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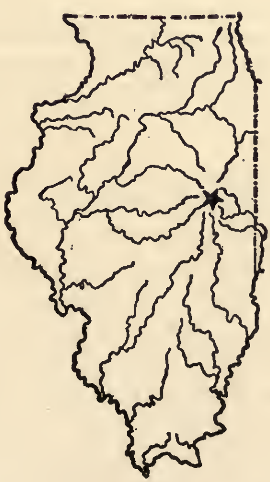
BULLETIN No. 202

IS SYMBIOSIS POSSIBLE BETWEEN LEGUME BACTERIA AND NON-LEGUME PLANTS?

By THOMAS J. BURRILL and ROY HANSEN



URBANA, ILLINOIS, JULY, 1917

Contents of Bulletin No. 202

Illustrations

FOREWORD

This bulletin reports the last work of Thomas Jonathan Burrill, who in 1880, thru studies of pear blight, first experimentally proved the fact that plant diseases are sometimes caused by bacterial invasion.

Symbiotic relationships early attracted the attention of Dr. Burrill, especially the relationship existing between certain nitrogen-gathering bacteria and legumes. In those days every discovery gave rise to new and fundamental questions, and the query whether such relation is necessarily confined to legumes was always in his mind, and was put aside only by the urgency of pressing duties.

When after retirement from active service, opportunity came to Dr. Burrill for following his inclination, his attention at once reverted to the old-time problem, and he fitted up a laboratory and employed an assistant for its study. Here he devoted the last three years of his life, and here the call came suddenly on April 14, 1916, forty-eight years to a month after his coming to the University of Illinois.

Especial credit is due the junior author for his faithful and hopefully successful attempt at accurately reporting the work as planned by his chief, and so far as is humanly possible correctly interpreting his ideas and convictions.

In this difficult task Mr. Hansen has been aided by Dr. A. L. Whiting with some special knowledge of the technical material involved, and by Professor C. F. Hottes, for a quarter of a century Dr. Burrill's close associate, who has read the manuscript with the view of insuring that so far as possible the spirit and thought of the pioneer investigator is expressed.

E. Davenport
Director

IS SYMBIOSIS POSSIBLE BETWEEN LEGUME BACTERIA AND NON-LEGUME PLANTS?

By THOMAS J. BURRILL, Professor of Botany, Emeritus, and
ROY HANSEN, Assistant in Nitrogen-Fixation Research

INTRODUCTION

The work reported in this bulletin deals with an attempt to develop a symbiosis between legume bacteria and non-legume plants similar to that which exists between legume bacteria (*Pseudomonas radicicola*) and legume plants.

Since the demonstration, in 1886, by Hellriegel and Wilfarth of the symbiotic fixation of atmospheric nitrogen by legume plants and certain microorganisms, no crop rotation has been considered rational that does not include a liberal use of legumes. The importance of this discovery to agriculture is generally appreciated. That it is applicable thruout the world makes it of especial value to mankind.

The benefit that would result could other ordinary farm crops be enabled to utilize atmospheric nitrogen would be inestimable; hence the importance of any success in this direction. In attempting to study this question it was fully realized that success might not be attained, but that it was in the realm of possibilities. It was nearly a quarter of a century ago that the first work was done under the direction of the senior author. Since that time a few attempts have been made by other workers to grow legume bacteria on mustard and grasses, but with negative results.

In returning to this problem, the authors found it necessary at first to spend considerable time in acquiring an intimate acquaintance with the organism concerned, especially in regard to its cultivation and identification. Attention was given to the special adaptations, or varieties, of the symbiotic bacteria in order to learn, first, whether these adaptations were constant or subject to change; and second, what factors were responsible for their existence. Histological studies of the nodule were undertaken with the view of learning something of the relations existing between the two symbionts. The nodules of certain non-legume plants (*Ceanothus, Cycas, Elaeagnus,* etc.), said to be concerned in the fixation of atmospheric nitrogen, were given some attention in the hope that perhaps here lay a start. Cross-inoculations of importance and interest were found and are reported as a part of this contribution. Some preliminary trials were made attempting the inoculation of non-legume plants with the legume organism.

Part I.—THE ORGANISM

ISOLATION AND CULTIVATION

Media.—Pseudomonas radicicola was cultivated on many kinds of media differing widely in composition, and it was found that it would thrive on most of them. For plating out, Harrison and Barlow's wood-ash agar was usually used, as it gave more uniform results. Many media were unsuitable for plating, yet permitted growth upon agar slants.

A list of media employed in these experiments for cultivating *Ps. radicicola,* together with the composition and reaction of each, is given in Table 1.

TABLE 1.—COMPOSITION AND REACTION OF MEDIA USED IN CULTIVATING *Pseudomonas radicicola*

Laboratory No.	Medium	Composition		Reaction[a]
100	Wood ash (Harrison and Barlow)	Wood-ash extract (15 gms. ashes to 1 liter tap water)	1000 cc.	Not changed; usually +7° to +10° to phenolphthalein
		Saccharose	10 gms.	
		Monopotassium phosphate	3 gms.	
101	Synthetic (Fred)	Distilled water	1000 cc.	Titrate to +10°
		Dextrose	20 gms.	
		Monopotassium phosphate	1 gm.	
		Magnesium sulfate	.1 gm.	
		Sodium chlorid	Trace	
		Ferrous sulfate	"	
		Manganous sulfate	"	
		Calcium chlorid	"	
102	Mannite (Ashby)	Distilled water	1000 cc.	Not changed
		Mannite	20 gms.	
		Dipotassium phosphate	.2 gm.	
		Magnesium sulfate	.2 gm.	
		Sodium chlorid	.2 gm.	
		Calcium sulfate	.1 gm.	
		Calcium carbonate	5 gms.	
103	Synthetic (Spratt)	Distilled water	100 cc.	Titrate to +10°
		Cane sugar	1 gm.	
		Dipotassium phosphate	.5 gm.	
		Magnesium sulfate	.02 gm.	
		Calcium carbonate	.1 gm.	
104	Asparaginate (Conn)	Distilled water	1000 cc.	Not changed; usually +6° to +8°
		Sodium asparaginate	1 gm.	
		Dextrose	1 gm.	
		Magnesium sulfate	.2 gm.	
		Ammonium phosphate[b]	1.5 gms.	
		Calcium chlorid	.1 gm.	
		Potassium chlorid	.1 gm.	
		Ferric chlorid	Trace	

[a]Fuller's scale in all cases.
[b]Used in place of mono-ammonium phosphate.

TABLE 1.—*Continued*

Laboratory No.	Medium	Composition	Reaction
105	Beef broth	Tap water 1000 cc. Witte's peptone 10 gms. Beef extract (Liebig's) 5 gms.	Titrate to +10°
106	Legume extract, using bean plant	Extract of bean plant (Heat 100 gms. roots and stems in 1 liter tap water ½ hour at 60° C.) 1000 cc. Cane sugar 20 gms.	Titrate to +10°
107	Bean-extract peptone	Same as 106, plus 1 percent peptone (Witte's)	Titrate to +10°
108	Legume extract, using sweet clover	Sweet-clover extract 1000 cc. Cane sugar 20 gms.	Titrate to +10°
109	Sweet-clover-extract peptone	Same as 108, plus 1 percent peptone	Titrate to +10°
110	Tomato infusion	Tomato extract (100 gms. plant substance to 1 liter water) 1000 cc. Cane sugar 20 gms.	Titrate to +10°
111	Tomato-infusion peptone	Same as 110, plus 1 percent peptone	Titrate to +10°
200	Wood-ash agar	Same as 100, plus 1 percent agar	Not changed
201	Synthetic agar (Fred)	Same as 101, plus 1 percent agar	Titrate to +10°
202	Mannite agar (Ashby)	Same as 102, plus 1 percent agar	Not changed
203	Synthetic agar (Spratt)	Same as 103, plus 1 percent agar	Titrate to +10°
204	Asparaginate agar	Same as 104, plus 1 percent agar	Not changed
205	Beef-broth agar	Same at 105, plus 1 percent agar	Titrate to +10°
206	Bean-extract agar	Same as 106, plus 1 percent agar	Titrate to +10°
207	Bean-extract-peptone agar	Same as 107, plus 1 percent agar	Titrate to +10°
208	Sweet-clover-extract agar	Same as 108, plus 1 percent agar	Titrate to +10°
209	Sweet-clover-extract-peptone agar	Same as 109, plus 1 percent agar	Titrate to +10°
210	Tomato-extract agar	Same as 110, plus 1 percent agar	Titrate to +10°
211	Tomato-extract-peptone agar	Same as 111, plus 1 percent agar	Titrate to +10°
300	Wood-ash gelatin	Same as 100, plus 12 percent gelatin	Not changed
305	Beef-broth gelatin	Same as 105, plus 12 percent gelatin	Titrate to +10°
420	Potato slant		
421	Tomato-stem slant	Fresh young tomato stems in distilled water	

Isolation.—In isolating the organism the following method adapted from that of Harrison and Barlow[32a] was used: Where choice is possible select a medium sized nodule appearing young and sound. In cutting it off leave two or three millimeters of the root on both sides of the nodule to permit handling it with forceps. Wash carefully, rinse in distilled water, and drop into a sterilizing fluid made as follows:

Distilled water	500 cc.
Bichlorid of mercury	1 gm.
Hydrocloric acid (C. P.)	2.5 cc.

Shake the nodule violently in this solution for one or two minutes, after which wash it three times with sterile distilled water. Then cover with about 1 cc. of sterile distilled water and crush with a heavy glass rod, previously flamed and cooled. Pour two or three drops of the cloudy suspension into a test tube of ash agar[b] at 45° C. Inoculate a second tube of the agar with five loops from the first, and pour plates. When a large nodule is used, inoculate the first tube with five loops of the suspension, and inoculate the second tube with five loops from the first, and pour plates. Only two plates are poured; a third was found unnecessary. Incubate plates at from 20° to 25° C. Replating is usually unnecessary, altho it is a safe practice with questionable plates. If the sterilization and washing are carefully done, foreign organisms seldom appear.

Cultivation.—For keeping stock cultures ash agar was used. Transfers were made once a month, tho cultures may easily be kept six weeks or two months between transfers. Plate colonies should be large enough for transfer in six to fourteen days, depending upon the host plant used and other conditions.

MORPHOLOGY

Agar Colonies.—In general the colonies appearing on agar plates may be divided into two types, buried and surface colonies.

Buried colonies are small and submerged, most frequently lens, or spindle shaped, with smooth and even edges. They are quite opaque, granular in structure, and in color are cream to a chalk white. They increase slowly in size, eventually appearing on the surface of the agar as surface colonies, when the growth becomes rapid. The

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[a]Superior figures are used to indicate the literature citations having special reference to this work which are given in the bibliographies.

[b]Obtain ashes from thoroly burned hard wood and run thru a fine sieve. (Little difference was found in different lots of ashes.) Use 15 grams to one liter of tap water and bring to a boil over a free flame, stirring at intervals. Allow solution to stand from five to ten minutes, then filter thru a double filter. To one liter of ash extract add 10 grams of saccharose, 3 grams of KH_2PO_4, and 10 grams of agar. Autoclave for fifteen minutes, filter thru absorbent cotton, and proceed as with other media. The reaction usually was $+7°$ to $+10°$ (Fuller's scale) to phenolphthalein, and was never changed.

lens colonies, however, remain visible for many days in the center of the new growth.

Surface colonies originate at or near the surface of the agar or develop from buried colonies. They are drop-form, watery, mucilaginous (in appearance, tho not always to the touch), gray-white to pearly white in color, glistening, and semitranslucent to opaque. The edges are smooth and even. Under the low power the interior is granular. They frequently attain considerable size, a centimeter or more in diameter.

Plates made direct from the nodule lack uniformity to a marked degree. The undiluted plate (first plate) begins to show a few colonies in two to four days. These colonies become extremely large in a very short time, their rapid growth being due to small pieces of nodule tissue or to clumps of bacteria carried over into the agar (see Plate I). In five or six days numerous colonies begin to make their appearance, most of them as submerged colonies, which later grow to the surface.

The dilution-plate (second-plate) colonies are always extremely slow in growth. Generally colonies are large enough for transfer in six to fourteen days, tho plates should not be discarded for two or even three weeks.

The rate of growth of colonies also varies with the organisms of different nodules (see Plate II). Among the fast growers are the organisms from the pea (*Pisum*), vetch (*Vicia*), lentil (*Lens*), sweet pea (*Lathyrus*), bean (*Phaseolus*), lupine (*Lupinus*), wild bean (*Strophostyles*), clover (*Trifolium*), sweet clover (*Melilotus*), alfalfa (*Medicago*), and fenugreek (*Trigonella*). The organisms appreciably slower in growth are those from the cowpea (*Vigna*), Japan clover (*Lespedeza*), tick trefoil (*Desmodium*), acacia (*Acacia*), partridge pea (*Cassia*), false indigo (*Baptisia*), dyer's greenweed (*Genista*), peanut (*Arachis*), soybean (*Glycine*), and hog peanut (*Amphicarpa*).

The Bacteria.—The life cycle of *Pseudomonas radicicola* from the soil thru the nodule and back to the soil is clouded in doubt because of the extreme variability of the organism under apparently the same conditions. While it has been isolated from soil (see Lipman[41 42]), there is no clue to the form in which it existed in the soil.

Observation of cowpea nodules showed that in the very young nodules there is considerable variation in size and shape of the organisms. Many of the small, oval forms, the swarmers described by Beyerinck,[9] are found. These forms and the normal rods predominate. Large club-shaped bacteroids are frequent; the characteristic branched forms are not so numerous. The bacteroids are best demonstrated when the young nodule is just beginning to show a reddish interior. At this stage they are extremely large and contain the maximum staining substance (see Plate III). The characteristic X and Y forms occur in great numbers; they show considerable vacuolation and unevenness in staining, especially when stained with carbolfuchsin.

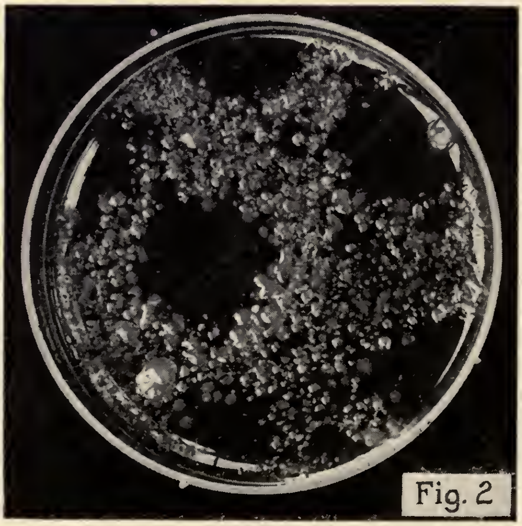

PLATE I

Fig. 1.—Ash-agar plate from bean (*Phaseolus vulgaris*), showing giant colonies in a thickly seeded plate

Fig. 2.—Ash-agar plate from perennial pea (*Lathyrus latifolius*); the clear spaces are due to sterilizing fluid carried over with pieces of nodule tissue

In the old, decomposing nodule the bacteroids are extremely vacuolated and ghost-like, showing small, oval, deep-staining bodies within. The inference is that these bodies are motile swarmers, which later free themselves from the ghost-like capsules, rather than bud off, as has been described by some writers. Frequently the swollen rods have a beaded appearance with unstained bands or areas. A few motile rods may sometimes be seen in hanging drops in this stage, and sometimes a bacteroid is seen to oscillate as tho swung about by some propelling force in one end. Division of the bacteroids into bacilli, as represented by Dawson,[23] may also occur.

