PHYSIOLOGICAL STUDIES ON
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PHYSIOLOGICAL STUDIES ON CEANOTHUS AMERICANUS

The Root Nodules and Some Characters of the Causal Organism

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TABLE OF CONTENTS

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The Root Nodules and Some Characters of the Causal Organism.

I. Introduction

The structure and functions of the root nodules of non-leguminous plants have engaged the attention of numerous investigators during a century. However, in no case has the causal organism been clearly isolated and tested.

The nodules of Ceanothus americanus L. have been studied by several investigators (6)* since Dr. Beal first called attention to them in 1890 (3); and Bottomley (4) claimed to have isolated a strain of Pseudomonas radicicola Beij. from specimens collected in America by Rosendahl and others. But Bottomley (4) did not test the ability of his isolated organisms to infect the host plant. Burrill and Hansen (6) shortly thereafter failed to isolate any organism after many attempts with a wide range of media; and on this account, the claims of Bottomley have been doubted by many investigators.

The historic interest of Ceanothus americanus, as well as its use in ornamental plantings, has made desirable a more detailed knowledge of the physiology of this plant. Furthermore, the general ecological significance of the root nodules of Ceanothus takes on added interest due to the important role discovered for analogous structures on legume roots; and this

*The parenthetical indices refer to the authors cited in the literature given after the conclusion of this paper.

interest is not lessened by the fact that similar functions have been attributed, even if on vague grounds, to the nodules of such non-legumes as Cycas (8), Podocarpus (9), Alnus (8), Myrica (4), Elaeagnus (8), and Ceanothus species (4).

The observations reported in this paper are the results of experiments undertaken with the purpose of discovering more substantial bases for the claims made for nitrogen assimilation of Ceanothus americanus L., and to determine some of the characteristics of the causal organism of the root nodules.

II. Historical

When Beal reported the presence of nodules on Ceanothus americanus to the American Botanical Society (3), Atkinson became much interested in them and immediately began the study of their structure and of the causal organism (2). He gave a description of the mature nodules to which little has been added by subsequent workers. Basing his findings upon the organism as it occurs in the host cells only, he referred it to a new species Frankia ceanothi.

Among the later investigators only Bottomley (4) and Spratt (16) dispute this classification.

Upon the role of these nodules Atkinson makes no correlative field observations on growth, and he dismisses the whole matter of symbiosis with the statement that "so far as can be seen they (parasite) cause no inconvenience to their host". He reports no attempts at the isolation of the causal organism. He also states that the older hyphae are devoid of protoplasmic contents.

Arzberger in 1910 (1) repeated the structural and taxonomic studies of Atkinson without reaching different conclusions, but he also investigated the enzymatic action of nodule sap. He found a decided ability of the juice to peptize certain proteids (blood fibrin) and from this concluded that the empty hyphae noted by Atkinson have in all cases had their contents digested by a proteolytic enzyme, which, as he states, is probably secreted by the host plant. He concludes that "Symbiosis" (nutritive mutualism is most probably meant) "exists, which is quite apparent in the early stage."

In 1915 Bottomley (4) reported briefly some studies on the structure and branching of the nodule and he also presented data bearing upon the classification and nitrogen-gathering ability of the causal organism. He claimed that the branching of nodules is lateral and not dichotomous. He also isolated an organism which he identified as a strain of *Pseudomonas radicicola* Beij., and from very limited experiments concluded that it could assimilate very small amounts of atmospheric (elementary) nitrogen. From these few tests and without any inoculation trials he concluded that "it is therefore evident that the root nodules of *Ceanothus* are definitely concerned with nitrogen assimilation". Neither Arzberger nor any other investigator of *Ceanothus* had, up until 1918, reported any effort to correlate general growth with degree of nodulation.

It was to be expected that investigators interested in legume nodule organisms would immediately try to confirm Bottomley's conclusions. Especially Burrill and Hansen (6) tried repeatedly to isolate the organism by employing a wide range of the best *Pseudomonas* media; but they always failed to get anything remotely suggesting Pseudomonas radicicola from the Ceanothus nodules, although the media used were always successful in isolating various strains of Pseudomonas from various leguminous species. Since Bottomley had used only ordinary "Pseudomonas culture solution", Burrill and Hansen judged that Bottomley's conclusions were of doubtful taxonomic reliability, and were more inclined to accept Atkinson's identification as the correct one.

