

7.

T H E S I S

Catabolic Processes in the Nodules
of Legumes
as Affecting the Nitrogen Content of the Plant.

By

FRANK W. BOUSON.

This thesis is submitted to the faculty of the Graduate School of the University of Tennessee in partial fulfillment of the requirements for the degree of Master of Science, May 15, 1917.

36274

C O N T E N T S .

HISTORICAL.

BIOLOGICAL.

Infection by the *Bacillus Radicicola*.

Types of Nodules.

Lupinus.

Robina.

Growth of Nodule.

Development of the Nodule after Infection
of the Root Hair.

Color of the Bacteroidal Tissue.

The Infecting Strand.

Description of the Nodule at Each Stage
of Growth.

BACTERIOLOGICAL.

Bacillus Radicicola.

Shape and Size.

Number of Flagella.

Method of Staining.

Bacteroids.

Method of Growth.

Production of Vacuoles.

NITROGEN FIXATION BY THE *BACILLUS RADICICOLA*.

As a Base for Rotation of Crops.

Method of Transfer of Free Nitrogen to the
Tissues of Legumes.

Maze Theory.

Buchanan's Contradiction.

EXPERIMENTAL.

Statement of the Problem.

Method of Proceedure.

Series 1 and 2.

Analyses for Ammonia in Nodules, Roots, Stems,
and Leaves.

Results of the Analyses.

Series 3 and 4.

Conditions Under Which Series 3 and 4
Were Grown.

Method of Sterilization of Seed.

Description of Apparatus.

Method of Operation.

Growth of Plants to Date.

Series 5 and 6.

Conditions under Which Series 5 and 6
Were Grown.

Description of Apparatus.

Method of Planting the Seed.

Growth of Plants to Date.

CONCLUSIONS.

H I S T O R I C A L .

As early as 1687 Malpighi observed the nodules on the roots of legumes, and like other investigators of that time, believed that they were results of pathological processes which were described as warts, lumps, knobs, and galls. It was not until 1853 that L. C. Treviranus established the theory that the nodule was a normal outgrowth on the legume plant.

A number of theories have been advanced as to the main function of the nodule; among these are such conceptions as that they were storage places for certain substances whereby a legume may be able to obtain nitrogen thru its leaves from the atmosphere.

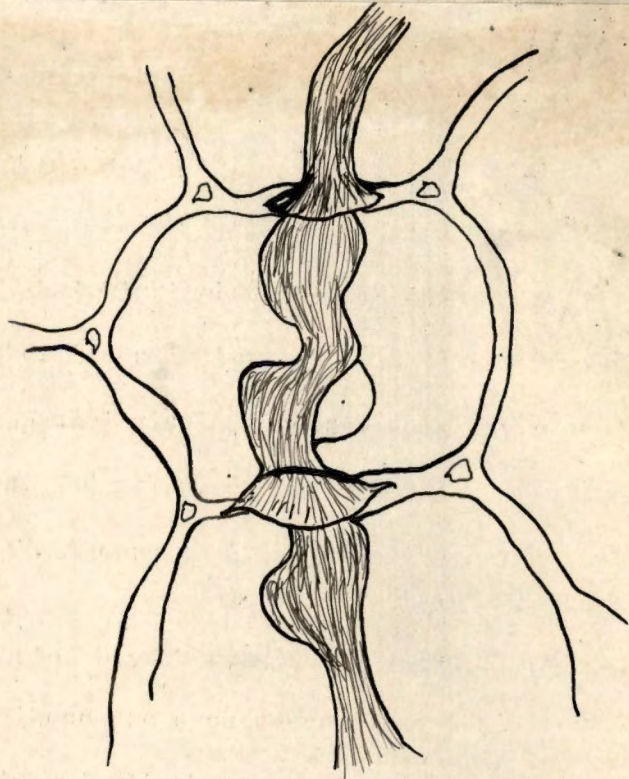
It was known as early as the days of the Roman Empire that legumes enriched the soil, but it was not until 1886 that Hellreigel and his co-worker Wilfarth made the discovery that legumes obtained nitrogen from the atmosphere thru the association of the microorganisms living in the nodules.

In 1887 Marshall Ward definitely proved that the formation of the nodules on legumes was due to an external infection. He was the first to successfully inoculate the roots of a legume by placing them in contact with old nodules.

