. "The value of litmus, brom-cresol purple and janus-green milk in a study of the nodule organisms of Leguminosæ," *Jour. Agr. Research* (U.S.), 1925, **31**, 997–1000.

Temple, J. C. .. .. "Studies of *Bacillus radicicola*: I.—Testing commercial cultures; II—Soil as a medium," *Ga. Agr. Expt. Sta. Bull.*, 1916, **120**, 65–80.

Thornton, H. G. .. .. "On the development of a standardized Agar medium for counting soil bacteria with especial regard to the repression of spreading colonies," *Ann. App. Biol.*, 1922, **9**, 241– .

Thornton, H. G. .. .. "Lucerne 'Inoculation' and the factors affecting its success," *Imp. Bur. Soil Sci., Techn. Commun.*, No. 20.

Walker, R. H. .. .. "Physiological studies on the nitrogen-fixing bacteria of the genus *Rhizobium*," *Iowa Agr. Expt. Sta. Bull.*, 1928, **113**.

Wright, W. H. .. .. "The nodule bacteria of soy-beans: I.—Bacteriology of strains," *Soil Sci.*, 1925, **20**, 95–129.