When first plated out, the young colonies consist of small rods which show considerable variation in length. No bacteroids are present, tho the rods are sometimes slightly club-shaped and sometimes show vacuolation. However, they never attain the size of bacteroids. With frequent transfers the rods become quite uniform in size and stain deeply and evenly, especially with aniline-gentian-violet.

In very old cultures (three months on ash agar, without transfer) the small, oval swarmers and the normal rods predominate, tho a few club-shaped and a few branched bacteroids are found. The bacteroids produced upon artificial media[a] are never so large nor so numerous as those seen in mounts direct from a young nodule.

Staining.—The organisms do not stain well with ordinary aniline stains. Carbol-fuchsin and aniline-gentian-violet (used steaming) are the most satisfactory stains. Tho carbol-fuchsin was preferred, aniline-gentian-violet stains were always used as checks, because the former stain accents the vacuolated appearance, particularly in bacteroids. Carbol-fuchsin is especially useful in staining bacteroids direct from the nodule and also old agar cultures. Kiskalt's amyl-gram stain, described by Harrison and Barlow,[32] is useful since the amyl alcohol clears up the field, leaving the bacteria stained, tho not so intensely. This stain, however, should not be considered a means of identifying *Ps. radicicola.*

Bacteroids.—While *Ps. radicicola* produces no spores, it produces bacteroids which are very evidently more resistant than the normal rods. Unfavorable conditions, such as unsuitable media, infrequent transfer, or addition of caffein to the medium, cause their appearance. This is in accord with what takes place in the nodule. In the growing nodule, when development is most rapid, the bacteroids are at their maximum; they enable the organisms to multiply rapidly in spite of the resistance offered by the plant cells. Transferred to favorable media from this stage the normal uniform bacilli are produced. The bacteroid, then, must be regarded as a normal and a very necessary

[a]From the writers' observations this is equally true of the bacteroids produced by adding caffein to a legume-extract-agar medium, according to the method of Zipfel[37] and Fred.[35]

stage in the life of the organism. Its significance in the actual fixation of nitrogen, however, is pure speculation.

Motility.—The motility of the organism is best seen in young agar-slant cultures, twenty-four to forty-eight hours old. The bacteria dart about with amazing rapidity, now tumbling end over end, now spinning violently on the shorter axis, and then sweeping across the field in a darting, jerky course.

Flagella.—Owing to the gum or slime produced by the organism, the demonstration of flagella is especially difficult. The lack of agreement among investigators as to the number is shown in Table 2. The organisms reported by these investigators were all the most abundant producers of gum.

TABLE 2.—RESULTS OF PREVIOUS WORK UPON FLAGELLA STAINS

Investigator	Source of organism	Flagella	Remarks
Beyerinck 1888		One polar flagellum	Inferred during slow motility and not seen
Smith, R. G.[20] 1899		Exceedingly thin, single, terminal flagellum about 2 microns long and bearing upon the distal end a tuft, like the lash of a whip	One photomicrograph
Harrison and Barlow 1907	Hairy vetch (*Vicia villosa*) Perennial pea (*Lathyrus sativus*) Bean (*Phaseolus vulgaris*)	Single polar flagellum	Several figures; mucilage, or negative method, by which slime is stained leaving flagella unstained; discredited by Kellerman
De Rossi[30] 1907	Broad bean (*Vicia faba*)	Bacillus	No figures; describes white, non-liquefying, non-infectuous intruder which has a polar flagellum
De Rossi[33] 1909	White clover (*Trifolium repens*) and other clovers	8 to 10 flagella; peritrichic	One photomicrograph of *Trifolium repens*; very good
Kellerman 1912	Garden pea (*Pisum sativum*) Lima bean (*Phaseolus lunatus*) Alfalfa (*Medicago sativa*)	Flagella fairly numerous; peritrichic	Three photomicrographs, none of which is convincing
Zipfel 1912		Numerous flagella; peritrichic	No figures
Prucha[43] 1915	Canada field pea (*Pisum sativum arvense*)	Peritrichic; largest number observed was six, but there may be more	No figures

Organisms from red clover (*Trifolium pratense*), broad bean (*Vicia faba*), hairy vetch (*Vicia villosa*), common bean (*Phaseolus vulgaris*), sweet clover (*Melilotus alba*), alfalfa (*Medicago sativa*), field pea (*Pisum arvense*), and sweet pea (*Lathyrus odoratus*) were stained for flagella, using several methods, but the gum stained so heavily that none could be seen. The production of gum by the organism, as will be shown later, depends more upon the plant species from which it is isolated than upon the culture medium. Attention was then turned to the organisms making less vigorous growth, which produce less gum. Successful stains were made of the organisms from cowpea (*Vigna sinensis*), tick trefoil (*Desmodium canescens*), dyer's greenweed (*Genista tinctoria*), velvet bean (*Mucuna utilis*), peanut (*Arachis hypogoea*), wild indigo (*Baptisia tinctoria*), Japan clover (*Lespedeza striata*), acacia (*Acacia floribunda*), partridge pea (*Cassia chamaecrista*),[a] soybean (*Glycine hispida*), and hog peanut (*Amphicarpa monoica*).

Loeffler's method of staining was used. The mordant[b] was made up as follows:

Solution of tannin (20 percent in water)	10 parts
Saturated (cold) aqueous solution of ferrous sulfate	5 parts
Saturated alcoholic solution of basic fuchsin	1 part

Transfer the organisms successively several times upon ash agar to hasten the growth. With a platinum needle transfer some of the organisms from the edge of a transfer two or three days old to a small drop of sterile water upon a clean cover slip. Spread slowly and carefully (only a few strokes are necessary), and allow to dry. Cover well with a mordant, bring to a steam, and allow to stand about one minute. Wash carefully with distilled water and apply carbol-fuchsin, bring to a steam, and again let stand one minute.

In examining the slide, look especially near the edges of the smear and close to the "drifts" of bacteria. Slime and stain deposits frequently interfere, tho not seriously. The organism has a single polar flagellum (see Plates IV and V). It was noted that the flagellum is rarely attached at the end, but rather at a corner.

CULTURAL CHARACTERISTICS

Ps. radicicola will grow between 0° and 50°C. The optimum temperature is 25° to 28° C., tho it will grow well at room temperature, or 20° to 25°C. The organism is aerobic. The diffused light

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[a]The organisms of these nine plants comprize a single group, i. e., they are indentical, as will be shown later. Isolations, however, were made from the host plants as named. Actually, then, but three distinct varieties were stained—*Vigna, Glycine,* and *Amphicarpa.*

[b]Filter the ingredients separately and mix in the order given. Filter direct upon the cover slip. The mordant is best used fresh.

of the laboratory is not harmful. Even exposure to direct sunlight for several months without transfer did not kill organisms when grown upon favorable media with precautions to prevent evaporation. Under such conditions a temperature of 47°C. in the flask was reached with the thermometer shaded.

Slight alkalinity to +20° to +25° acid (Fuller's scale) with phenolphthalein is tolerated; neutral to +10° is best. Growth is generally better in gelatin or agar media than in liquid media of the same composition.

In an agar stab a typical drop-form colony is produced at the surface. A thin, gray growth follows the line of stab.

Maltose as a source of carbon has little if any advantage over saccharose or dextrose. Mannite is also suitable as a source of carbon.

In standard beef broth the growth of the organism is slow. The liquid becomes cloudy, a gray-white ring is formed, and a thin membrane covers the surface. Later a flocculent precipitate settles to the bottom of the tube.

In standard beef-broth gelatin the growth of the organism is at first funnel-shaped and then stratiform. The gelatin slowly liquefies, the process sometimes requiring two or three months for completion. In gelatin stabs the growth sometimes seals over the stab with a drop-form growth and liquefaction does not occur. If inoculated tubes are kept for several weeks at a temperature just allowing the gelatin to remain liquid, upon cooling it will be found that the gelatin refuses to solidify, whereas the gelatin in uninoculated check tubes does solidify. The enzyme causing liquefaction is present.

On ash-agar plates the presence of *Penicillium glaucum*, which occasionally intruded, seemed to benefit the colonies of *Ps. radicicola* that were in close proximity. Ash agar upon which *Penicillium glaucum* had been allowed to grow for two weeks and which had then been sterilized and filtered, had a noticeable advantage over untreated ash agar, especially with the slower growing organisms, such as those of *Vigna, Glycine,* and *Genista.*

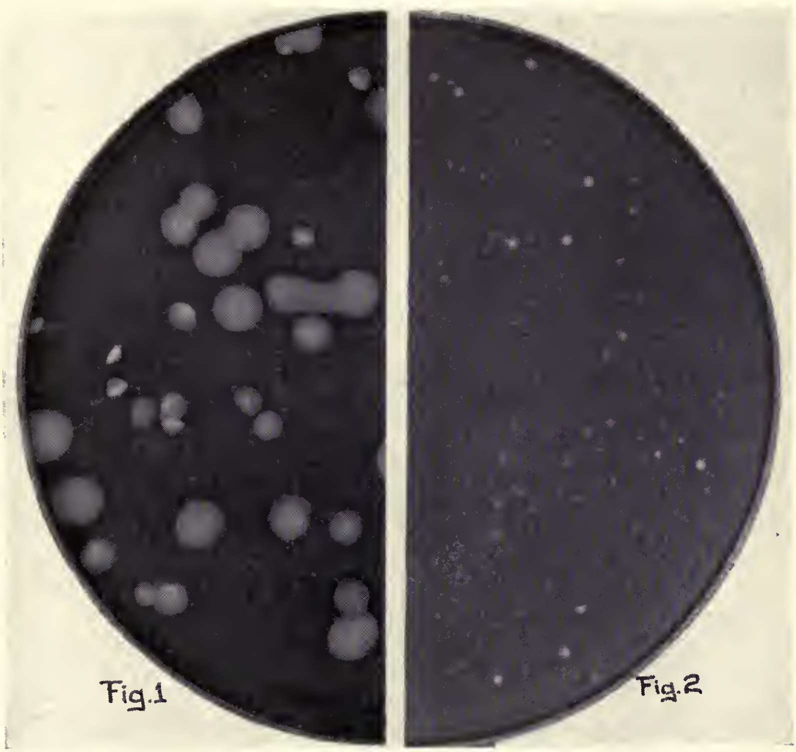

PLATE II

Fig. 1.—Ash-agar plate from pea (*Pisum sativum*), seven days old
Fig. 2.—Ash-agar plate from dyer's greenweed (*Genista tinctoria*), twenty-five days old

PLATE III

Fig. 1.—Bacteroids from a very young nodule of pea (*Pisum sativum*), showing swarmers among the bacteroids × 1080
Fig. 2.—Bacteroids from young, growing nodule of hairy vetch (*Vicia villosa*) × 1080
Fig. 3.—Bacteroids from an older nodule of hairy vetch (*Vicia villosa*), showing vacuolization × 1080

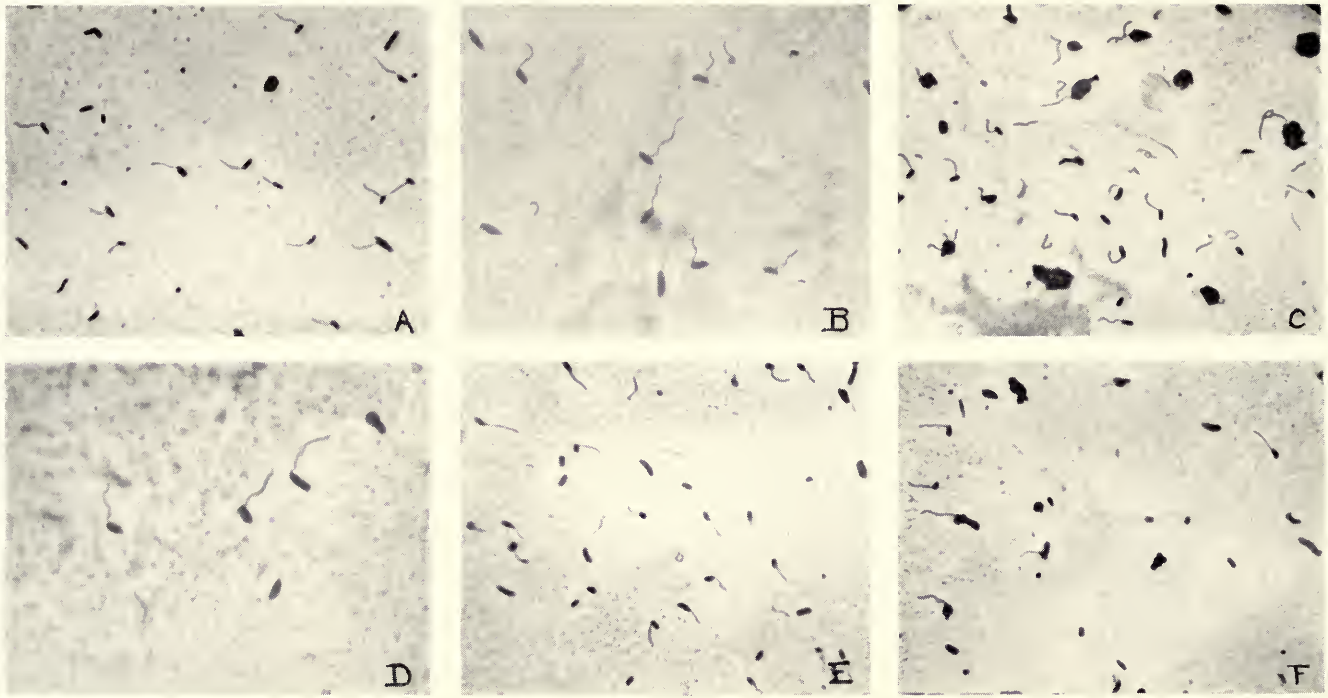

PLATE IV

Pseudomonas radicicola, showing polar flagellum: A.—Cowpea (*Vigna sinensis*) × 1080; B.—Cowpea (*Vigna sinensis*), more highly magnified; C.—Partridge pea (*Cassia chamaecrista*) × 1080; D.—Acacia (*Acacia floribunda*) × about 1500; E.—Tick trefoil (*Desmodium canescens*) × 1080; F.—Japan clover (*Lespedeza striata*) × 1080