I N F E C T I O N .



Root hair showing the
infecting strand.
(After Prazmowski)



Root cell, showing infecting
strand passing thru it.
(After Prazmowski)

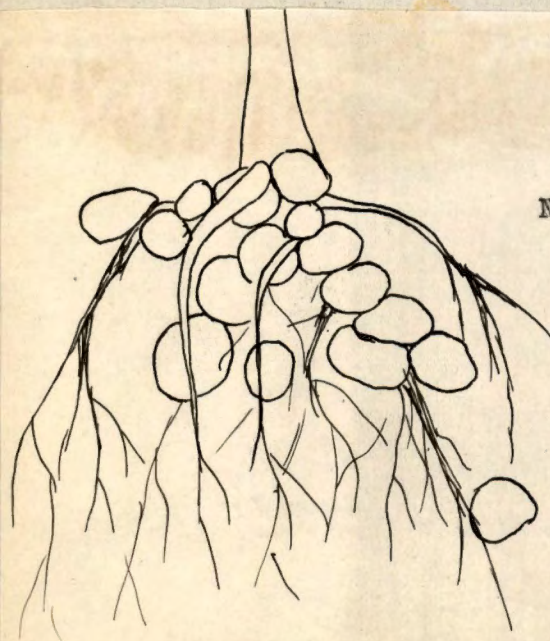
A legume may be readily inoculated by bringing the root into contact with the *Bacillus radiculicola*, and the result of such an inoculation is the formation of the nodules. The first infection is thru the root hairs as the young roots make their way thru the soil. Thus coming in contact with the *B. radiculicola*, they are attacked by the organism. Just what governs the infection of the root hairs has not been determined as yet, but the theory of chance inoculation is now generally accepted.

Upon coming in contact with the root hair, this

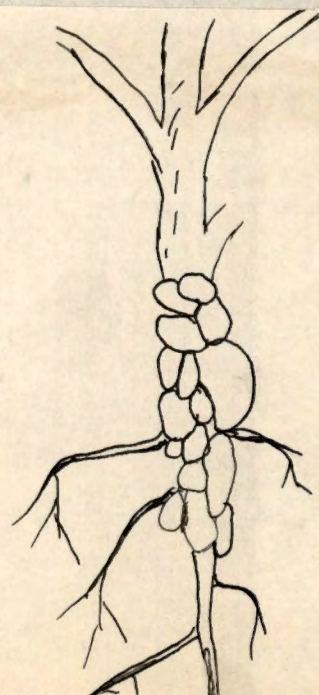
organism enters. Reproduction then takes place, and during its life process excretes enzymes which soften the cell wall allowing further entrance of the organism into the root tissues. At this point they further multiply forming the infecting strand which passes from one cell to another. It will be noticed from the drawing showing the passing of the strand from one cell to another, that when the strand comes into contact with the cell wall, there is a tendency to widen out; but upon entering the next cell, the strand again reduces in size.

TYPES OF NODULES.