These investigators were not able to grow the wild plants or their seedlings successfully in sand cultures, nor does Bottomley or any other investigator, except the present writer (14), report the successful growth of this somewhat fastidious plant when grown under artificial conditions. Burrill and Hansen succeeded in sprouting only five per cent. of the seeds even after treating ten minutes with concentrated sulfuric acid, while the writer at the beginning of this investigation (1920) (14) succeeded in obtaining regularly from forty-five to seventy-six per cent. with the sulfuric acid treatment and thirty-seven to sixty-five per cent. by ordinary hand scarification with sandpaper. Part of this wide divergence may be explained, in the experience of the writer, by locality of seed production, and part may be due to differences in methods of sterilization and germination. In the paper referred to, the writer (14) also reported effects of sterilized soils upon the sprouting and further growth of Ceanothus seedlings. These effects are of importance in cultural work with this species, but they need not be summarized here.

Thus the role of the nodules, the correlation of their rate and mode of growth with general growth, as well as the isolation and identification of the causal organism, were all very uncertain when the following experiments were begun. Moreover, the successful growth of sterile seedlings, and especially in sterile sand cultures with and without nitrogenous fertilizers -- matters of the greatest importance in physiologic studies of this nature -- had not been attempted.

III. Experimental

A. Growth and Distribution of Nodules.

In order to correlate general growth with nodulation it is first necessary to find a method of determining the age and annual growth rate of nodules. Otherwise, a positive correlation between nodulation and growth in a perennial of this type would lead to error in case a plant had only slight nodulation till late in life. The mode of branching in nodules may also be related to the determination of their age. The following observations on these points were made during two growing seasons.

1. Rate of Growth and Factors Concerned.

a. Annual Growth and the Glass Plate Method of Observation.

About two hundred nodules on twenty different plants were carefully exposed. These were covered by glass plates according to the method of McDougal (9), to provide for later examinations without disturbing the root system. Small constrictions were noted on most nodules and these were searched for in other plants. The significance of these constrictions was at first not appreciated, but by counting them and comparing with the age of the plant as shown by the annual rings of its hypocotyl, it was found that they agreed in most cases where the oldest nodules of plants from two to five years were considered. All discrepancies were due to branching of the nodules in the first year's growth, and a previous failure to note that in such cases there is no constriction at the bases of the branches.

This discovery later proved to constitute a valuable criterion of rate of nodule growth in different parts of a given root system for different years and it later formed the basis for comparisons of nodule growth in different soils and habitats.

b. Observations on Nodules in situ.

During the autumn, winter, and spring of 1918-19 and only autumn and spring of 1919-20, observations were made at intervals of two weeks. During the first half of the first winter, which was a mild one, about thirty per cent. of the nodules nearly doubled their growth as measured by that of the summer, while forty per cent. only added from one-fifth to three-fifths as much. The remainder grew slightly or not at all. Those nearest the tap root grew most rapidly.

No growth was noted during February or March, but in mid-April it was resumed again without stopping, till the next October when a severe winter set in. This did not allow measurement again till January 1920 when no increase over the October's measurements could be noted. Late in the following April slow growth was resumed again.

c. Observations on Nodule Growth of Plants taken to the greenhouse.

Similar observations were made daily on the nodules of five plants brought to the greenhouse from the same habitat. Here some fifty nodules made a more or less continuous growth for nearly two years. Each increase in growth of sufficient size to be measured took place during and immediately following considerable periods (1-2 weeks) of sunshine. No other factor could be satisfactorily correlated with nodule growth in these five plants.