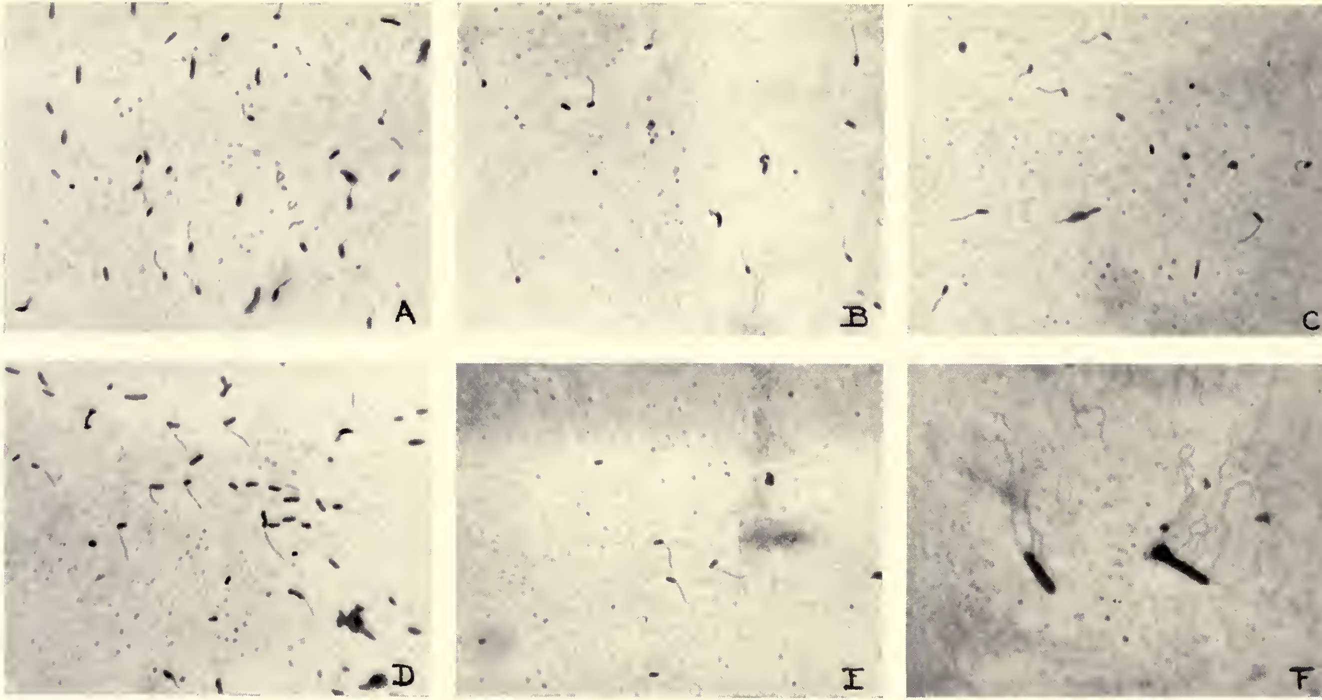

PLATE V

Pseudomonas radicicola, showing polar flagellum: A.—Velvet bean (*Mucuna utilis*) × 1080; B.—Peanut (*Arachis hypogoea*) × 1080; C.—Wild indigo (*Baptisia tinctoria*) × 1080; D.—Hog peanut (*Amphicarpa monoica*) × 1080; E.—Soybean (*Glycine hispida*) × 1080; F.—*B. subtilis*, introduced for comparison × 1080

Part II.—CROSS-INOCULATION: VARIETIES OF NODULE BACTERIA

CROSS-INOCULATION INVESTIGATIONS

It was early recognized that certain legumes require one specific organism for inoculation. For example, to inoculate soybeans it had been found necessary to import soil upon which soybeans had grown, as the bacteria from other legumes were not capable of causing infection. A few cross-inoculations which occur under field conditions were also early recognized. The bacteria of alfalfa and sweet clover[a] were known to be identical, as were those of the cowpea and partridge pea. A third group the bacteria of which were known to be interchangeable included pea, vetch, sweet pea, and lentil.

Several investigators, notably Laurent,[14] Mazé,[21] Moore,[28] and Kellerman,[36] claimed to have produced cross-inoculations which do not occur naturally. Little credence can be given these claims, however, since these men apparently did not fully appreciate what has been frequently referred to as the *ubiquity* of *Ps. radicicola*. No doubt their technic was at fault.

Methods Used in Cross-Inoculation Work.—For testing cross-inoculations bacteria were isolated from as many genera and species of legumes, both wild and cultivated, as could be obtained. Great care was taken in their isolation and in the maintenance of purity. Two methods of testing crosses were used—the pot-culture method and the agar test-tube method of Garman.[38] In the pot-culture method, plants were grown in one-gallon pots of limed white quartz sand watered with a nutrient solution less nitrogen, as described by Hopkins and Pettit.[b] The sand was not sterilized, as it was dry and clean, and sterile so far as legume bacteria were concerned, as proven by the record of the check pots. The pots were washed clean and exposed to sunlight in the greenhouse a week before using, being turned several times. A number of dry, clean pots were always on hand so that no

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[a]Hopkins.[26]

[b]From Hopkins and Pettit Laboratory Manual for Soil Fertility, page 34.

Solution No. 1.—Nitrogen: Dissolve 80 grams of ammonium nitrate in 2500 cc. of distilled water.

Solution No. 2.—Phosphorus: Dissolve 25 grams of mono-calcium phosphate in 2500 cc. of ammonia-free water.

Solution No. 3.—Potassium: Dissolve 50 grams of potassium sulfate in 2500 cc. of ammonia-free water.

Solution No. 4.—Magnesium: Dissolve 20 grams of magnesium sulfate in 2500 cc. of ammonia-free water.

Solution No. 5.—Iron: Dissolve .1 gram ferric chlorid in 250 cc. of ammonia-free water.

Use 10 cc. each of Solutions Nos. 1, 2, 3, and 4, and 1 cc. of Solution No. 5 per liter of water. When nitrogen is omitted the fact is so stated.

PLATE VI

Seedlings of partridge pea (*Cassia chamaecrista*) inoculated with bacteria from cowpea (*Vigna sinensis*) loss of time resulted when organisms were to be tested. The seeds were sterilized by shaking them violently in Harrison and Barlow's sterilizing fluid, previously described, allowing them to remain in the fluid for ten minutes, and then washing in distilled water. Usually five seeds were planted in a pot. Inoculation was made at the time of planting by adding the contents of an agar slant mixed with sterile distilled water. One pot in each four was left uninoculated as a check.

Occasionally scattered nodules did appear on checks and in pots which when repeated gave negative results. The use of open pots in a greenhouse frequented by many people, together with the presence of occasional insects, etc., cannot but result in some chance inoculations. A chance inoculation, however, is easily distinguished from a true one, for in the former case the nodules are few in number and widely scattered, whereas in a true inoculation the nodules are numerous and clustered in a mass about the tap root. This pot-culture method was used for growing plants with large seeds, such as *Vigna, Glycine, Pisum, Vicia, Lathyrus,* and *Phaseolus.*

In the second method used, that of Garman,[38] seeds were planted in test-tubes (6″ x ¾″) containing a medium composed of .65 percent agar in distilled water. No nutrients were added. The agar was inoculated at 42° to 45°C. Seeds (usually three to a tube), sterilized as before, were dropped upon the agar and set apart with a flamed platinum needle. Generally the nodules resulting were not numerous, but where the seeds germinated well, results were always positive and dependable. This method of testing crosses is especially adapted to smaller seeds, such as *Melilotus, Medicago,* and *Trifolium.* Large seeds give trouble, as they are difficult to sterilize.

Vigna × *Cassia.*—The inoculation of the cowpea by bacteria from the partridge pea was first reported by Hopkins.[34] In the other cross-inoculations mentioned above (alfalfa and sweet clover; pea, sweet pea, vetch, and lentil), the plants having a common organism stand in close botanical relationship,[a] while *Vigna sinensis* and *Cassia chamaecrista* are widely separated. Moreover, the former is a plant introduced from Asia, while the latter is a native.

The first cross-inoculation experiments in these investigations were conducted with the partridge pea (*Cassia chamaecrista*), inoculating it as shown in Table 3. Partridge-pea seeds and nodules were obtained from plants found upon virgin prairie in a wild locality where in all probability cowpeas had never been grown. Cultures were obtained from cowpea nodules grown in the greenhouse. Thus the sources of the organisms were wide apart.

The seeds were planted on October 16, 1915, three in each pot; the plants were examined and photographed on November 19 (see Plate VI). The results appear in Table 3. The number of nodules reported

[a]Engler und Prantl: Die Naturlichen Pflanzenfamilien, III.

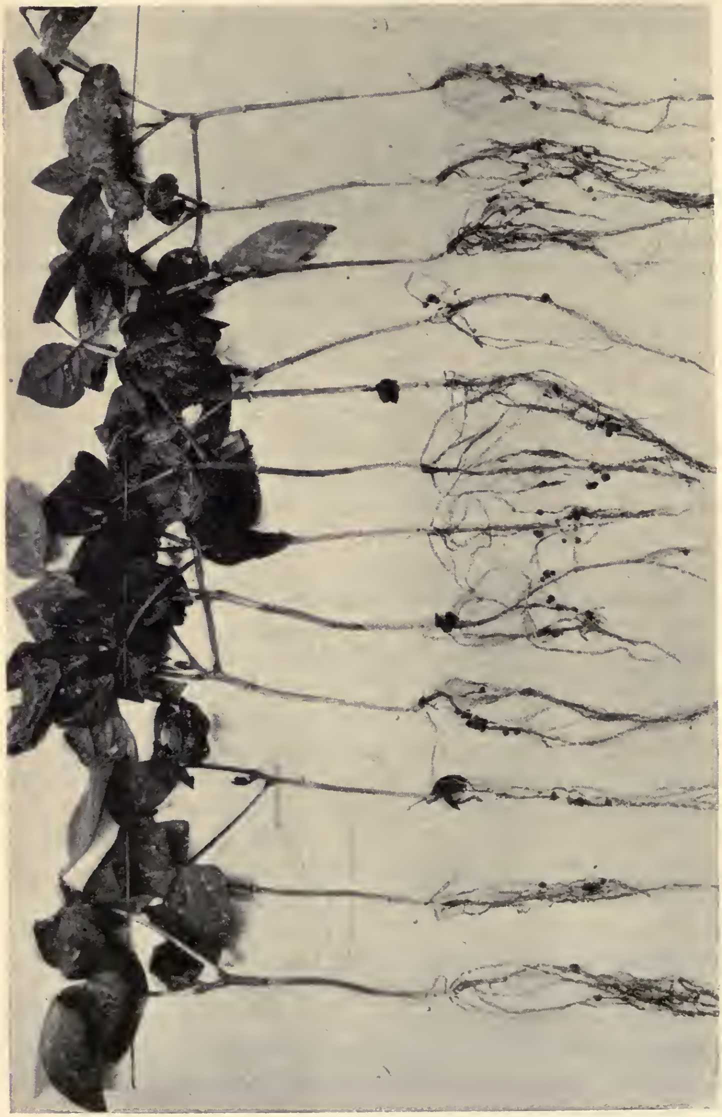

PLATE VII

Seedlings of cowpea (*Vigna sinensis*) inoculated with bacteria from partridge pea (*Cassia chamaecrista*) may be somewhat low, as it is difficult to count the smaller nodules. The checks were examined with great care and found to be free from nodules.

TABLE 3.—PARTRIDGE PEA × COWPEA (*Cassia chamaecrista* × *Vigna sinensis*)

Pot No.	Plant	No. of plants	Source of inoculation	Nodules	Results + or —
4593	Partridge pea	3	Partridge pea No. 4608	17, 15, 9	+
4594	” ”	3	” ” No. 4609	3, 11, 7	+
4595	” ”	3	” ” No. 4611	5, 5, 5	+
4596	” ”	3	” ” No. 4613	3, 3, 11	+
4597	Partridge pea	3	Check	0, 0, 0	—
4598	” ”	3	”	0, 0, 0	—
4599	” ”	3	”	0, 0, 0	—
4600	” ”	3	”	0, 0, 0	—
4601	Partridge pea	3	Cowpea No. 4615	12, 3, 8	+
4602	” ”	3	” No. 4617	8, 10, 7	+
4603	” ”	3	” No. 4619	7, 8, 6	+
4604[a]	” ”	3	” No. 4621	0, 0, 0	—

[a]For some unknown reason the plants in this pot produced no nodules.

The reciprocal was then tried. Five seeds of cowpea were planted in each pot and inoculations made as shown in Table 4. The seeds were planted on November 29, 1915; the plants were examined and photographed on January 3, 1916 (see Plate VII). The results are also shown in Table 4.

TABLE 4.—COWPEA × PARTRIDGE PEA (*Vigna sinensis* × *Cassia chamaecrista*)

Pot No.	Plant	No. of plants	Source of inoculation	Nodules	Results + or —
5023	Cowpea	5	Cowpea Nos. 4398 and 5042	Abundant	+
5024	”	5	” Nos. 4614 ” 5039	”	+
5025	”	5	” Nos. 4616 ” 5040	”	+
5026	”	5	” Nos. 4618 ” 5041	”	+
5027	Cowpea	5	Check	None	—
5028	”	5	”	”	—
5029	”	5	”	”	—
5030	”	5	”	”	—
5031	Cowpea	5	Partridge pea Nos. 4605 and 5035	Abundant	+
5032	”	5	” ” Nos. 4607 ” 5036	”	+
5033	”	5	” ” Nos. 4610 ” 5037	”	+
5034	”	5	” ” Nos. 4612 ” 5038	”	+

Vigna × *Acacia*.—Great interest had been taken in some preliminary trials which had given evidence that a cross exists between cowpea and acacia. Accordingly cowpea plants were inoculated with cultures from six species of *Acacia*, and later with a culture from a seventh. These were *Acacia armata, floribunda, linifolia, longifolia, semperflora,* and a species the nodules of which had been received from

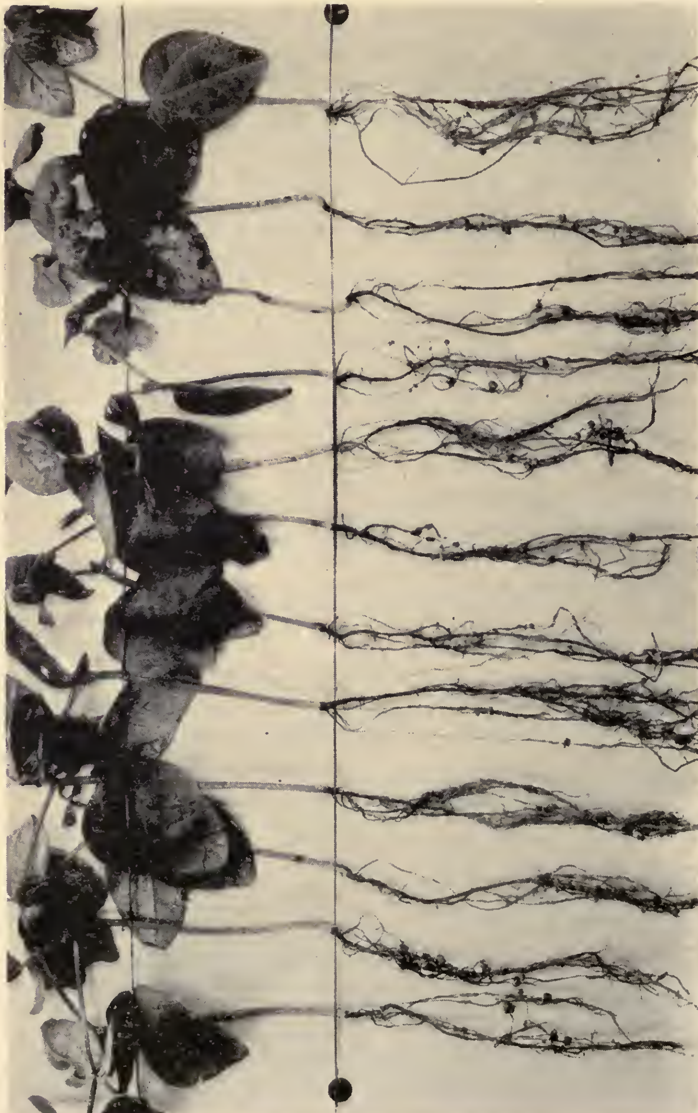

PLATE VIII

Seedlings of cowpea (*Vigna sinensis*) inoculated with bacteria from six species of *Acacia*

California but of which nothing else was known except that it is an ornamental tree. Cultures of the first five species were obtained from nodules of plants grown in the horticultural greenhouse on this campus. Cultures from a seventh species, *Acacia melanoxylon*, which was later grown in this greenhouse, behaved exactly like the other six. Seeds were planted on January 13, 1916; the plants were examined and photographed on February 21 (see Plate VIII). The results are shown in Table 5.