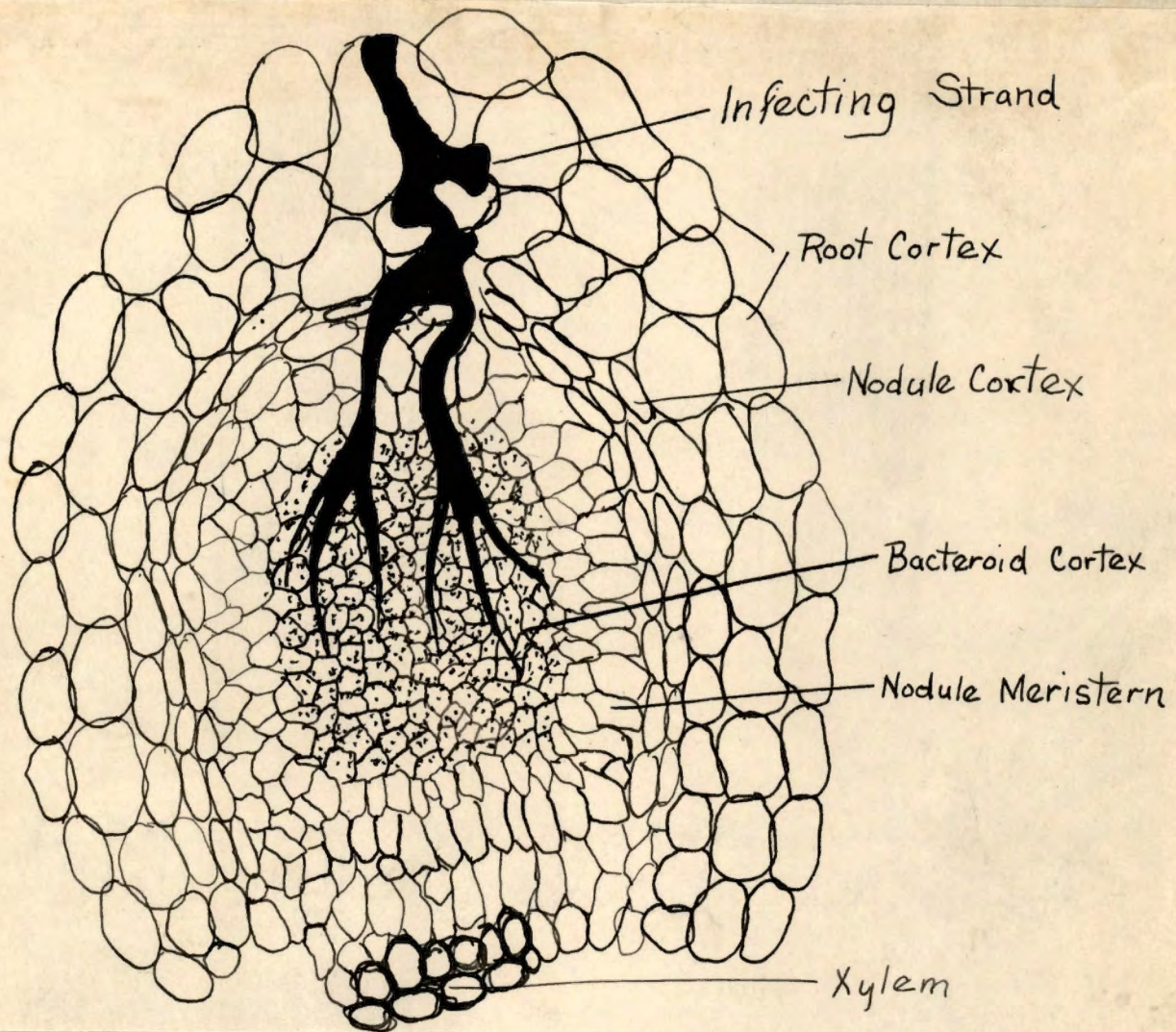
Tschirch recognized two distinct types of nodules which are known as the Lupinus type and the Robina type. These two types differ greatly in their morphological appearance.



Nodules of the
Robina Type.



Nodules of the
Lupinus Type.



Young Nodule, Showing the Beginning of the Differentiation of its Tissues

G R O W T H O F N O D U L E .

After the entrance of the *B. radiculicola* into the tissues of the roots, there is a rapid cell division of the pericycle. The protoplasmic content of these cells is increased, causing the cortical parenchyma and the epidermis to be pushed out, thus forming the nodule. We find that the nodule contains the various tissues which are present in the plant. The bac-

teroidal tissue is found in the center of the nodule and to vary in color from ochre to gray according to the age of the plant and the nodule. In the nodule, the process of the formation of the infecting strand may be observed. This strand in passing thru the cell does not impede the growth of the cell, but the power of cell division is destroyed.

Nodules on a young plant are firm, and the internal cells seem to be filled to their capacity, causing an internal pressure to be noticed upon mashing a nodule between the fingers. As the plant and nodules grow older, the nodules lose their firmness, and there seems to be a general breaking down of the entire structure of the nodule.

B A C I L L U S R A D I C I C O L A .

The *B. radicumicola* has been known by a number of different names. They are rod-shaped at time of entrance into the cell of the root hair. During the process of division and the formation of the infecting strand, this organism becomes greatly enlarged. In older nodules we find the bacteroidal shapes. The *B. radicumicola* varies in length from 1 to 5 microns, and in width from .5 to .6 microns. In Bulletin 179 of the Illinois Station, Albert L. Whiting describes the *B. radicumicola* as being peritrichous, having from 6 to 20 flagella. In studying the *B. radicumicola* from a culture obtained from the Bureau of Plant Industry at Washington, D.C., I found that this strain possessed but a single polar flagellum.