Summarizing briefly, these observations on Rate of Growth indicated (1) that nodule growth continues beyond the normal growth period of the tops if the autumn and winter are mild and that growth is stopped by very low temperatures, (2) that active nodule growth attends and extends beyond a period of photosynthetic activity and therefore most probably depends largely upon the products of photosynthesis, (3) that nodule growth is isodiametric or without notable constrictions, when the conditions are favorable to general growth over long periods (one or more years), (4) that the annual constrictions of nodules make possible comparisons of nodule and general growth in different parts of the same root system throughout the different years of the plant's life, and finally (5) that the effect of different habitats on nodule and general growth can thus be more accurately judged.

2. Mode of Branching in Nodules.

In determining the age of a nodule, the branching must be considered, as has been previously pointed out. All writers except Bottomley agree that branching is dichotomous or polychotomous. Bottomley (4) states that as many as five branches may be formed in one year but that they are successive. Such an interpretation of the mode of branching is inconsistent with the formation of annual constrictions as noted above, because branches do not occur between constrictions, except rarely in the first year's growth, and here the coloration and other evidences of simultaneous branching are too strong to escape notice. The following observations were therefore made upon several hundred nodules to determine upon what ground, if any, such an interpretation as that of Bottomley might rest. Three methods were followed: the first consisting of measurements of the length of di- and polychotomous branches; the second of longitudinal and cross sections of incipient branchings, and the third, applicable only in the case of the oldest nodules, a comparison of actual age of the plant with the age of nodules as indicated by the constrictions detailed above.

The results of these observations are as follows: First, in all cases (five hundred in number) of young nodule branches measured, their length was practically identical within the group (Pls. I and III); while in older branchings (six hundred in number), uneven growth in a branch in some succeeding year, or a total cessation of growth in one or more old branches of a group, gave the appearance of lateral outgrowth, inasmuch as these old branches were often thinner and were crowded out of position by the more sturdy branch of equal age which had greater capacity for growth.

The second method of examination included about five hundred nodules whose earliest branching stages were longitudinally, or in some cases transversely, sectioned. Some of these were stained after being passed through the usual paraffin technique schedule. All showed the same method of branching in which the growing point flattened or became lobed, depending upon what the future position and number of branches was to be. Each lobe therefore becomes a new branch and thus they must be simultaneous in origin even if they should not grow to equal length subsequently. The figure shown by Bottomley (4) is certainly that of a section which does not follow the long axis of each branch (in a pair), hence one seems older and more developed than the other. Only the careful following of a complete series of such sections of a whole nodule, or the use of younger stages of branching could avoid misjudgment in this connection.

The general or longitudinal type (Pl. I, fig. 2) of infection and nodule formation which was rarely found by the writer, produced as many as five branches simultaneously and in a row. In these as in other cases, branching of the plerome and stele is often accomplished before the apex of the nodule shows much deformation.

In using the third method of determining the mode of branching, the oldest nodules of plants from one to five years old were found most suitable. The age of the plant usually was first determined. The annual constrictions were then taken into account. Thus in over two hundred cases examined at random the ages of branches agreed exactly.

When this test is applied in the reverse order, the first part; viz., age of nodule, in a small percentage of cases, appears greater than that of the plant by one year, as determined by the annual rings of the wood. This is due to the fact mentioned previously that in the vigorous growth of some young plants the main body of a nodule or of a branch divides while it is still actively elongating. But in such cases the constriction at the point of branching is not evident as it is in all other cases where branching usually occurs early in the growing season. Moreover, the uniformity in color of such exceptional branches, with their branch of origin, persists for at least two years.

Briefly stated, these observations furnish no support for the claims of Bottomley that branching of Ceanothus nodules is not simultaneous; but on the contrary, they indicate that one may misjudge the type of branching, due to (1) faulty sections when not studied in series; or (2) failure of some nodule branches to grow, while their contemporaries monopolize the food and push them out of position; and finally (3), that these facts may cause inaccurate age comparisons to be made.

3. Distribution of Nodules.

Microscopic examination of nodules has always disclosed the fact that the cortical cells are nearly filled with starch. If the metabolism of the parasite requires starch or starch-forming sugars and if it liberates materials of benefit to the host, it would be interesting to see if nodule distribution conforms to the most efficient location of such a double function. The following observations on field and greenhouse seedlings were made to determine whether nodulation has some regularity or not, and if regular, whether this can be correlated with other habits of growth or facts concerning food distribution.