TABLE 5.—COWPEA × ACACIA (*Vigna sinensis* × *Acacia*)

Pot No.	Plant	No. of plants	Source of inoculation	Nodules	Results + or —
5464	Cowpea	5	*Acacia armata* Nos. 5578 and 5649	Abundant	+
5465	”	5	*Acacia floribunda* Nos. 5579 and 5650	”	+
5466	”	5	*Acacia linifolia* Nos. 5580 and 5651	”	+
5467	Cowpea	5	Check	None	—
5468	”	5	”	”	—
5469	”	5	”	”	—
5470	”	5	”	”	—
5471	Cowpea	5	*Acacia longifolia* Nos. 5581 and 5652	Abundant	+
5472	”	5	*Acacia semperflora* Nos. 5582 and 5653	”	+
5473	”	5	*Acacia* ? (from California) Nos. 5583 and 5654	”	+

Cowpea × Several Generic Groups.—Tests made from time to time with the cowpea had shown that infection could be produced with bacteria from eight different generic groups besides the cowpea organism. An experiment was then conducted to bring together the results of these previous trials. The results are given in Table 6.

TABLE 6.—COWPEA (*Vigna sinensis*) × SEVERAL GENERIC GROUPS

Pot No.	Plant	No. of plants	Source of inoculation	Nodules	Results + or —
6309	Cowpea	5	Check	None	—
6310	”	5	Acacia (*Acacia melanoxylon*)	Abundant	+
6311	”	5	Lead plant (*Amorpha canescens*)	None	—
6312	”	5	Hog peanut (*Amphicarpa monoica*)	Several	—
6313	”	5	Check	Several	—
6314	”	5	Peanut (*Arachis hypogoea*)	Abundant	+
6315	”	5	Wild indigo (*Baptisia tinctoria*)	Abundant	+
6316	”	5	Partridge pea (*Cassia chamaecrista*)	Abundant	+
6317	”	5	Check	None	—
6318	”	5	Tick trefoil (*Desmodium canescens*)	Abundant	+
6319	”	5	Dyer's greenweed (*Genista tinctoria*)	Abundant	+
6320	”	5	Japan clover (*Lespedeza striata*)	Abundant	+
6321	”	5	Check	None	—
6322	”	5	Common locust (*Robinia pseudo-acacia*)	Several	—
6323	”	5	Velvet bean (*Mucuna utilis*)	Abundant	+
6324	”	5	Cowpea (*Vigna sinensis*)	Abundant	+

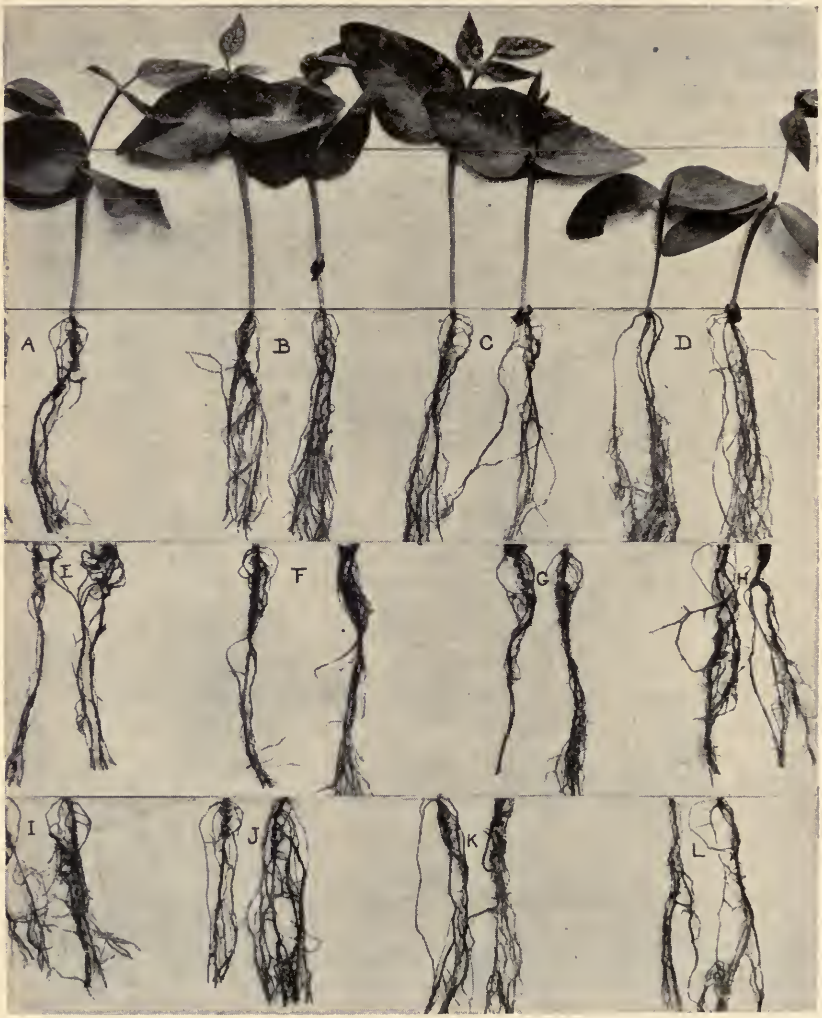

PLATE IX

Seedlings of cowpea (*Vigna sinensis*) inoculated as follows: A.—Partridge pea (*Cassia chamaecrista*); B.—Tick trefoil (*Desmodium canescens*); C.—Dyer's greenweed (*Genista tinctoria*); D.—Check; E.—Japan clover (*Lespedeza striata*); F.—Velvet bean (*Mucuna utilis*); G.—Cowpea (*Vigna sinensis*); H.—Check; I.—Acacia (*Acacia melanoxylon*); J.—Peanut (*Arachis hypogoea*); K.—Wild indigo (*Baptisia tinctoria*); L.—Check

In Plate IX two plants from each pot are shown; those which were negative have been omitted. The results confirmed those of the earlier tests. The plants in three of the negative pots (those crossed with *Amphicarpa, Robinia,* and a check) had several nodules, but these were no doubt accidental as they were very scattered. In a previous trial, inoculations with *Amphicarpa* and *Robinia* had both given negative results.

Lens × *Several Generic Groups.*—Another set of similar experiments is of interest. Lentils were planted on March 17, 1916, and inoculated with bacteria from several generic groups. The seedlings were examined on April 14. The results are shown in Table 7.

TABLE 7.—LENTIL (*Lens esculenta*) × SEVERAL GENERIC GROUPS

Pot No.	Plant	No. of plants	Source of inoculation	Nodules	Results + or —
6386	Lentil	3	Scarlet runner bean (*Phaseolus multiflorus*)	None	—
6387	"	3	Common bean (*Phaseolus vulgaris*)	None	—
6388	"	3	Trailing wild bean (*Strophostyles helvola*)	None	—
6389	"	3	Perennial pea (*Lathyrus latifolius*)	Abundant	+
6390	"	3	Common garden pea (*Pisum sativum*)	Abundant	+
6391	"	3	Field pea (*Pisum arvense*)	Abundant	+
6392	"	3	Broad bean (*Vicia faba*)	Abundant	+
6393	"	3	Check	None	—

In a similar experiment with seedlings of *Trigonella foenum-graecum,* it was found that they could be infected with the bacteria from *Melilotus alba* and *Medicago sativa.* In fact, many inoculation trials were made with all available bacteria. Many results were negative, as is shown in Table 9.

Tests with Garman's Method.—The cross-inoculation trials made by Garman's method together with the results obtained are shown in Table 8. Four inoculated tubes and one check were used for each culture tested. In Plate X are shown seven cultures tested upon *Medicago sativa* (only one tube of each of the seven series is shown) together with two checks. In all, this photograph represents thirty-five tubes, twenty-eight inoculated and seven uninoculated.

Results of Cross-Inoculation Trials.—Table 9 gives in full the results of the cross-inoculation experiments conducted. All available cultures were tried upon seedlings of *Anthyllis vulneraria* and *Mimosa pudica,* but no nodules were produced. It is assumed that the organisms of these plants are distinct from any of those used.

TABLE 8.—CROSS-INOCULATIONS BY GARMAN'S METHOD

Source of inoculation		Plants					
Common name	Botanical name	Lespedeza (*Lespedeza virginica*)	Black medick (*Medicago lupulina*)	Alfalfa (*Medicago sativa*)	Sweet clover (*Melilotus alba*)	Serradella (*Ornithopus sativus*)	Red clover (*Trifolium pratense*)
Acacia	*Acacia floribunda*	+	—	—	—		—
Lead plant	*Amorpha canescens*	—	—	—	—		—
Hog peanut	*Amphicarpa monoica*	—	—	—	—		—
Peanut	*Arachis hypogoea*	+	—	—	—		—
Wild indigo	*Baptisia tinctoria*	+	—	—	—		—
Partridge pea	*Cassia chamaecrista*	+	—	—	—		—
Tick trefoil	*Desmodium canescens*	+	—	—	—		—
Tick trefoil	*Desmodium illinoense*	+	—	—	—		—
Dyer's greenweed	*Genista tinctoria*	+	—	—	—		—
Soybean	*Glycine hispida*	—	—	—	—	—	—
Sweet pea	*Lathyrus odoratus*	—	—	—	—		—
Lespedeza	*Lespedeza virginica*	+	—	—	—		—
Japan clover	*Lespedeza striata*	+	—	—	—		—
Wild lupine	*Lupinus perennis*	—	—	—	—		—
Yellow trefoil or black medick	*Medicago lupulina*	—	+	+	+		—
Alfalfa	*Medicago sativa*	—	+	+	+		—
White sweet clover	*Melilotus alba*	—	+	+	+		—
Wild yellow sweet clover	*Melilotus indica*	—	+	+	+		—
Yellow sweet clover	*Melilotus officinalis*	—	+	+	+		—
Velvet bean	*Mucuna utilis*	+	—	—	—	—	—
Bean	*Phaseolus vulgaris*	—	—	—	—	—	—
Pea	*Pisum sativum*	—	—	—	—	—	—
Black or common locust	*Robinia pseudo-acacia*	—	—	—	—		—
Trailing wild bean	*Strophostyles helvola*	—	—	—	—	—	—
Red clover	*Trifolium pratense*	—	—	—	—		+
Fenugreek	*Trigonella foenum-graecum*	—	+	+	+		—
Hairy vetch	*Vicia villosa*	—	—	—	—		—
Cowpea	*Vigna sinensis*	+	—	—	—	—	—

TABLE 9.—COMPLETE TABLE OF CROSS-INOCULATIONS

Plants		Source of inoculation																							
Common name	Botanical name	*Acacia* (seven species)	*Amorpha canescens*	*Amphicarpa monoica*	*Arachis hypogaea*	*Baptisia tinctoria*	*Cassia chamaecrista*	*Desmodium canescens* and *illinoense*	*Genista tinctoria*	*Glycine hispida*	*Lathyrus odoratus* and *latifolius*	*Lens esculenta*	*Lespedeza virginica* and *striata*	*Lupinus perennis*	*Medicago* (four species)	*Melilotus* (three species)	*Mucuna utilis*	*Phaseolus* (three species)	*Pisum arvense* and *sativum*	*Robinia pseudo-acacia*	*Strophostyles helvola*	*Trifolium* (six species)	*Trigonella foenum-graecum*	*Vicia* (five species)	*Vigna sinensis*
Acacia	*Acacia*	+																							
Hog peanut	*Amphicarpa*			+						—	—	—													
Kidney vetch	*Anthyllis*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Peanut	*Arachis*	+	—	—	+	+	+	+	+	—	—	—	+	—	—	—	+	—	—	—	—	—	—	—	+
Partridge pea	*Cassia*	+	—	—	+	+	+	+	+	—	—	—	+	—	—	—	+	—	—	—	—	—	—	—	+
Tick trefoil	*Desmodium*	+	—	—	+	+	+	+	+	—	—	—	+	—	—	—	+	—	—	—	—	—	—	—	+
Soybean	*Glycine*	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sweet pea	*Lathyrus*	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—	—	—	+	—
Lentil	*Lens*	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—	—	—	+	—
Lespedeza	*Lespedeza*	+	—	—	+	+	+	+	+	—	—	—	+	—	—	—	+	—	—	—	—	—	—	—	+
Lupine	*Lupinus*	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
Alfalfa	*Medicago*	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—
Sweet clover	*Melilotus*	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—
Sensitive plant	*Mimosa*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Velvet bean	*Mucuna*									—	—	—					+								+
Serradella	*Ornithopus*	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
Bean	*Phaseolus*	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—
Pea	*Pisum*	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—	—	—	+	—
Trailing wild bean	*Strophostyles*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
Clover	*Trifolium*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
Fenugreek	*Trigonella*	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—
Vetch	*Vicia*	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	+	—	—	—	—	+	—
Cowpea	*Vigna*	+	—	—	+	+	+	+	+	—	—	—	+	—	—	—	+	—	—	—	—	—	—	—	+

Based upon the trials made, the nodule organisms are divided into the following groups according as they are interchangeable for the purposes of inoculation:

GROUP I

Mammoth red clover, *Trifolium pratense perenne*
Alsike, or Swedish clover, *Trifolium hybridum*
Crimson clover, *Trifolium incarnatum*
Berseem, or Egytian clover, *Trifolium alexandrianum*
White clover, *Trifolium repens*
Zigzag, or cow clover, *Trifolium medium*

GROUP II

White sweet clover, *Melilotus alba*
Yellow sweet clover, *Melilotus officinalis*
Wild yellow sweet clover, *Melilotus indica*
Alfalfa, *Medicago sativa*
Alfalfa, *Medicago falcata*
Bur clover, *Medicago hispida*
Black medick, or yellow trefoil, *Medicago lupulina*
Fenugreek, *Trigonella foenum-graecum*

GROUP III

Cowpea, *Vigna sinensis*
Partridge pea, *Cassia chamaecrista*[a]
Peanut, *Arachis hypogoea*
Japan clover, *Lespedeza striata*
Slender bush clover, *Lespedeza virginica*
Velvet bean, *Mucuna utilis*
Wild indigo, *Baptisia tinctoria*
Tick trefoil, *Desmodium canescens*
Tick trefoil, *Desmodium illinoense*
Acacia, *Acacia armata*
Acacia, *Acacia floribunda*
Acacia, *Acacia linifolia*
Acacia, *Acacia longifolia*
Acacia, *Acacia melanoxylon*
Acacia, *Acacia semperflora*
Acacia, *Acacia ?*, from California
Dyer's greenweed, *Genista tinctoria*

GROUP IV

Common garden pea, *Pisum sativum*
Field pea, or Canada field pea, *Pisum sativum arvense*
Hairy vetch, *Vicia villosa*
Spring vetch, *Vicia sativa*
Broad bean, *Vicia faba*
Narrow leaved vetch, *Vicia angustifolia*
Vetch, *Vicia daysiecarpa*
Lentil, *Lens esculenta*
Sweet pea, *Lathyrus odoratus*
Perennial pea, *Lathyrus latifolius*

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[a]Cultures isolated from nodules of *Cassia nictitans* and tested on seedlings of *Cassia chamaecrista* and *Vigna sinensis* failed to produce nodules. The cultures had been on hand some time when tried, and it was suspected that an error had been made. At a later time a number of seedlings of *Cassia medsgeri* were grown and inoculated with the *Cassia nictitans* cultures, as well as with bacteria from

GROUP V

Soybean, *Glycine hispida*

GROUP VI

Garden bean, *Phaseolus vulgaris*
Garden bean, *Phaseolus angustifolia*
Scarlet runner bean, *Phaseolus multiflorus*

GROUP VII

Lupine, *Lupinus perennis*
Serradella, *Ornithopus sativus*

GROUP VIII

Hog peanut, *Amphicarpa monoica*

GROUP IX

Lead plant, *Amorpha canescens*

GROUP X

Trailing wild bean, *Strophostyles helvola*

GROUP XI

Black, or common locust, *Robinia pseudo-acacia*

GROUPING BY SEROLOGICAL TESTS AND BY CULTURAL DIFFERENCES

Review of Results with Serological Tests.—In order to throw light upon the kinship among the various nodule bacteria, Zipfel,[37] in 1912, made use of the agglutination method. From his results he concluded that the nodule bacteria were not varieties of the same species, but that distinct species existed.