The Loeffler method of staining was used in connection with both analine gentian-violet and carbol-fuchsin.

This organism was successfully grown on mannite agar, while no growth was obtained on nutrient agar. The *B. radicicola* is a non-spore producer, and the way it lives in the soil has not as yet been determined.

Ball found that the organism becomes "Nitrogen Hungry" when cultivated thru several generations on nitrogen free media. This organism is not very sensitive to the reaction of the media whether acid or alkaline.



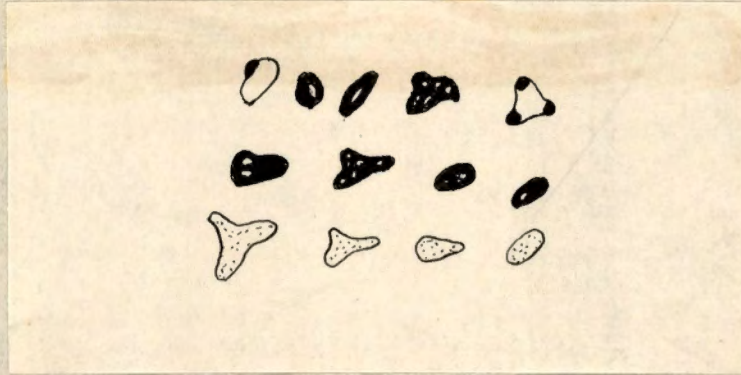
B. radicicola,
showing Polar Flagellum.

BACTERIODS .

Bacteroids are involution forms of the bacteria which may occur in the nodule or when grown on culture media. The first sign in the formation of the bacteroid is the thickening of one end of the organism. This club-shaped form then splits, taking a forked or "Y" form. Later these branched forms produce rounded outgrowths which are known as vacuoles. These vacuoles increase in size until they nearly fill the cell. These different forms have a definite period of growth, but

10.

this is not a sign of polymorphism of the bacteroid.



Bacteroids, showing shape and occurrence
of vacuoles.

NITROGEN FIXATION BY

THE B. RADICICOLA.

The accumulation of nitrogen by the *B. radiculicola* living in the nodules of legumes, has attracted universal attention. This fact is one of the bases upon which rotation of crops is based. While such crops as corn, wheat, and potatoes exhaust the soil of nitrogen, the legume plants such as clovers, beans, and vetches enrich the impoverished soil.

The question of how the possession of nodules on the roots of legumes enables this class of plants to transfer the nitrogen of the atmosphere to its tissues is still an undecided problem. It has been proven that the *B. radiculicola* has the power to fix free nitrogen in the absence of the legume plant.

Maze' based an interesting theory of nitrogen fixation upon the great viscosity of the *B. radiculicola*. This viscosity is due to the presence of a gum which was apart of the capsule or outer cell wall of the organism. Maze' was mistaken in his idea that this gum was a nitrogenous substance. Buchanan has shown that this gum contains no nitrogen, but is a carbohydrate.

Pot 1.	lined	Inoculated with culture from fernogera
Pot 2.	"	"
Pot 3.	"	"
Pot 4.	"	"
Pot 5.	"	Not inoculated.
Pot 6.	Not lined	"

E X P E R I M E N T A L .

An attempt is made in this paper to prove the theory that the *B. radiculicola* takes up free nitrogen from the atmosphere and builds it to protein for its body tissues, and afterwards this protein is broken down into amino compounds and thence to ammonia, which in turn is nitrified to the nitrate before it may be used by the host plant in its process of tissue building.

The soy bean strain of the *B. radiculicola* and the soy bean as the host plant were used thruout this study.

In series 1 and 2 the plants were grown in nitrogen free sand. The sand was brought into this condition by heating and washing with distilled water until the washings gave no reaction with Nessler's solution. No attempt was made to sterilize the complete outfit. The first four pots of each of these two series were inoculated with a culture gotten from a Farmogerm culture for the soy bean.

OUTLINE OF SERIES 1 AND 2.

Pot 1.	limed	Inoculated with culture from Farmogerm				
Pot 2.	"	"	"	"	"	"
Pot 3.	"	"	"	"	"	"
Pot 4.	"	"	"	"	"	"
Pot 5.	"	Not inoculated.				
Pot 6.	Not limed	"	"			

These plants were fed the following nutrient solution.