Over one thousand root systems were carefully studied, over seven hundred of which came from three different types of habitat soils, two hundred were greenhouse seedlings from seven to nine months old grown in several types of soils, while seventy were plants of all ages transferred from field to greenhouse. The nodules were removed from these latter plants which soon produced new nodules on new roots.

a. Of the seven hundred plants studied in the field two hundred of all ages were dug from a fine clay soil. These had most of their nodules at a depth of from three to ten cm.; and the largest, i.e., those of most rapid growth, were located in the upper part of this stratum and mostly on or very near the primary root. Below a fifteen cm. depth the nodules were small in diameter and of slow growth.

Three hundred plants dug from sandy soils showed a slightly deeper distribution than was found in clay soil. Below a fifteen cm. depth the nodules were slightly larger and more numerous than in the clay soil. A few were found at one hundred fifty cm. below the surface of the soil which is about seventy-five cm. deeper than the deepest found in the clay soil.

Over two hundred plants from a sandy loam soil had essentially the same distribution of nodules as those in the sandy soil. As found for the other soils, the lowest nodules here showed the smallest diameters and shortest annual growths.

b. The seventy plants brought to the greenhouse conformed in every way to the distributions just mentioned. The nodules were removed and the longer roots were pruned to fit them for planting in pots having from fifteen to twenty cm. of soil. They formed many new roots and nodules, and the tops grew luxuriantly. The bottom root growth was especially heavy due to bottom heat and good aeration, but the nodules were larger and more numerous in the upper half of the root system. However, the differences between the lowest and highest strata were not nearly so great as in the field conditions of these plants.

c. Three hundred greenhouse seedlings naturally infected by habitat soil showed most nodule growth in the upper two to eight cm. These plants had ample opportunity for a different distribution. Priority of infection and a possible acquired immunity are not factors here, because the higher (Pl.VII) lateral roots bore nodules, while the lower roots did not.

The explanation for this distribution of nodules as well as for that of the field plants, it would seem, does not lie in the variation of oxygen supply as all were well aerated, except those from the clay soils which had numerous nodules at depths known to be poorly aerated. Nor does it lie in the water supply for the regions favoring root growth most; i.e., those of optimum water supply were not the most highly nodulate regions of the root systems.

d. In sectioning the root crown to determine age it was always found to be excessively thick for a plant of such size, age, and woody character. This extra thickness tapers off very rapidly in the upper ten to fifteen cm. of its length. Here the largest amounts of food are deposited as shown by the thickness of the annual rings and here nodule growth is always most rapid and plentiful.

It would seem then, that this region of food deposit would be most favorable for the nourishment of parasites and at the same time most favorable for the distribution of useful foods or stimulants made by them, to roots and shoots of the host plant. The high degree of correlation found between this lodgment place for food and location of most nodule growth amounts to certainty or causation. A corresponding degree of certainty might be anticipated for a reciprocal distribution of the effect of the parasite upon the host if such could be demonstrated.

B. Correlation of Nodulation with Growth.

1. Field Studies.

As previously noted, Atkinson (2) did not consider the

root nodule organisms of *Ceanothus americanus* detrimental to growth, while Arzberger (1) probably intended to convey the opinion that a mutualistic symbiosis exists. Bottomley (4) claimed that these nodules are definitely associated with atmospheric nitrogen assimilation, without giving proof of the specificity for nodule production of the microörganism isolated. Burrill and Hansen (6) failed to isolate an organism, and on this account, as well as because even *Pseudomonas radicicola* synthesizes only minute amounts of organic nitrogen *in vitro*, they considered a mutualistic symbiosis involving nitrogen assimilation in these nodules, to be of very doubtful existence.

If such a relationship does exist in natural habitats, then careful comparisons among many plants of different degrees of nodulation should give some positive indications that the possession of nodules is of some advantage to *Ceanothus*, as is found to hold in the case of leguminous plants. In view of these considerations and of the foregoing detailed methods of making exact comparisons between the annual growth of nodules and general growth, the following observations were made on the total growths found in the field.