Klimmer and Krüger[39] two years later used serological tests to distinguish species. They used the agglutination method principally; and complement-binding and precipitation for confirmation and control. Working with organisms from eighteen legume species, they divided the bacteria, according to their methods, into nine species, which they asserted differed sharply from one another.

Simon,[40] in 1914, tested various cultures upon seedlings of several legume species, and compared the results with those obtained by using Zipfel's agglutination method. He found that the results of both methods agreed substantially. His grouping of the nodule bacteria is in general agreement with that of Klimmer and Krüger.[a] He concluded, however, that "the root bacteria of legumes are rather to be conceived as more or less constant adaptations of the species *Bacillus radicicola.*"

Cassia chamaecrista and *Vigna sinensis,* but no nodules were produced. This suggested that perhaps there is more than one adaptation affecting *Cassia.* The evidence is not conclusive, however.

[a]It should be noted that the pure cultures used by Klimmer and Krüger were supplied by Simon.

PLATE X

Seedlings of alfalfa (*Medicago sativa*) grown in agar tubes after Garman's method, showing inoculation by several cultures

Grouping by Cultural Differences.—While no serological tests were made in these experiments, a possible basis of distinguishing varieties or species was observed in the striking differences among the organisms upon culture media. When grown upon ash-agar slants, three quite distinct types of growth were noted. The organisms were divided upon this basis into three groups as follows:

Group I.—The organisms are distinguished by the thin, scant growth upon the slant; the streak is a dull gray-white. They are exceedingly slow growers, colonies on agar plates being especially slow. They are not sticky to the touch, and spread quite easily in water upon the cover slip. Flagella are quite easily demonstrated, since there is little gum to interfere. The group includes *Vigna, Cassia, Acacia, Lespedeza, Desmodium, Baptisia, Genista, Arachis, Mucuna, Glycine,* and *Amphicarpa.*

Group II.—The organisms of this group grow more rapidly than those of Group I. The growth is moderate to abundant with but little tendency to spread. The streak is raised, glistening, opaque, and pearly white in color. Tho not usually very sticky to the touch, there is considerable gum, which seriously interferes in attempts to stain flagella. *Melilotus, Medicago,* and *Trigonella* make up this group.

Group III.—The growth of these organisms is very fast, and there is a strong tendency to spread. The streak is watery and semi-translucent, being quite different from the opaque growth of Group II. The surface is not so shiny as in that group. Further, the organisms are quite slimy and usually quite sticky to the touch. The excessive amount of gum prevents staining of flagella and holds the organisms in clumps so that they cannot be spread easily on the cover slip. The organisms of *Vicia, Pisum, Lens, Lathyrus, Trifolium, Phaseolus,* and *Strophostyles* are included in this group.

While the descriptions above apply more directly to growth upon ash agar, the differences hold true for growth on other agar media, especially Fred's synthetic and Conn's asparaginate agar. The latter is especially suited to the organisms of Group I, growth upon it being considerably better than upon ash agar. It was further noted that these differences did not disappear with the aging of the cultures, but on the contrary became more pronounced. Cultures which were two years old showed in young transfers the differences noted in freshly isolated cultures.

Varieties vs. Species.—While the grouping of the various nodule bacteria is perhaps best determined by actual plant inoculations, yet the results of serologic tests show that the various organisms are different and that these differences are permanent. Likewise in certain cultural characteristics herein described we find differences which also are permanent. Furthermore, the adaptations as tested by actual inoculations upon plants are constant. For example, the soybean organism not only retains its individuality as tested by serologic methods and by cultural characteristics, but it also retains its special adaptation to the soybean plant, in spite of imposed conditions designed to break this adaptation. These facts form perhaps a legitimate basis for the belief that distinct species exist among the nodule bacteria. In numerous other characteristics, however, these bacteria are so much alike, and as a whole they differ so widely from any other species of bacteria, that it seems more consistent to regard the adapted forms as varieties of the single species *Pseudomonas radicicola.*

Experiments in cross-inoculation brought out the fact that in many cases in which a single organism is capable of infecting several plant genera, the host plants stand in close botanical relationship. In Group III, however (see page 136), is a striking exception to this. In *Acacia, Cassia,* and *Vigna,* we have each of the three sub-families of the *Leguminosae* represented, yet the same organism produces nodules upon all three. Obviously botanical relationship is not responsible in this case.

Mazé[21] claimed that the reaction of the soil was responsible for the special adaptations. He divided the nodule bacteria into two groups, those infecting plants which have become accustomed to acid soil and those infecting plants which have become accustomed to alkaline soil. By gradually accustoming a bacterium from the alkaline-soil group to an acid medium, he claimed to have so modified it that it would produce nodules upon lupines, which belonged to his acid-soil group. However, observations do not bear out Mazé's statements in regard to the two groups. The reaction of the soil does not appear to have any significance in determining the groups, of which there are certainly more than two. Furthermore, the reaction of artificial media does not break or change the special adaptations, nor is the organism modified at all so far as its power to produce nodules is concerned.

Evidently, then, the adaptation is between the root-sap of the plant and the bacteria. It may be a case of specific enzymes produced by the bacteria, or of differences in the root-sap which cannot be detected by chemical methods.

Part III.—HISTOLOGY OF THE NODULES OF THE LEGUMINOSAE

Technic.—Sections were made, for the most part, from nodules of plants grown in normal soil, for it was desired to study especially the nodules as they occur in nature. When sections from young nodules were desired, however, plants were grown in quartz sand, watered with the nutrient solution.[a]

The paraffin method of imbedding was used entirely. Sections were usually cut five or six microns thick; thinner sections were tried but abandoned owing to the difficulty in getting good mounts. In the earlier attempts, material was fixed by immersing from four to six hours in a picric fixing agent.[b] Haidenhain's iron-haematoxylin was usually used in conjunction with the picric fixative. Sections were mounted in series and stained upon the slides, after removing the paraffin with xylol, etc. The mounts obtained were not entirely satisfactory, but were valuable for comparison with mounts stained otherwise. Differentiation with the ferrous-ammonium-sulfate solution can be carefully controlled, which is the great advantage of this method.

Flemming's method was later adopted for most of the work. Nodules were fixed in Flemming's weaker solution (chrom-osmic-acetic solution), and the triple stain[c] then applied. Sometimes the triple stain was used with material fixed in picric acid, and altho results were not so good as with material fixed in the chrom-osmic-acetic fluid, still very satisfactory mounts were obtained. With haematoxylin mounts, cedar oil was used for clearing; with the triple stain, clove oil was preferred.

Considerable difficulty was experienced with thin sections when the triple stain was used, owing to the rapid loss of stain when dehydrated with ethyl alcohol. Dehydration and differentiation with ethyl alcohol was not wholly unsatisfactory; however, by using amyl alcohol instead of ethyl alcohol, more densely stained mounts were obtained.

For demonstrating starch, slides were stained with Flemming's triple stain and differentiated with ethyl alcohol, removing all excess stain. They were then transferred to distilled water to remove the alcohol, after which they were mounted in distilled water to which a small quantity of Lugol's iodine solution was added. The iodine was not concentrated enough to discolor the field, but gave the characteristic blue to the starch. Water was added during examination when

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[a]See footnote, page 125.

[b]The solution was made up as follows:

Corrosive sublimate	5 gms.
Glacial acetic acid	5 cc.
Saturated solution of picric acid in 70 percent alcohol	100 cc.

[c]Safranin, gentian-violet, orange G.

necessary to prevent drying out. Such mounts are not permanent, but they may be made so by restaining and mounting in balsam, tho the blue color is not retained by the starch.

A useful modification of Flemming's triple stain is to follow the gentain-violet with Lichtgrün[a] instead of orange G.

ORIGIN OF THE NODULE

Whether the active motility of *Ps. radicicola* in certain stages has any bearing upon the infection of the root-hairs, or young roots, of legumes, is not known. The vigorous root system of the *Leguminosae* in general, however, is probably a big factor.

The entrance of the organism into the root thru the root-hair, as described by previous investigators, was not studied in these investigations. According to these workers,[b] the infection first appears as a bright spot at or near the tip of the root-hair. The bacteria gain entrance by dissolving the cell wall, probably thru the agency of an enzyme. Infection is followed by a distinct bending of the root-hair, the response to the irritation set up. The organisms multiply and form a zoogloeal strand, which makes its way down the root-hair into the root-cortex. In the innermost layers of the cortex, the irritation is set up which gives rise to the nodule. The cells are stimulated to rapid growth, a meristem is formed, and the young nodule emerges from the root epidermis as a mass of parenchymal cells. In origin, then, the nodule is similar to the lateral root, but here the similarity ends.

While it is well established that infection takes place in this way, still it is evident that it is not thru the root-hairs alone that the bacteria gain entrance. The root epidermis itself may be penetrated, as with seedlings grown in agar test-tube cultures (Garman's method), in which case no root-hairs are produced. The same is found true of seedlings grown in sand saturated with a nutrient solution and not inoculated until the roots have made several inches of growth. On allowing time for infection and nodule production, it will be found that nodules are produced abundantly around the tap root above the region of root-hairs.

STRUCTURE OF THE NODULE

In Plate XI is shown the gross structure of nodules of *Trifolium pratense* which are well developed but still growing. Fig. 1 represents a longitudinal section near the central part of a nodule. The greater portion, it will be seen, is made up of bacteroidal cells, which occupy

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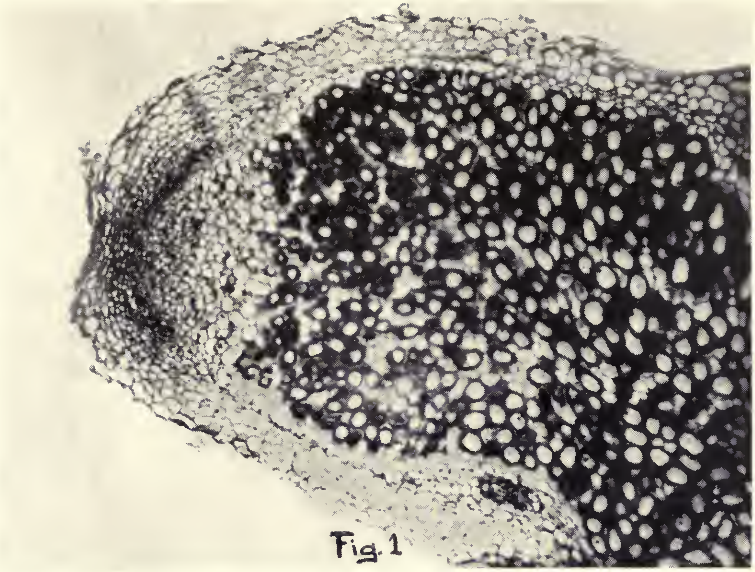
[a].5 gram in 200 cc. of alcohol.

[b]Marshall Ward,[8, 13] and Peirce[25] should be consulted. Other important contributions are the following. Woronin,[1, 2] Eriksson,[3] Frank,[4] Beyerinck,[9] Tschirch,[7] Vuillemin,[11] Prazmowski,[6, 12, 15] Schneider,[16] Atkinson,[17] and Dawson.[22, 23]



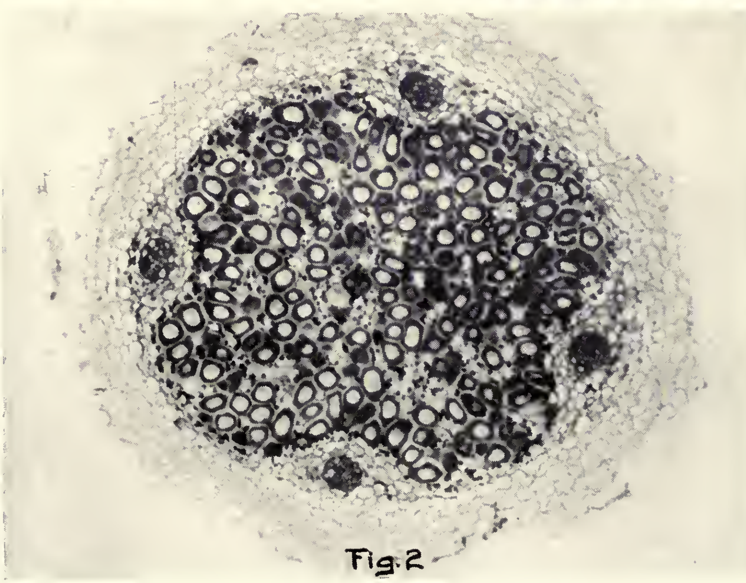
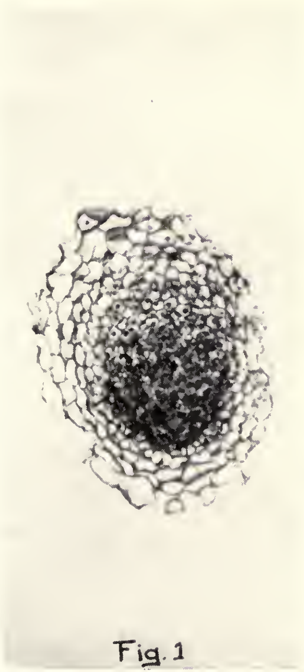
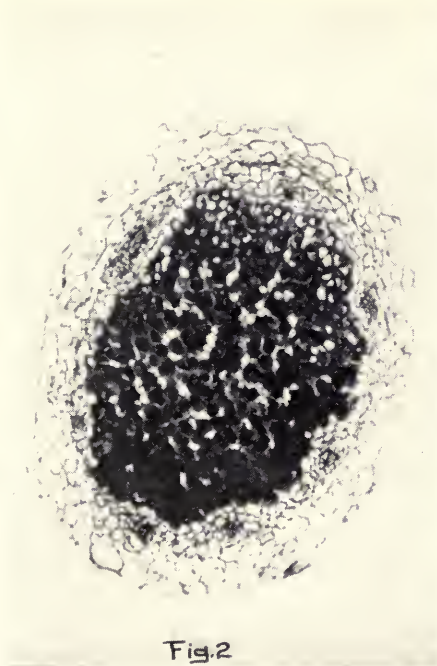


PLATE XI

Fig. 1.—Longitudinal section of a nodule of red clover (*Trifolium pratense*) which was well advanced but still growing, showing vacuolated bacteroidal cells, vascular tissue, nodule cortex, and meristem. Stained with Flemming's triple stain with amyl alcohol dehydration × 100

Fig. 2.—Cross-section of a similar nodule of red clover (*Trifolium pratense*). Stained with Flemming's triple stain and mounted in dilute iodine solution to show the starch. The fibro-vascular bundles are especially prominent × 100