$2\frac{1}{2}$ cc solution of K_2SO_4 containing 50gm per 2500cc of water.

$2\frac{1}{2}$ cc " " $MgSO_4$ " 20gm " " " "

$2\frac{1}{2}$ cc " " $CaH_4(PO_4)_2$ " 25gm " " " "

$2\frac{1}{2}$ cc " " $NaNO_3$ " 75.3gm " " " "

$\frac{1}{4}$ cc " " $FeCl_3$ " 1/10gm " " " "

Four grams of Calcium Carbonate were added to the first five pots of each series.

The above solutions were made up to 250cc before adding to the pots.

SERIES 2.

Pot 1.	Limed	Inoculated with culture from Farmogerm
Pot 2.	"	" " " "
Pot 3.	"	" " " "
Pot 4.	"	" " " "
Pot 5.	"	Not inoculated.
Pot 6.	Not limed	" "

The same nutrient solution at the same rate with the exception that sodium nitrite was substituted for the sodium nitrate.

61 grams of Sodium Nitrite to 2500cc of water.

$2\frac{1}{2}$ cc of this solution was used as in the other formula.

4gms of calcium carbonate were added to each of the first five pots.

At the end of six weeks, the entire plants were carefully removed from the pots.

At the end of two weeks the plants in Pot 6 of both series had died. This was undoubtedly due to the absence of lime.

The plants from pot 5 in each series were found to have been accidentally inoculated.

An analysis for ammonia in composite samples of the Nodules, roots, stems, and leaves of the plants from the first four pots of each series. Each of these samples were ground in a mortar containing a little distilled water and a little ammonia free sand. These samples were then decolorized and filtered. Analyses were made by Nesslerizing the solutions. Results are reported in the following outlines.

SERIES 1.

Part of Plant	Weight	Standard	Ratio to Standard.
Nodules	.488	1	1.3
Roots	7.231	1	1.0
Stems	6. 690	1	1.5
Leaves	8.020	1	3.0

SERIES 2.

Nodules	.103	1	4.8
Roots	1.456	1	4.2
Stems	7.573	1	1.3
Leaves	5. 738	1	6.0

From the results of these analyses, it can plainly be seen that the nodules are rich in nitrogen in the form of ammonia. The presence of ammonia in the nodules in such quantities may be due to at least three conditions.

It may have been absorbed from the atmosphere as ammonia.

Decomposition may have taken place in the nodule due to some other organism than the *B. radicicola*.

This ammonia may be a direct product of the decomposition of the protein of the bacterial cells of the *B. radicicola*.

To prevent the first two conditions from existing, and to prove the third, it necessitates the growing of the legume under sterile conditions and in the absence of ammonia.

A special apparatus was designed by the author in which to grow plants under controlled conditions. This apparatus was used in Series 3 and 4.

SERIES 3 AND 4.

These series consists of five plants each, grown in the apparatus designed to grow plants in an atmosphere free of ammonia and under controlled bacteriological conditions. Series three was inoculated with a soy bean strain of the *B. radicicola* received from the U.S. Bureau of Plant Industry. The plants of series 4 were grown under sterile conditions.

The medium in this experiment was nitrogen free sand. The sand was brought into this conditions by heating until all the organic matter was decomposed and then washing with

distilled water until the washings were free from ammonia.

The same nutrient solution as used in series 1 and 2 was used in these two series. This solution showed no trace of ammonia by Nesslerizing.

M E T H O D O F S T E R I L I Z I N G T H E S E E D .

A number of methods of sterilizing the soy beans for the following series were tried. Calcium Hypochlorite and Bichloride of Mercury were used with little success for either the method was not successful or the germinating power of the seeds was weakened.

The following experiments with the use of the Ultra-violet rays were tried and the results reported below. It has been known that the Ultra-violet rays have the power of sterilizing a thin layer of water. Thus by treating seeds with a solution of bichloride of mercury or with water we surround the seed with a liquid film.

Outline of Treatments.

Sample No.	Preliminary treatment for 20 minutes.	No. of Minutes in the Ultra-violet Rays.
1.	1gm bichloride to 500cc water	20
2.	" " " " "	20
3.	" " " 1000cc "	20
4.	" " " " "	20

Outline of Treatments Continued.