Actual correlative studies were preceded by a general survey of ecologic conditions such as topography, type of soil, and associated plants. The plants were not dug at random on account of the great age of some and on account of lack of very young plants in the first habitat due to excessive pasturing. Equal sampling in different types of soil and a fair representation of the different ages from one to ten years were always sought so far as the growing material would allow. Before digging, the individuals were studied in a general way as to size, vigor, color, and branching of stems, and their blossoming or fruiting habits. After careful digging, the age of the plant, disposition of nodules, and comparisons of their annual growths with those of the annual rings in the root crown, were made and in most cases rough weights were also taken. The data are presented by habitats as follows:

A. First Habitat

This habitat is a low gently sloping hill whose flat top consisted of fine calcareous clay and whose sandy sides were gullied in such a way as to prevent the deposition of the clay washed off the top. The exposure is to the south and east. Small clumps of poplar, white oak, hickory, and sumac occur over this twenty-acre tract. but the open spaces have been occupied during the last two decades by pear trees. The areas occupied by Ceanothus are low in nitrogen (below 0.05 per cent.) and variable in acidity (Ph. 6.5 to 6.9). The clay soils are so close-textured that drouth effects often appear.

The numbers and ages of plants dug from this Habitat are as follows: thirty were from five to six years old; eighty from from seven to eight years old; seventy from nine to ten years old, and twenty were from ten to twelve years old. Their distribution according to age and relative amount of nodulation is shown in Table I.

Table I. Distribution of *Ceanothus americanus* according to age, size, and nodulation in Habitat 1.

Age of seedlings*	Total number of seedlings	Type of nodulation and relative size of plants dug.			
		Poorly nodulate. Small plants	Medium nodulation. Medium plants	Heavy nodulation. Large plants	Very heavily nodulate. Very large plants.
5 years**	10	3	6	1	.....
6 "	20	2	5	11	2
7 "	30	2	7	16	5
8 "	55	3	7	35	10
9 "	45	2	5	30	8
10 "	25	1	3	15	6
11 "	11	1	2	5	3
12 "	9	1	1	3	4
Totals	205	15	36	116	38

* Seedlings, as distinguished from plants made by stolon-like growth from very old plants.

** About 100 seedlings from two to three years old are not included, because atypical, due to shade and crowding.

It will be noted that in this total of two hundred and five plants examined, fifteen were poorly nodulate, thirty-six were of medium nodulation, one hundred and sixteen were heavily nodulate, while thirty-eight were very heavily nodulate. The poorly nodulate plants were all small and their average weight at the same age was about half of that of the next group, each of which had from one to three fair-sized nodules (Pl. III) as old as the plant itself; i.e., infection and nodule growth began soon after the sprouting of the seed. The nodules of the smaller plants were so new that they indicated late infection. The one-hundred sixteen heavily nodulate plants had from three to ten very large and much-branched nodules on the main root and many smaller ones among the secondary roots. These plants averaged over twice the weight of those in the medium nodulate group owing to profuse branching of tops (Pl. II, top) and heavier root system. Young plants of this group were frequently supposed to be several years older than the age shown by the number of annual rings and bud scars. The very heavily nodulate group of thirty-eight plants (Pl. II) had their nodules distributed as in the heavily nodulate group, but the nodule branches were densely packed, and these large dense nodules covered the tap root as well as the large lateral roots to such an extent that the observer would expect a detrimental effect on top growth if the relationship were purely parasitic. Yet the size and weight of tops alone ranged from two to three times that of the heavily nodulate groups, while the root systems bore a similar ratio down to a depth of over one metre.

In this habitat, rapidity of nodule and general growth go hand in hand, and the total growths are vastly different in the different groups based on amount of nodulation and age. In general, the plants of clay soil were smaller than those of sandy soil. They had fewer nodules, due possibly to a limitation placed upon infection by this close-textured soil -- a phenomenon also noted for greenhouse seedlings when planted in a clayey soil which had been compacted by wet sterilization.