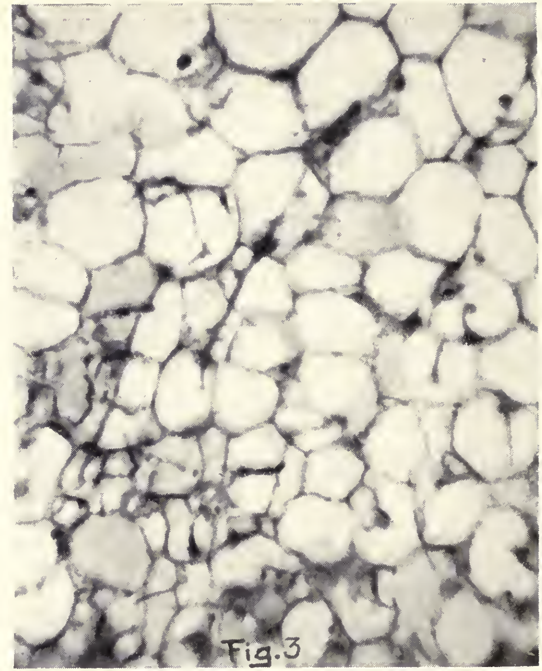
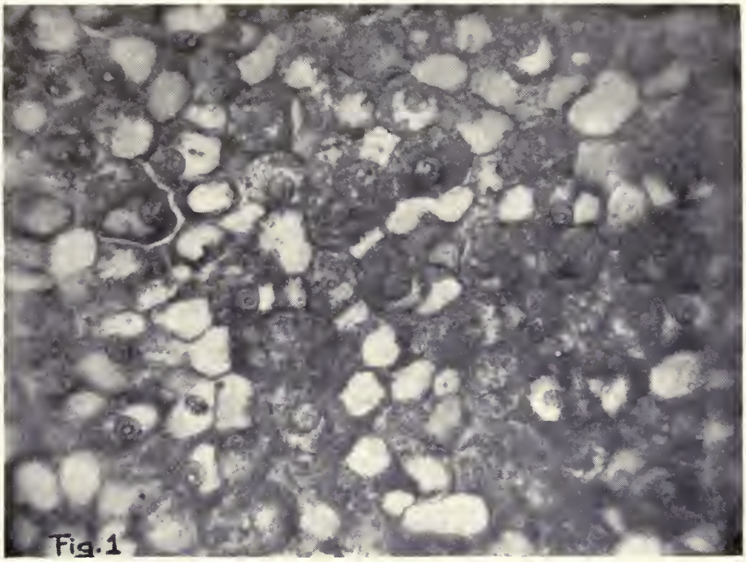


Plate XII

Fig. 1.—Cross-section thru the meristem region of a nodule of hairy vetch (*Vicia villosa*) $\times$ 100
Fig. 2.—Cross-section thru the same nodule some distance back from the meristem $\times$ 100
Fig. 3.—Infection threads in the cortex cells of a nodule of red clover (*Trifolium pratense*) $\times$ 1080



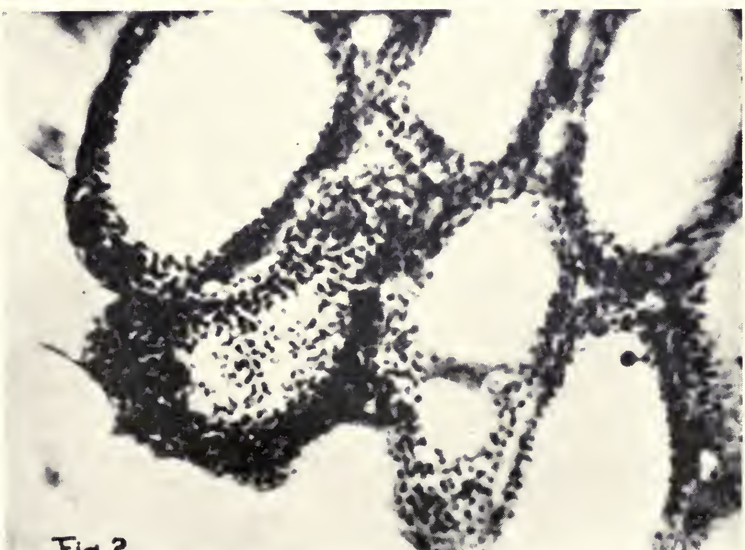


PLATE XIII

Fig. 1.—Young infected cells of a nodule of hairy vetch (*Vicia villosa*) (same as Fig. 2, Plate XII). Stained with Flemming's triple stain. The bacteria do not show distinctly × 430

Fig. 2.—Bacteroidal cells of red clover (*Trifolium pratense*) in a well advanced but growing nodule. Cells have become vacuolated and filled with bacteroids. Flemming's triple stain with amyl dehydration × 1080

Plate XIV

Root nodules of *Ceanothus americanus*

the middle area. The fibro-vascular system is immediately outside this, inclosed in the cortex layer of cells. At the tip is a well defined meristem made up of small, rapidly dividing cells, containing large nuclei. Few bacteria or bacteroids are found in the meristem or in the cortex; they are confined mostly to the bacteroidal cells. Aside from possessing bacteroidal tissue, the nodule differs from the lateral root in that it has no central cylinder. Further, it has no root-cap and no epidermis, but is inclosed by a protective band of corky cells.

Fig. 2, Plate XI, shows a cross-section of a nodule of *Trifolium pratense* at about the same stage of development as that shown in Fig. 1. This section was stained with Flemming's triple stain and then mounted in dilute iodine solution in order to show the location and distribution of starch. The fibro-vascular bundles may be seen especially well.

The bacteroidal cells of younger nodules are strikingly different from those of older ones. In the young nodule the cells are full and closely packed. Plate XII shows sections of a young nodule of *Vicia villosa.* Fig. 1 of this plate shows the meristem, and Fig. 2 a section some distance back. The latter figure should be compared with Fig. 2 of Plate XI. These young infected cells are shown more highly magnified (× 430) in Plate XIII, Fig. 1. The cells are filled with cytoplasm in which are dispersed myriads of bacteria. These bacteria occur chiefly as swarmers or as small vacuolated rods, so that it is difficult to resolve them in the cytoplasm. The nuclei are quite prominent.

As the nodule becomes older, the bacteria are more in evidence, the bacteroids becoming especially large and numerous. The nucleus becomes distorted and is pushed to one side of the cell, tho sometimes it disintegrates and disappears entirely, giving way to a large central vacuole, which is inclosed by a band containing mostly bacteroids. Fig. 2, Plate XIII, shows a few cells (× 1080) in which this has taken place. (This figure shows a few cells of Fig. 1, Plate XI, more highly magnified.) It is in this stage of development that the large, branched bacteroids, such as those shown in Plate III, are found.

The fibro-vascular system extends from the meristem region to the base of the nodule, where the elements unite and communicate with the central cylinder of the lateral root. As the nodule becomes older, the bacteria further devastate the cells and probably automatically shut off the food supply from the root, whereupon the nodule decays and sloughs off.

The so-called infection threads (*Fäden,* of Tschirsch; *Infektionschlauche,* of Prazmowski) so frequently found, especially in young nodules, were the objects of much study. Contrary to the opinions of Dawson[23] and Peirce,[25] the infection threads are *not* zoogloeal strands made up of small bacilli, but are solid hyphae-like structures bearing a remarkable resemblance at times to tubes, which in fact some earlier investigators believed them to be (see Plate XII, Fig. 3). No septae were found. The threads were more frequently seen in the meristem or in the cortex cells near the apex of the nodule. The longest one observed in a single section traversed six consecutive cells. Shorter threads were frequently encountered in the bacteroidal tissue. Frequently the threads were found branched, and invariably they were growing directly toward the cell nucleus or sending a branch to it. In passing thru the cell walls the thread becomes peculiarly thickened or flattened, producing a funnel-like appearance. This also occurs when the thread approaches the nucleus.

With the view that the infection threads are not zoogloeal strands composed of separate bacilli, they become more difficult to explain. A possibility is that they are due to unusually stimulated bacteroids or to a number of bacteroids which fail to divide but remain attached with the resorption of the cell wall between. However, this is pure speculation.

Part IV.—NON-LEGUMES SAID TO BE CONCERNED IN THE FIXATION OF ATMOSPHERIC NITROGEN

Historical

The demonstration by Hellriegel and Wilfarth[5 10] of the fixation of nitrogen by legume plants and the isolation by Beyerinck of the organism from the legume nodules, stimulated interest in the root nodules found upon non-legume plants. Of the groups of non-legumes which possess these structures, those which have received the most attention are *Ceanothus, Elaeagnus, Alnus, Podocarpus, Cycas,* and *Myrica.*

The earlier investigators[a] for the most part held that nodules were of fungous origin. Arzberger[49] held that the causal agents (*Frankia ceanothi* and *Frankia subtilis*) in *Ceanothus* and *Elaeagnus* were quite similar, but that that (*Frankia brunchorstii*) of *Mryica* was quite different, being of the nature of an *Actinomyces.*

Previous to Arzberger, Hiltner[45] in 1896 claimed a fixation of nitrogen by *Alnus* and *Elaeagnus.* In 1899, Nobbe and Hiltner[46] claimed the same for *Podocarpus,* the nodules of which were said to be due to an endotrophic mycorrhiza.

Bottomley[48] [b] claims to have demonstrated in 1907 the presence of nitrogen-fixing bacteria in the nodules of *Cycas.* In 1912 the same writer[51] reported the isolation of an organism identical with *Ps. radicicola* from *Myrica gale* and claimed fixation of nitrogen by young *Myrica* plants.

In the same year Spratt,[52] working in Bottomley's laboratory, reported a similar isolation from *Alnus* and *Elaeagnus* and also from *Podocarpus.*[53]

More recently Bottomley[54] has reported the isolation of *Ps. radicicola* from *Ceanothus,* and shown the fixation of nitrogen in culture solutions by the organism isolated.

Ceanothus americanus

Attempts to Isolate the Causal Organism.—As *Ceanothus americanus* grows close at hand, material for study was easily obtained. Efforts to isolate a causal organism were persistent, covering a period from early spring to late fall. Nodules in all stages were plated, special effort being made upon the extremely young ones. With ash agar alone ninety plates were poured in duplicate. Legume nodules were frequently plated as checks upon the method, the same procedure being used in each case. Legume nodules nearly always gave good plates; *Ceanothus* nodules failed always. Variation in the seeding of the

[a]For a review of the subject Arzberger should be consulted.
[b]The paper written in 1907 to which Bottomley refers was not found.

plates was tried. Sometimes the entire nodule was crushed with several cubic centimeters of sterile water and poured with the plate; sometimes several loops of infusion were used; and sometimes the nodules were cut open and the tissue scraped out for plating. Ash-agar plates were inoculated direct with nodule tissue and with crushed infusion.

Other media were tried. The list included Fred's agar (No. 201), Ashby's (No. 202), Spratt's (No. 203), beef-broth agar (No. 205), Conn's asparaginate agar (No. 204), *Ceanothus*-extract agar (similar to No. 206), a mixture of *Ceanothus*-extract agar and ash agar, potato agar, oatmeal agar, cornmeal agar, Loeffler's blood-serum agar, and Koch's blood-serum agar. Many plates were poured and many direct slants tried. Liquid media were not extensively employed as this means of isolation is objectionable. However, Spratt's medium (No. 103) and beef broth (No. 105) were tried.

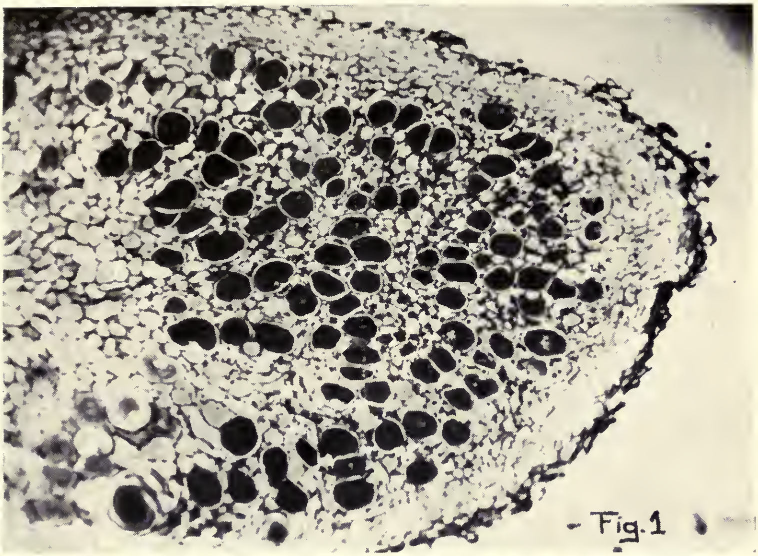
In no case did a typical plate resembling those obtained in plating legume nodules result. For the most part the plates were blank except for an occasional mold or yeast. Bacterial colonies sometimes grew, but never did a single organism persist that upon examination in any way resembled *Ps. radicicola.*

Almost invariably slants made direct from nodule tissue or crushed infusions failed to show growth. As with the plates, there was nothing to suggest a causal agent, either like or unlike *Ps. radicicola.* Little reliance was placed on the liquid cultures; most of them showed no growth.

Nitrogen-Fixation by Ceanothus americanus.—In order to test the fixation of nitrogen by *Ceanothus,* thirteen young plants were washed clean and planted in clean quartz sand to which lime had been added. Seven of the plants were given a nutrient solution without nitrogen, and inoculated abundantly with an infusion of crushed nodules. Six were given the same solution plus nitrogen, but were not inoculated. None of the plants fully recovered or made very vigorous growth. After ten months all those not receiving nitrogen were dead. Two of those receiving nitrogen still survived but were not doing well. All the plants produced nodules.

Seeds of *Ceanothus americanus* were obtained in the fall of 1915. By immersing them in commercial sulfuric acid for ten minutes a germination of about five percent was obtained. Three series of six pots were then filled with white quartz sand and four seedlings planted in each pot. Nutrient solutions were added as shown in Table 10. The plants in Series III were inoculated with an infusion of crushed *Ceanothus* nodule.