Sample No.	Preliminary treatment for 20 minutes.	No. of minutes in the Ultra-violet Rays.
5.	1gm. bichloride to 2000cc water	20
6.	" " " " "	20
7.	" " " 40000cc "	20
8.	" " " " "	20
9.	Sterile water	20
10.	" "	20
11.	" "	40
12.	" "	40

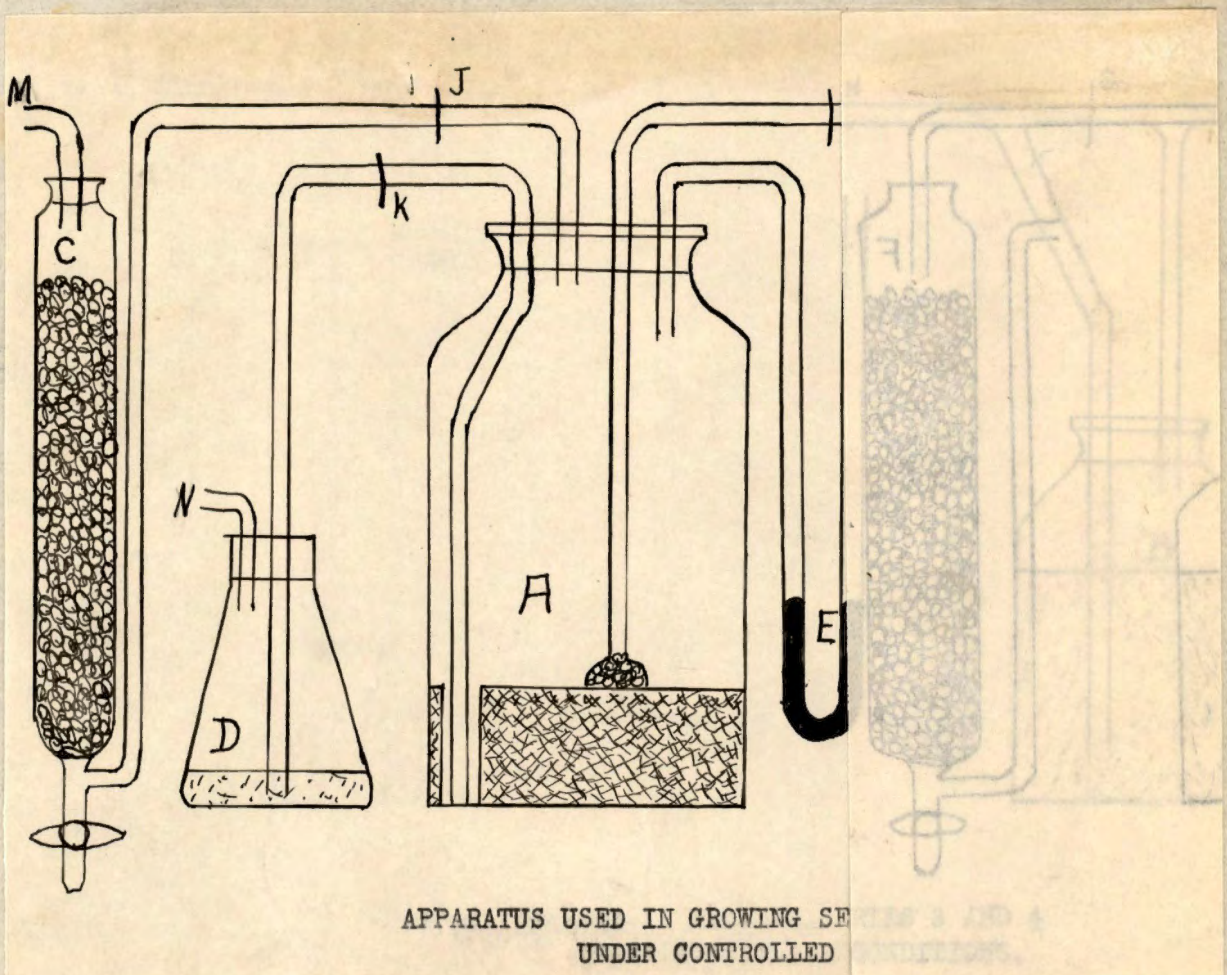
After the preliminary treatment and exposure to the ultra-violet light, each sample of five seeds was transferred to a sterile flask containing 1% agar. Due to the season of the year these flasks were placed in the incubator with a constant temperature of 20 degrees.