B. Second Habitat.

This tract of about six acres lies on the sides of a narrow vale or glen trending north and south. The soil consists of fine sand only, and is roughly covered on the east side by brambles, sumac, and rock rose, while the west side is covered by red and white oak and hickories standing in rather open formation. The total soil nitrogen content of this area (o.06 to 0.07 per cent.) is only slightly higher than that of the first habitat. Both sides of this valley receive much less sunlight than the first habitat and growth, in general, is notably less in amount even though the humidity is slightly higher.

The numbers of plants dug from the second habitat and studied as in the first habitat are as follows: two hundred and seventy-eight were from one to two years old; two hundred and sixty-seven from three to four years old; one hundred and fifty-eight from five to six years old; eighty from seven to eight years old; and thirty-three from nine to twelve years old. Their distribution, according to degree of nodulation, is shown in Table II.

Amongst this total of eight hundred and sixteen plants forty are poorly nodulate; two hundred are of medium nodulation; three hundred and ninety-six heavily nodulate; and one

Table II. Distribution of Ceanothus americanus according to age, size, and nodulation in Habitat 2.

Age of seedlings.	Total number of seedlings	Type of nodulation and relative size of plants dug.			
		Poorly nodulate Small plants	Medium nodulation Medium plants	Heavy nodulation Large plants	Very heavily nodulate. Very large plants.
1 year	111	7	19	63	22
2 years	167	11	38	83	35
3 "	163	9	41	79	34
4 "	104	5	27	44	28
5 "	81	4	22	34	21
6 "	77	3	20	35	19
7 "	53	1	15	25	12
8 "	27	..	8	14	5
9 "	26	..	9	16	1
10 "	5	..	1	2	2
11 "	1	..	..	..	1
12 "	1	..	..	1	..
Totals	816	40	200	396	180

hundred and eighty very heavily nodulate. The distribution is better here among the younger plants than in the first habitat, because pasturing has apparently been very light within the previous four or five years.

The differences among the younger seedlings were not so apparent, as between heavily nodulate and poorly nodulate older plants (Pl. III), but careful measurement showed that they were relatively almost as great as among the older groups. In this habitat (Table II), precisely the same relations hold as those found in the first habitat.

The distribution over this area is much more regular than in the first habitat and it is difficult to believe that these plants are hereditarily so different amongst themselves, so far as vegetative thriftiness is concerned, as to cause such great variations in size independently of, or rather with, the handicap of the nodules which are known to store quantities of foods which they seem never to disgorge, and to harbor a parasite which must feed on organic foods.

In this habitat, heavily nodulate plants blossomed more copiously and uniformly and fruited more heavily than the poorly nodulate plants. The annual growths in thickness of the tap root and attached nodules paralleled that of the neighboring area, while the nodules at greater depths than fifteen or twenty cm., made much less growth in length and thickness. Some unthrifty seedlings of one season's growth were found with very minute nodules or none. Some dead ones with no nodules were found in the same areas where sufficient moisture and sunshine were present. These seem to indicate a survival value for nodules which could not, however, be established with the small numbers; viz., seventeen non-nodulate and dead, and twelve plants with minute nodules, which barely survived their first winter.

Summarizing the data from these field studies it is found that:

1. Plants are larger, heavier, and more thrifty, in proportion to the number of large nodules found on their roots.

2. These nodules are found in the upper part of the root system near to or upon the primary root.

3. Seed bearing is heavier and fruits ripen later in the heavily nodulate plants than in poorly nodulate plants.

4. Very young seedlings which were heavily nodulate withstood winter conditions and competition better than poorly nodulate plants, and a few very small non-nodulate seedlings died from no apparent cause.

2. Greenhouse Experiments.

A. Sprouting of Dormant Field Plants.

The positive correlations between growth and nodulations found in the field studies soon raised the question whether a correlation between forced growth or sprouting from the dormant condition might not be found and whether the complete removal of nodules would have a notable effect upon sprouting from dormancy. Several series of field plants were tested in the greenhouse. The plants were dug very early in the spring and were tested in two series as follows: Series I consisted of plants of different degrees of nodulation; Series II consisted of fifty pairs of plants, each pair being alike and one plant of each pair having all its nodules removed before sprouting.