After seven months, Series II and III had made a very weak growth; there was no choice between them. Series I also had not made a very vigorous growth, but the plants were noticeably larger and greener than those of the other series. A further observation of im-



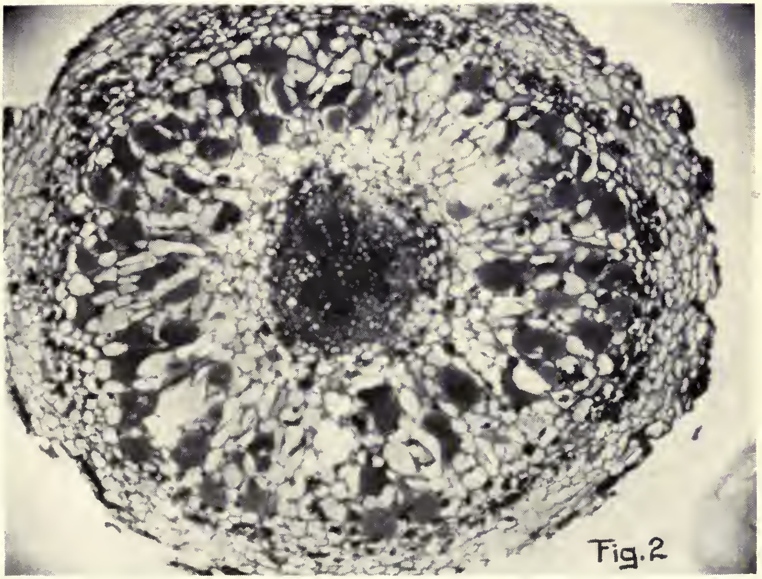


Plate XV

Fig. 1.—Longitudinal section of a *Ceanothus americanus* nodule, showing parasitized zone. Stained with Flemming's triple stain and mounted in iodine to show the starch × 100

Fig. 2.—Cross-section thru a similar nodule of *Ceanothus americanus,* showing central cylinder, some parasitized cells, starch, etc. × 100

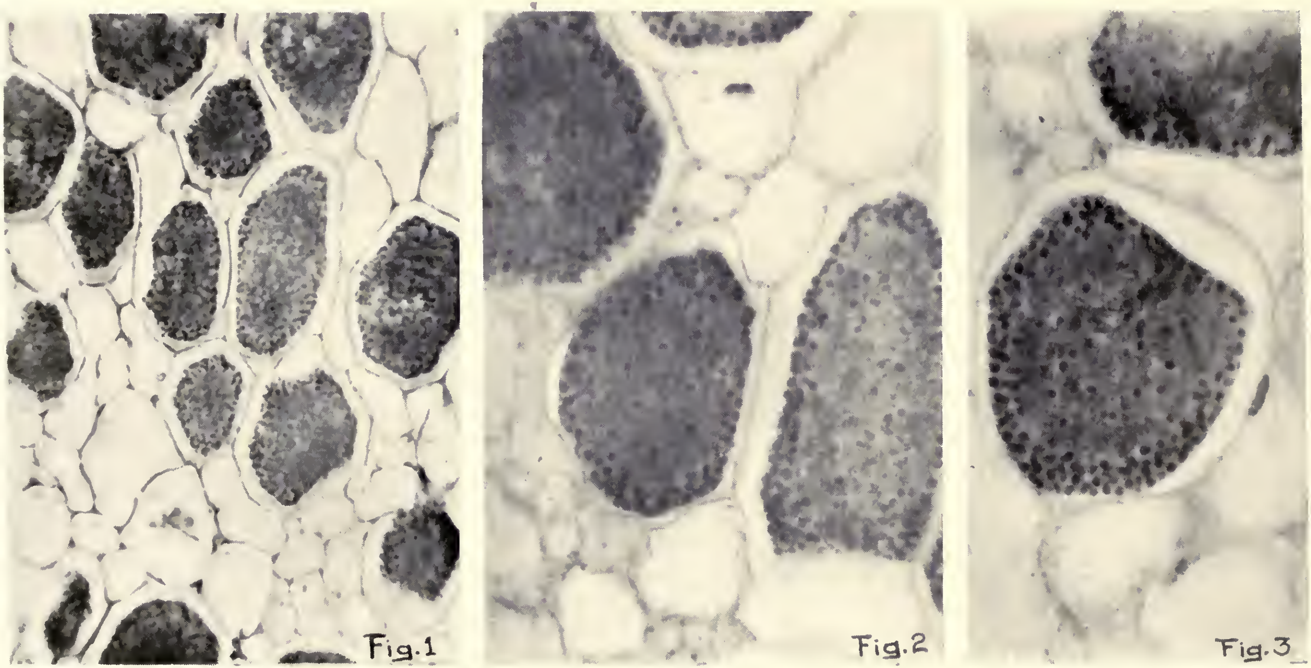


PLATE XVI

Fig. 1.—Parasitized cells of a *Ceanothus americanus* nodule × 430
Figs. 2 and 3.—Same more highly magnified. There is a strong suggestion of fungous threads, especially in Fig. 3 × 1080

portance was that Series III had no nodules, in spite of the fact that the young seedlings had been abundantly inoculated with an infusion of crushed nodules.

TABLE 10.—EXPERIMENT IN NITROGEN-FIXATION WITH SEEDLINGS OF CEANOTHUS AMERICANUS

Series	No. of plants	Inoculation	Treatment	Nodules
I	24	None	Full nutrient solution	None
II	24	None	Nutrient solution without nitrogen	None
III	24	Infusion of crushed *Ceanothus* nodule	Nutrient solution without nitrogen	None

While the evidence submitted is not conclusive, it at least throws doubt upon the ability of *Ceanothus* to fix atmospheric nitrogen. Apparently either quartz sand is not favorable or the solutions used were not best suited to this plant. It must be considered, however, that *Ceanothus* is a slow-growing shrub, and hence the demonstration would not be so easy as with quick-growing legumes.

Histology of the Nodules of Ceanothus americanus.—An extensive description of the nodules of *Ceanothus americanus* is not intended here, but it is desired to show enough of the structure so that a fair comparison with legume nodules may be made. For further information Bottomley, Spratt, and especially Arzberger, should be consulted.

In Plate XIV are shown some *Ceanothus* nodules. When extremely young, they are white or nearly so, and are round or slightly oval. They grow mostly along the longer axis, and become distinctly club-shaped, lacking entirely the plumpness of legume nodules. Branching commonly occurs; one nodule divides to form two or three, and later these branches divide, the whole ultimately forming a cluster of considerable size. The structures are perennial, making new growth and sending out new branches each year. In appearance, as well as to the section knife, they are quite woody, a point that further distinguishes them from legume nodules.

The parasitized zone of a *Ceanothus* nodule may be seen in Plate XV, Fig. 1. The section was mounted in iodine solution, as before described, to show the starch. The meristem and central cylinder do not show. Fig. 2 of the same plate shows a cross-section thru a similar nodule. The central cylinder is well developed and possesses a well defined endodermis. In the nodule cortex surrounding the endodermis, there are first several layers of small, apparently vacant cells, and then a zone of parasitized cells, which are rather loosely scattered in this area. The accumulation of starch in the cells surrounding the parasitized zone shows clearly. As in the legume nodule, there is no epidermis and no root-cap, but there is a protective layer of corky cells.

Plate XVI shows some of the parasitized cells. Fig. 1 is magnified 430 diameters, and Figs. 2 and 3, 1,080 diameters. The dark bodies are said by Arzberger to be sporangia of a fungus, the hyphae of which may be seen within the cells at certain stages. He designates the fungus as *Frankia ceanothi* Atkinson. While not agreeing with Arzberger in all the details, the writers accept the fungous conceptions as the true ones. The parasitized cells are tough and horny in consistency. When nodules were crushed for plating, these cells remained intact and were frequently seen distributed thruout the agar plates, where they were at first mistaken for colonies. No growth was produced by them, however. These parasitized cells clearly bore no resemblance to the bacteroidal cells of the legumes.

Summarizing, the nodules of *Ceanothus* are unlike those of the *Leguminosae* in the following points:

1. The *Ceanothus* nodules differ in external appearance from legume nodules; also, they are quite woody.
2. They are perennial structures, making new growth and producing new branches each year. The mode of branching is different from that of legume nodules.
3. They contain a well developed central cylinder, resembling in this respect a lateral root, of which they may be considered as a modification.
4. The parasitized cells are not closely packed as in the case of legume nodules, the characteristic bacteroids of legume nodules are not present, and the cells do not develop a central vacuole as do bacteroidal cells. Instead, the parasitized cells bear every indication of containing fungous hyphae.
5. No infection threads were found in the nodules of *Ceanothus*.

CYCAS REVOLUTA

Attempts to Isolate the Causal Organism.—Repeated attempts were made to isolate the causal organism from nodules of *Cycas revoluta* obtained from a greenhouse plant. The results were not wholly without success. The nodules of *Cycas* differ from those of the other five groups of non-legumes producing nodules in that the older ones become infected with a blue-green alga,[a] undoubtedly a secondary infection, which renders the nodule less solid and compact. In a cross-section cut from one of these older nodules the algal zone can easily be seen with the unaided eye.

From several algal-infected nodules three forms of bacteria were isolated, none of which resembled *Ps. radicicola*. Two were small, deeply staining rods, and the third was a larger rod. These three organisms were tried in sand pot cultures upon *Pisum arvense, Vicia villosa, Trifolium pratense, Medicago sativa, Melilotus alba, Phaseolus*

[a]See Spratt [50] [55]; also Life.[47]

vulgaris, Vigna sinensis, Glycine hispida, Lupinus perennis, Arachis hypogoea, Trigonella foenum-graecum, Desmodium canescens, Amphicarpa monoica, Ornithopus sativus, and *Onobrychis sativa.* Only one plant produced nodules—*Trigonella,* on which three appeared. This fact, however, is not regarded as significant, since *Melilotus* and *Medicago* were without nodules. (The nodules of *Trigonella, Melilotus,* and *Medicago,* it has been shown, are produced by the same organism.) Nodules due to chance inoculation are to be expected when open pots are used. It was further observed that the roots of most of the plants were distinctly brown and unhealthy, as tho attacked by a brown rot. The roots of *Pisum, Vicia, Phaseolus,* and *Lupinus* seemed especially to be injured. A small piece of an unhealthy lupine root was teased apart and examined, disclosing a host of motile bacteria.

Young *Cycas* nodules in which the algal zone was not present were plated, but without success. This seemed to indicate that the organisms first isolated were not causal agents, but that they followed the alga. Examination of the algal infected nodules disclosed a very loose, open structure; indeed, the whole nodule lacks the compactness of a *Ceanothus* nodule. The chance for entrance by the alga and later by foreign bacteria is very great. It would be surprising indeed if bacteria were not found in these older nodules.

ALNUS, ELAEAGNUS, AND MYRICA

Spratt,[52] after pointing to the demonstration by Hiltner[45] of the fixation of nitrogen by *Alnus* and *Elaeagnus,* reported the isolation of *Ps. radicicola* from the nodules of these plants. The results reported in connection with the experiments are subject to the following criticisms:

First: The demonstration by Hiltner of the fixation of nitrogen by *Alnus* and *Elaeagnus* is not nearly so convincing as Hellreigel's and Wilfarth's[10] discovery of the symbiosis between *Ps. radicicola* and legumes.

Second: Spratt's method of isolation is at fault. Spratt sterilized nodules and dropped them into flasks containing a liquid medium, incubating them two days. Obviously a single foreign organism in two days becomes a multitude, and no doubt a pure culture. It is not clear whether her agar plates were made from fresh nodule infusions or incubated material as described above, tho it appears that the latter was the case. The method is unsafe unless carried out on a very extensive scale (and then it is questionable), but Spratt used but one culture flask for *Alnus* and one for *Elaeagnus,* leaving one check, which may as well have been omitted. (Agar plates poured from legume nodules as before described, seldom fail to give good plates in this laboratory. If *Ps. radicicola* is present in *Alnus* and *Elaeagnus,* this method should easily demonstrate it.)

Third: The Kiskalt amyl-gram stain described by Harrison and Barlow and used by Spratt does not identify *Ps. radicicola.* Numerous gram-negative soil organisms lose the stain (aniline-gentian-violet) in ethyl alcohol, but retain it when amyl alcohol is used.

Fourth: The coccoid form described by Spratt is not analogous to the bacteroids of *Ps. radicicola.* There is no evidence to show that *Ps. radicicola* ever assumes the shape or characteristics described by Spratt. The writers have never observed it. The only form of *Ps. radicicola* approaching a coccus form is the extremely small, oval *schwärmer* of Beyerinck. The form described by Spratt is entirely too large for the *schwärmer.*

Fifth: The fixation of nitrogen in culture solutions is neither a test for *Ps. radicicola* nor a proof of symbiosis. With the legume organism, the fixation of nitrogen in culture solutions is not significant when compared with fixation in the nodule. Besides, many soil organisms have been attributed this power of fixation in nutrient solutions.

Attempts to Isolate the Causal Organism.—Nodules from *Alnus glutinosa* and *Myrica gale* were obtained and plated out. The results were negative. However, the trials were not extensive because the available material was limited. Ash agar, Spratt's agar, and beef-broth agar were used as media.

CONCLUSIONS

From the foregoing discussion, the following conclusions are drawn:

1. The root-nodules of *Ceanothus, Cycas, Alnus,* and *Myrica* are not caused by *Ps. radicicola.*

2. It is conceivable that *Ps. radicicola* might enter the nodules of *Cycas* as a secondary infection and function symbiotically, but its presence was not demonstrated.

3. The evidence that *Elaeagnus* and *Podocarpus* nodules are caused by *Ps. radicicola* is not conclusive.

4. Proof that these six groups of plants are concerned with the fixation of atmospheric nitrogen is wanting.

Part V.—ATTEMPTS TO DEVELOP A SYMBIOSIS BETWEEN LEGUME BACTERIA AND NON-LEGUME PLANTS

Previous Attempts.—In 1893, Schneider,[18] at the Illinois Experiment Station, under the direction of the senior author, cultivated nodule bacteria from *Phaseolus vulgaris* upon bean-extract agar, then upon a mixture of bean-extract and corn-extract agar, and finally upon pure corn-root-extract agar. Transfers were made every sixth day. After the cultures had grown for a month upon the pure corn-root extract, they were applied upon germinating seeds of corn and oats. Tho the inoculated corn plants produced no nodules, Schneider claimed that they were more thrifty than the uninoculated plants. He described and figured the infection of some of the root-hair cells, as well as some of the epidermal and parenchymal cells. No effect was noted upon oats.

That the senior author was intensely interested in this problem of developing a symbiosis between legume bacteria and non-legume plants is shown by the fact that when the opportunity presented itself after over twenty years, he took up the problem where Schneider* had left it.

Other attempts in this direction have been reported. Stutzer, Burri, and Maul[19] inoculated mustard plants with nodule bacteria which had gradually become accustomed to a mustard-plant medium, but without success. Grosbüsch[31] experimented with *Graminae,* but his results were negative.

Lemmermann[27] studied the difference in nutrition between the *Leguminosae* and the *Graminae.* He believed that the reasons for the existence of bacterial symbiosis in the *Leguminosae* and not in the *Graminae* are, namely, the smaller transpiration current, the higher acidity of the root sap, and the greater root development of the former as compared with the latter.

Preliminary Discussion.—How long ago symbiosis between the *Leguminosae* and the nodule bacteria began cannot be estimated. The conditions under which the first infection took place are but a matter of conjecture. Only a mass of contradictory literature concerning this symbiosis existed as a basis in attempting to develop a symbiosis between these bacteria and non-legume plants. There seemed, however,

*The following excerpts are quoted from a footnote by the senior author introducing Schneider's work in 1893.

"Can the organisms be made to grow upon these roots (grasses or cereals) by artificial means?

"It must be confessed that it would have been exceedingly hazardous for any one to have expressed an affirmative opinion upon this question; but the vast importance of the matter made it desirable to try anything which gave the least promise of success. . . . . While little direct evidence has been gained in favor of ultimate success, it is desirable to publish an account of the work so far done, with the hope of being able at some future time to add greatly to the information now obtained."

to be several points of attack which offered possibilities. There was some hope that the organism might be modified or changed. By accustoming it to media containing juices of non-legume plants, it was thought that it might become so modified that it would infect such plants. Injury to the plant, especially nitrogen starvation, was a possibility. Mechanical injury, however, seemed useless, since if it would develop a symbiosis the cultivation of crops would long since have accomplished it. The non-legume plants bearing nodules, such as *Ceanothus, Elaeagnus,* and *Cycas,* offered another possibility. It was hoped that if *Ps. radicicola* was present in the nodules, the organism would be more adaptable to other non-legume plants. In addition, the fact that the symbiosis appeared not to be confined to the *Leguminosae* alone was a great encouragement. It became evident, however, on investigating nodules of non-legumes that *Ps. radicicola* was not the causal organism; and furthermore, it seemed very doubtful if these plants were concerned with fixation of atmospheric nitrogen.

Another plan was to obtain a non-legume plant standing in close botanical relationship to the legumes, and attempt to inoculate it with legume bacteria. The organism from cowpea nodules offered the greatest possibilities, since it seemed less particular in its selection of a host plant.

EVIDENCE OF CONSTANCY OR CHANGE IN THE ORGANISM

Moore,[28] in 1905, reported that by inoculating legumes with nodule bacteria from *Pisum sativum* which had been grown for two weeks upon nitrogen-free media, he was able to produce nodules upon many genera. He stated that this was but a single demonstration of numerous successful cross-inoculations. It appears from his work that it was necessary only to grow the organisms upon nitrogen-free media in order to break the special adaptations.

Nobbe and Hiltner,[24] in 1900, claimed that they were able to make the nodule bacteria from peas produce nodules upon the roots of beans, and vice versa.