The following week these flasks were examined, and all of the samples were found to be sterile. Those samples which were treated with the solution of bichloride of mercury, in all cases showed its effect upon the germination of the seeds. The samples which were treated with the solution of one gram of bichloride of mercury to five hundred cubic centimeters of water did not germinate at all. Treatments Nos. 9 and 10 yielded the strongest looking seedlings. These had been treated with sterile water until they had swollen past the stage where the cotyledons begin to absorb water, and had been exposed to the ultra-violet rays for a period of twenty minutes.

Care was taken with all the samples to see that during part of the time all parts of the seeds had been exposed to the ultra-violet rays. This was accomplished by placing the seed in a sterile petri dish without covering due to the fact that the ultra-violet rays do not have the power to penetrate glass.

This method was used thruout the experiments and in each case with good results.

DESCRIPTION OF APPARATUS.



Apparatus used in growing series 3 and 4.

This apparatus was designed by the author to be used in growing plants under sterile conditions, and in studying the the gaseous products.

Two units of this apparatus were made and used in series 3 and 4. In these two series the following ideas were taken into consideration.

1. To grow plants under controlled soil flora conditions.
2. To grow plants in ammonia-free air.
3. To collect all ammonia as a product from the plants or bacteria.

The apparatus consists mainly of a Camp absorbtion tower used to free the air, taken in to the apparatus, of ammonia(This tower is half filled with a solution of sulphuric acid); a bottle containing the nutrient solution, a growing chamber, a resovoir for collecting the drainage, and a second Camp absorbtion tower for collecting the ammonia given off in the growing chamber. The parts are lettered on the diagram as follows:-

- F. Camp Absorbtion tower containing sulphuric acid.
- B. Bottle containing nutrient solution.
- A. Growing-chamber.
- D. Resovoir to hold drainage.
- C. Camp tower containing sulphuric acid to absorb ammonia which may be formed in A.
- E. Barometer to partially care for differences in pressure which may take place in A.

Stop cocks are placed at K, J, H, and G.

METHOD OF OPERATION .
S E R I E S 3 A N D 4 .

The two units of apparatus for the series 3 and 4 was sterilized in the autoclave. Some trouble was encountered in sterilizing due to the fact that the growing-chamber and the nutrient solution container are ordinary glass bottles, and in the procees of sterilization in the autoclave, the steam was applied too rapidly, resulting in the breaking of the bottles. After several attempts, sterilization of the units without breaking was obtained.

Sterile seedlings were transferred to the growing-chamber in the inoculating room after the walls had been sprayed with a solution of bichloride of mercury.

Series 3 was not inoculated with the *B. radicicola* at this time due to the fact that the culture ordered from the Bureau of Plant Industry had not arrived. This series was inoculated two weeks later. Series 4 was used as a check to series 3. The plants of this series were not inoculated, and were allowed to grow under sterile conditions.

Operation of the Apparatus During the Growth
of the Plants.

The gasses from the growing-chamber were aspirated off every other day thru the Camp tower C which, containing sulphuric acid, would absorb all ammonia present. This was done by having the stop cocks K closed and J, H, and G

open. The suction was applied at M of the tower C.