Series I.

The forty plants of this series were selected to represent the various degrees of nodulation found in the field studies described above. Only plants four, five, and six years old were represented on account of convenience in potting. Habitat soils and greenhouse compost were used in an equal number of cases, and bottom heat was used to shorten the dormancy period.

The sprouting data are as follows: twelve heavily nodulate plants sprouted one week earlier than fifteen plants of medium nodulation, and there was no difference as between habitat soil and greenhouse compost. Thirteen poorly nodulate plants were not only from one to two weeks slower in sprouting than the medium nodulate, but they had fewer and much smaller shoots. Later field examinations to the number of over one hundred, showed similar differences. The extremes of these differences must give the advantage of an early start, equivalent to two or three weeks of growth in the spring, before shading in forested areas occurs.

Series II.

In order to see if living nodules exercise a direct or immediate effect upon sprouting, the following three sub-series consisting of one hundred dormant field plants were sprouted in the greenhouse. These were carefully selected in pairs of equal size, nodulation, age, and habit (Pls. II and III). All nodules were carefully removed from one plant of each pair before potting.

Sub-series A.

The first sub-series consisted of fifteen pairs of one-year old seedlings carefully dug and carried to the greenhouse in moist soils. After carefully matching the pairs and pruning off the nodules with a sharp scalpel the whole root system was potted. The pairs were numbered (Pl. V) and the denodulated plant was labeled "-N". Six of these pairs were heavily nodulate and nine were of medium nodulation. After four weeks of sprouting, notable differences between nodulate and denodulate plants of each pair existed. The leaves of the nodulate ("+N", Pl. V) were in all cases somewhat larger, more numerous, and more thrifty in appearance than those of the denodulate plants and the new stems (scarcely visible in Pl.V) were from two to five times as long in the nodulate as in the denodulate plants. There was no exception in this sub-series, all plants of which came from a small area of uniform soil and other conditions. The nodulate plants showed larger differences in growth according to nodulation than the denodulate plants. No injuries to roots due to removal of nodules could be found several weeks later, and all wounds were covered by healthy callous tissue. In this sub-series the nodules seem to exert a very noticeable effect upon the rate of sprouting.

Sub-series B.

The second sub-series consisted of twenty pairs of plants from three to six years old (Pl. II, lower figure). These were heavily top- and root-pruned after carefully matching pairs. The nodules were removed as in sub-series A, but the plants were put into suitably larger pots holding from one to two kilograms of soil. These, as in sub-series A, were carefully top watered with rainwater and kept at thirty to fifty per cent. of saturation.

In a few weeks the differences were even more notable as between nodulate and denodulate plants than in the first sub-series and the differences continued to increase for more than two months. While the root systems of these were very severely reduced, the number of potential shoots or buds was probably even more reduced (to three nodes) by top pruning, thus probably having the effect of exaggerating the differences in the pairs due to the presence and absence of nodules. It is scarcely probable that the extra wounding due to the careful removal of nodules could make such a difference between plants that had been so equally severely wounded in both tops and roots. All root wounds were well calloused over.

Sub-series C.

The third sub-series consisted of older plants than those of sub-series B. The fifteen pairs ranged from five to eight years old and were pruned and otherwise treated as sub-series B, except that three of the denodulate plants were watered more heavily than their nodulate mates.

No differences due to extra water or to richer soils could be noted and the whole sub-series behaved exactly like the second sub-series of which it may be considered a continuation. The small fluctuations in water content throughout all these sub-series is very probably of small consequence. But the correlation of more rapid sprouting with the presence of nodules, or the converse,-- the consistent retardation of sprouting accompanying the removal of nodules, is present throughout, being more marked in the older and more severely wounded pairs.

From these data it seems clear that Ceanothus receives not only a general benefit to growth from the possession of nodules, but that there is an immediate benefit or advantage secured during the sprouting period. Whether this effect is due to hormones, to nitrogenous or carbohydrate foods stored or made in the nodules is not as yet clear.