Laurent,[14] Mazé,[21] and Kellerman, [37] among others, have reported similar successful cross-inoculations.

EXPERIMENT I: COMPARISON OF NITROGEN AND NITROGEN-FREE MEDIA FOR THE GROWTH OF PS. RADICICOLA

In order to compare nitrogen and nitrogen-free media for the growth of *Ps. radicicola,* bacteria from *Melilotus alba* and *Trifolium pratense* were transferred to Freudenreich flasks containing standard beef-broth agar (No. 205) and Fred's synthetic agar (No. 201). The cultures were kept in the incubator at room temperature for thirty months without transfer. Duplicate cultures were transferred to test-tube slants once a month. At the end of the thirty months all cultures were transferred to ash-agar slants for comparison. All were alive and *capable of producing nodules* upon plants in test-tube cultures after Garman's method. Furthermore, it is important to note that the *cultures retained* their *special adaptation* to the *original host* plant and their cultural individualities as described on pages 136 and 139.

EXPERIMENT II: COMPARISON OF ASH AGAR AND BEEF-BROTH AGAR FOR THE GROWTH OF PS. RADICICOLA

Ash agar and beef-broth agar were compared as in Experiment I. Cultures of *Trifolium pratense, Melilotus alba, Vigna sinensis, Glycine hispida, Robinia pseudo-acacia,* and *Arachis hypogoea* were transferred to Freudenreich flasks containing ash agar and beef-broth agar. Duplicate test-tube cultures transferred once a month were kept. The culture of *Robinia pseudo-acacia* upon beef-broth agar was lost because of a mold. After seventeen months the cultures were transferred to ash-agar slants for comparison. All were alive (except the *Robinia*) and all *retained their individual habit of growth. Trifolium pratense* and *Melilotus alba* were tested by Garman's method and the others in sand pot cultures. (The remaining *Robinia* cultures were not tested except for growth.) All those tested were *capable of producing nodules* upon their *original hosts.* A few cross-inoculations were tried but failed. No difference in virulence was noted.

EXPERIMENT III: GROWTH OF PS. RADICICOLA ON TOMATO-STEM SLANTS

For the purpose of infecting tomato plants, cultures of *Melilotus alba* and *Trifolium pratense* were grown upon tomato-stem slants (No. 421) placed in tubes containing standard beef broth. Transfers were made once a month. After several months, distilled water was substituted for the broth. At the end of twenty-three months the cultures were transferred to ash-agar slants. Eight cultures of *Melilotus alba* and two of *Trifolium pratense* were examined. All grew readily when transferred to ash-agar slants. Tested in agar-tube cultures, all *produced nodules* upon their *original hosts. Melilotus alba* bacteria, however, failed to inoculate *Trifolium pratense* plants, and *Trifolium* bacteria failed with *Melilotus* seedlings.

EXPERIMENT IV: COMPARISON OF ASH AGAR AND CONN'S ASPARAGINATE AGAR AND THE EFFECT OF SUNLIGHT ON GROWTH AND VIRULENCE OF PS. RADICICOLA

This experiment was designed not only to compare nitrogenous and non-nitrogenous media, but also to note the effect upon growth and virulence of exposure to direct sunlight. Cultures of organisms from various legumes were transferred to Freudenreich flasks of 25-cc.

capacity, two sets containing Conn's asparaginate agar and two ash agar. All cultures were incubated for three days at room temperature, after which one set of the asparaginate-agar flasks and one of the ash-agar were removed to the greenhouse and left exposed to the sunlight. The slopes were turned toward the south to give maximum exposure. The checks, fewer in number, were left in the incubator. The experiment covered three months—from March 2 to June 2, 1916. The temperature in the greenhouse varied from 18° to 42.5° C.[a] The highest temperature recorded within a similarly prepared flask was 47° C. (thermometer shaded). In Table 11 is shown the arrangement of the experiment.

TABLE 11.—ARRANGEMENT OF CULTURES TESTED ON ASH AGAR AND CONN'S ASPARAGINATE AGAR IN SUNLIGHT AND IN DARKNESS: EXPERIMENT IV

Source of organism		In sunlight		In darkness	
Common name	Botanical name	Ash agar	Conn's agar	Ash agar	Conn's agar
		Flask No.	*Flask No.*	*Flask No.*	*Flask No.*
Acacia	*Acacia melanoxylon*	6011 (died)	6026	....	....
Tick trefoil	*Desmodium canescens*	6012	6027	....	....
Dyer's greenweed	*Genista tinctoria*	6013	6028	....	....
Soybean	*Glycine hispida*	6014	6029	....	....
Sweet pea	*Lathyrus odoratus*	6015	6030	6016	6031
White sweet clover	*Melilotus alba*	6017 (lost)	6032	6018	6033
Bean	*Phaseolus vulgaris*	6019	6034	....	....
Trailing wild bean	*Strophostyles helvola*	6020	6035	....	....
Red clover	*Trifolium pratense*	6021	6036	6022	6037
Broad bean	*Vicia faba*	6023	6038	....	....
Cowpea	*Vigna sinensis*	6024 (died)	6039 (died)	6025	6040

At the end of three months all cultures were transferred to ash-agar slants. Flask No. 6017 had been broken; the cultures in Nos. 6011, 6024, and 6039 had died. Of the surviving cultures those which had been kept in darkness recovered the most quickly. It was also noted that the organisms grown upon Conn's asparaginate agar were the most vigorous. The ash-agar cultures which had been exposed to sunlight were the slowest in recovery. After several transfers upon ash agar, the cultures resumed their normal appearance. They were then tested out for virulence (except *Phaseolus* and *Strophostyles*), and it was found that the *ability to produce nodules had not been affected.* The cultures of *Melilotus* and *Trifolium* were tested by Garman's method for ability to cross-inoculate seedlings of *Trifolium* and *Melilotus* respectively, but the cultures were virulent *only* upon the *original* host.

[a]The spring was cool and cloudy for the most part, tho there were some clear, hot days.

EXPERIMENTS ATTEMPTING THE INFECTION OF NON-LEGUME PLANTS WITH PS. RADICICOLA

The following experiments attempting the infection of non-legume plants with *Ps. radicicola* were but preliminary. In examining inoculated plants, attention was given only to any unexplained vigor and to the presence or absence of abnormal root conditions. Histological technic was not employed.

EXPERIMENT V: ATTEMPTED INFECTION OF TOMATO SEEDLINGS WITH SWEET-CLOVER BACTERIA

Very young tomato seedlings were transferred from flats of soil to one-gallon pots of limed white quartz sand. There were in all one hundred and fifty plants. Half were given a full nutrient solution,[a] and half were given a similar solution but without the nitrogen. Copious inoculations were made frequently with bacteria from sweet clover which had been grown for three weeks, with frequent transfers, upon a decoction of whole tomato plants plus two percent cane sugar and one percent peptone (Medium No. 111). After one month the plants were carefully washed free from sand and examined. Those which had been receiving nitrogen were decidedly more thrifty than the others. No abnormal conditions were observed in the roots.

EXPERIMENT VI: ATTEMPTED INFECTION OF TOMATO SEEDLINGS WITH SWEET-CLOVER BACTERIA IN THE PRESENCE OF COPPER SULFATE

Tomato seedlings which had been grown in flats of soil were transferred to paper boxes containing limed white quartz sand, one plant to each box. These boxes (2″ x 2″ x 4¾″) were arranged in a wooden frame, sixteen rows of sixteen each, making two hundred and fifty-six in all. Nutrient solutions made up as before were used, except that the nitrogen was varied as indicated in Table 12. Inoculations were made at the time of transplanting and again after two weeks with

TABLE 12.—TREATMENT APPLIED TO TOMATO SEEDLINGS: EXPERIMENT VI

Section No.	No. of plants	Nitrogen treatment	Copper-sulfate treatment
738	32	Full nutrient solution[a] (10 cc. stock solution per liter water)	None
739	32	Full nutrient solution	50 cc. of 1:2500 solution
740	32	Full nutrient solution	50 cc. of 1:1000 solution
741	32	Full nutrient solution	50 cc. of 1:500 solution
742	32	Nutrient solution without nitrogen	None
743	32	Double nutrient solution	None
744	32	Nutrient solution without nitrogen	50 cc. of 1:1000 solution
745	32	Double nutrient solution	50 cc. of 1:1000 solution

[a]See footnote, page 125.

bacteria from sweet clover which had grown upon tomato-infusion peptone (No. 111). After ten days copper sulfate was applied to the sections indicated in amounts intended to stimulate growth, to just hinder growth, and to seriously retard growth. The treatment is shown in Table 12.

The plants were examined after four weeks. Those which had received the normal amount of nitrogen showed the best development. The 1:2500 solution of copper sulfate stimulated both root and top development; the 1:1000 solution was slightly injurious; and the 1:500 damaged the plants seriously. No abnormal conditions of the roots were observed, except where the 1:500 copper-sulfate solution was applied, in which cases the injury was apparent.

EXPERIMENT VII: ATTEMPTED INFECTION OF TOMATO SEEDLINGS WITH SWEET-CLOVER BACTERIA IN SOIL AND IN SAND WITH VARIED NITROGEN TREATMENT

Tomato seedlings growing in sand and in soil in an arrangement similar to that of Experiment VI were inoculated with bacteria from sweet clover which had been grown for ten months upon tomato-stem slants (No. 421). Bacteria were applied at the beginning of the experiment and at intervals of a week thereafter. The nitrogen treatments used were varied as shown in Table 13.

TABLE 13.—TREATMENT APPLIED TO TOMATO SEEDLINGS IN SOIL AND IN SAND: EXPERIMENT VII

Section	Sand or soil	No. of plants	Treatment	Remarks
A	Sand	256	Full nutrient solution	
B	Sand	256	Nutrient solution without nitrogen	A small amount of nitrogen was added later, as the plants were starving. With the exception of a few very weak plants, however, all died
C	Sand	224	Harrison-Barlow wood-ash (No. 100)	
D	Soil	288	Tap water	Plants grew very vigorously and were cut back. 160 plants were given 1:500 copper sulfate solution to further check the growth
E	Sand	128	Nutrient solution with 1/10 nitrogen	
F	Sand	112	Nutrient solution with nitrogen trebled	
G	Soil	112	Tap water	Plants were cut back
H	Soil	112	Nutrient solution with nitrogen trebled	Plants were cut back

In general it may be said that the plants in soil were the most vigorous. Those in sand without nitrogen made very weak growth or died. Those watered with ash solution also made very little growth, probably because of a lack of nitrogen. The roots of the plants bore no abnormal structures.

EXPERIMENT VIII: ATTEMPTED INFECTION OF COMMON MORNING GLORY WITH SWEET-CLOVER BACTERIA

The plan was much like that of Experiment VII, except that common morning glory (*Convolvulus major*) was used. Seeds were planted in sand and in soil, using the paper boxes before described. There were 1,556 plants in all. Inoculations were made at the time of planting and again in two weeks with nodule bacteria of sweet clover which had been grown for one month, with frequent transfers, in an infusion of morning-glory plants plus two percent cane sugar and one percent peptone. Unfortunately some of the records were lost, but Plate XVII gives a general idea of the experiment. The inoculation produced no visible effect. The roots were carefully examined but showed no unusual conditions.

EXPERIMENT IX: ATTEMPTED INFECTION OF TOMATO SEEDLINGS WITH SWEET-CLOVER BACTERIA AND WITH A COMPOSITE INFUSION OF MANY LEGUME BACTERIA

The experiment involved 1,268 tomato seedlings grown in paper boxes filled with limed white quartz sand. These were divided into two equal sections. Those in one section were given the full nutrient solution, while those in the other were given the nutrient solution without nitrogen. Half the plants in each section were inoculated with sweet-clover bacteria which had been grown for twelve months upon tomato-stem slants (No. 421). The other half were inoculated with a composite of all the cultures of nodule bacteria on hand. There were cultures from forty-five plant species, including twenty different generic groups. Some were recent isolations, but most of them had been kept as stock cultures for one to two years. All had been grown upon ash-agar slants. The inoculations were in every case without apparent effect.

EXPERIMENT X: ATTEMPTED INFECTION OF STRAWBERRY PLANTS WITH SWEET-CLOVER BACTERIA AND WITH A COMPOSITE INFUSION OF BACTERIA FROM SEVEN SPECIES OF ACACIA

Young strawberry plants, one hundred and twenty in all, were planted in one-gallon pots of sand and of soil and treated as shown in Table 14. Half the plants in each series were inoculated with sweet-

PLATE XVII

Experiment VIII: Morning-glory plants grown in sand and in soil inoculated with sweet-clover bacteria grown for one month in morning-glory infusion media

TABLE 14.—TREATMENT APPLIED TO STRAWBERRY PLANTS IN SAND AND IN SOIL: EXPERIMENT X

Series	No. of plants	Medium	Treatment
A	30	Good potting soil	Tap water only
B	30	1 part soil to 4 parts sand	Tap water only
C	30	Washed yellow sand	Full nutrient solution
D	30	Washed yellow sand	Full nutrient solution without nitrogen

clover bacteria; the other half were inoculated with a composite infusion of bacteria from seven species of *Acacia.* Heavy inoculations were made frequently; the bacteria used had been grown upon ash-agar slants.

The plants did well at first, but later became infested with red spiders and in spite of sprays did not make a satisfactory growth. The results were negative.

SUMMARY

1. The nodule bacteria studied were found to be true *Schizomycetes,* actively motile by means of a single polar flagellum.

2. These bacteria may be divided into groups according to the host plants to which they become specifically adapted. In addition to the cross-inoculations previously known, many new ones were found to exist. These are given under Group III, page 136.

3. In addition to these special adaptations, there are among the various nodule bacteria serological and cultural differences which are permanent, giving perhaps a legitimate basis for the belief that distinct species exist. In numerous other characteristics, however, the nodule bacteria are so strikingly alike, and as a whole they differ so widely from any other species of bacteria, that it seems more consistent to regard the adapted forms as varieties of the single species *Pseudomonas radicicola.*

4. The legume nodule originates in the root-cortex, much as does the lateral root, but here the similarity ends. The nodule consists chiefly of a mass of parenchymal cells which are devastated by the nodule bacteria giving way to the bacteroid forms of the invading organism, which then make up the greater part of the cell contents.

5. The nodules of the non-legumes *Ceanothus, Cycas, Alnus,* and *Myrica,* said to be concerned with the fixation of atmospheric nitrogen, are not caused by *Pseudomonas radicicola.* The nodules of *Ceanothus* are wholly different morphologically from those of the *Leguminosae.* The evidence that the nodules of *Elaeagnus* and *Podocarpus* are caused by these organisms is not conclusive. Furthermore, the proof that any of these six groups of plants are concerned in the fixation of atmospheric nitrogen is not conclusive.

6. The adaptations of the nodule bacteria are constant. Such factors as the use of organic or inorganic substances in the medium, the acidity or alkalinity of the medium, and the presence or absence of combined nitrogen in the same, do not affect the virulence nor break the special adaptations. The virulence and specificity are bound up with the life of the organism.

7. The preliminary experiments here reported attempting the infection of non-legume plants with nodule bacteria failed.

8. No conclusions can be drawn as to the possibility or probability of developing or finding nodule bacteria that will grow on non-legume plants. The constancy of the special adaptations and the fact that no plants other than legumes harbor the organisms in question, as had been supposed, have been discouraging and to some degree limit the hope of ultimate success.

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[1]In the preparation of these bibliographies assistance was also rendered by Albert L. Whiting, Associate in Soil Biology, Warren R. Schoonover, First Assistant in Soil Biology, and William A. Albrecht, Fellow in Agronomy.

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