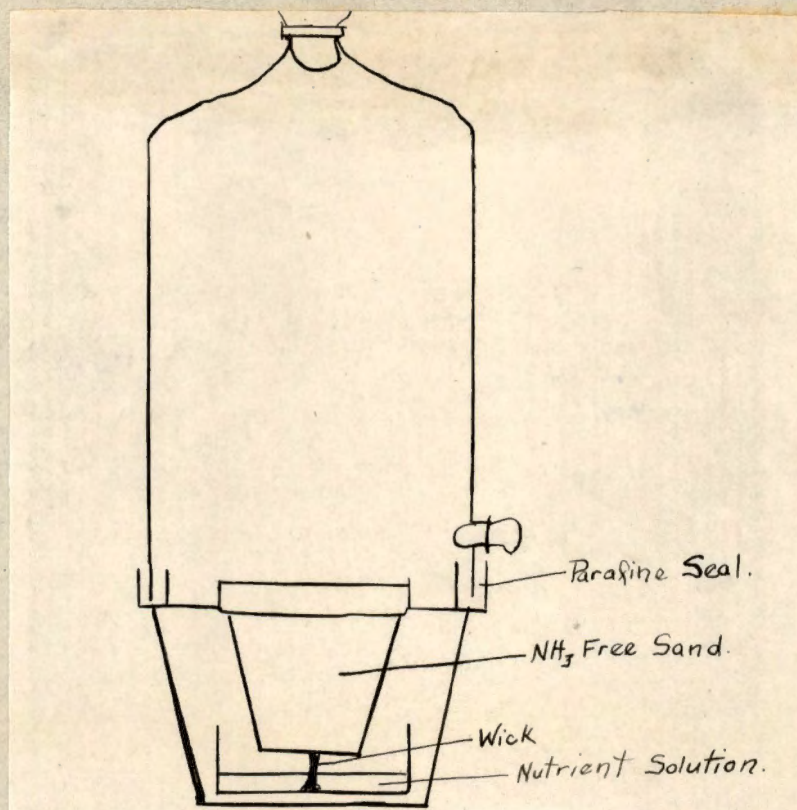
The drainage water was removed by opening the stop cocks K, H, G; and with the stop cocks J closed. Suction was applied at N.

The plants were watered by closing stop cocks G and K, and opening J and H. Suction was applied at M.

The condition of the plants in series 3 and 4 up to the present time are good. Series 4 has shown signs of a fungus growth on the fallen leaves. This may have been due to incomplete sterilization at the beginning.

S E R I E S 5 A N D 6

Series 5 and 6 were of the same nature as series 3 and 4, and will be used as a check. The apparatus used in this series was designed by Prof. Mulvania of the Tennessee Experiment Station. A full description will be found on the next page.



Apparatus designed by Prof. Mulvania for the
Growing of Plants under Sterile Conditions.

The essential parts of this apparatus consist of a large bell jar with a hole in the top and one in the side; a large galvanized iron pot with a groove arrangement in the top to allow the bell jar to fit therein. An ordinary flower pot with a wick fitted in the bottom to absorb the nutrient solution which is placed in a dish at the bottom of the galvanized pot.

After the flower pot had been filled with ammonia free sand and placed in its brackets, the bell jar was fitted and a seal made with paraffine. The entire outfit was placed in the autoclave and sterilized under a pressure of 15 pounds

for twenty minutes.

With the use of a small copper spoon, the seedlings which were grown from sterile seeds, were placed to a depth of an inch in the sand. This was accomplished thru the opening in the side of the bell jar. Both openings were closed with a cotton stopper.

Series 5 and 6 consisted of two units of this apparatus. After a week's growth, the sand of series 5 was inoculated with a pure culture of *B. radicicola*. Series 6 was left uninoculated, and the plants are making fair growth under sterile conditions.

Condition of Plants to Date.

Series 5 and 6.

Up to the present time both of these series are making good growth. Series 5 which had been inoculated with a pure culture of the *B. radicicola* seem to have been hindered in its growth as compared with Series 6 which was left uninoculated and sterile. At the time series 5 was inoculated, the author noticed the fact that the seedlings of both series were of the same size and vigor. During the three weeks following series 5 seemed to have made no growth at all. At the present time series 5 seems to be producing stronger plants than series 6 while the plants of series 6 are growing spindly.

The only explanation to be offered for the above observation is that immediately after inoculation, the *B. radicicola* is acting as a parasite on the soy bean plants. The same distinctions were noticed in series 3 and 4, but the differences

in the growth between these two series after the inoculation of series 3, was not so marked as the differences in the growth between series 5 and 6.

S U M M A R Y .

O F C O N C L U S I O N S .

1. The nodules of the soy bean are rich in ammonia as compared with the stems, roots, or leaves of this plant.
2. Soy beans will not grow in the absence of lime.
3. The absence of the usual soil flora, shows no very marked effect on the growth of the soy bean. This class of plants may be grown under sterile conditions.
4. When soy beans are grown under sterile conditions and are then inoculated with a pure culture of the *B. radiculicola*, there is a temporary check in the growth of the plants.

The author takes this opportunity to express his gratitude to Prof. Maurice Mulvania, who guided the work on this problem; and to all others who have been so kind in giving their aid and suggestions.