B. Sterile and Nodulate Seedlings.

The experiments and field observations already given do not answer the question as to whether Ceanothus can grow normally without nodules nor do they indicate what the amount of growth would be in case non-nodulate plants could be grown artificially. In order to get some light on these questions the following series of seedlings were grown in two different soils. One was the soil of the second habitat previously studied, while the other was a fertile, fine-textured greenhouse compost containing 0.21 per cent. total nitrogen, thus being three times as fertile in respect to nitrogen as the habitat soil used.

Seedlings were from seeds that had been carbonized by treating ten minutes with pure sulfuric acid. These were planted in sterilized soils and transferred after a week's growth to pots of sterile soil, by the use of a specially constructed planting tweezers with broad blades, which removed the unbranched primary root without injury.

For the production of nodulate seedlings (14) the seeds were planted in habitat soil taken from a region thickly covered by mature wild Ceanothus plants. To avoid soil effects, this sprouting soil was carefully washed from the roots when transplanting.

The pots were well packed with moist soil and sterilized at various pressures, subsequent results showing that heating at a pressure of two pounds per square inch was sufficient to prevent nodule formation. The following seedlings were grown in the two soils named: in the greenhouse compost, eighty-eight were in sterilized soil and eighty-five in unsterilized soil. Of the twenty-two per cent. of infected plants grown in this soil only a few were accidental, the remainder having been infected in the unsterilized habitat soil during germination. Sterile sprouted seedlings developed no nodules in this soil, although it was well-infected by Pseudomonas radicicola strains, as shown by the growth of red, white, sweet, and alsike clovers with nodules. Recalling Bottomley's identification of the causal organism, it is clear that even if it were a Pseudomonas the variety or strain which attacks Ceanothus certainly was not present in this soil. Although the nearest Ceanothus habitat was only about two miles distant, a wider distribution of Pseudomonas, by wind, might be expected. This leads to the idea that the causal organism may be a less easily distributed one and therefore not a variety of Pseudomonas radicicola, as Bottomley (4) claimed. The plants grown in the compost soil and their degree of nodulation are given in Table III, their weights are given in Table IV, and their nitrogen content, in Table V.

Table III

Laboratory Seedlings Found Infected

Plants and soils	Total number plants studied	Plants with nodules	Heavily infected no nodules	Not visibly infected
Fertile compost				
Sterilized	88	16	3	69
Not sterilized	85	17	2	66
Totals	173	33	5	135
Habitat soil				
Sterilized	105	13	7	85
Not sterilized	161	127	22	12
Totals	266	140	29	97
Totals, both soils	439	173	34	232

Of the two hundred and sixty-six plants grown in habitat soil, one hundred and five were in sterilized and one hundred and sixty-one in unsterilized pots. Of the twenty per cent. infected plants in the sterilized soil, all but two were intentional. The data for nodulation, weight, and nitrogen content, respectively, are given for comparison, with those of the plants grown in compost soils, in Tables III, IV, and V.

This table shows the considerable number of two hundred and thirty-two non-nodulate plants which were grown for more than eight months continuously. The heavily nodulate plants were few and mostly in inoculated soil, whereas the medium nodulate plants were sufficiently numerous for fair comparison with other groups.

The fact that all these plants had grown only one season and that a special type of infection not found in the field, appeared in considerable numbers made necessary a slightly different classification from that employed for field seedlings. This special type or group named "many infections" in Table III, had its roots literally covered by infections which did not seem able, for some unknown reason, to develop into nodules. Very few infections (Pl. IX) had started to produce minute nodules at the age of eight months, but judging by their position on the older roots as well as on the younger ones, these infections must have taken place as the roots were formed. The only factors which correlate with this failure to develop nodules are bottom heat and thorough mixing of the soil before planting, which, however, is not applicable to the ten plants in sterile soils. Two of the five plants with many infections in compost are accidental, while three were sprouted in infected soil, and even though washed when transplanted, must have carried over some soil as inoculum. This may also explains the occurrence of the seven heavily infected plants in the sterilized habitat soil.

It will be noted that there is a decided correlation between growth and degree of nodulation as found in the field studies. Moreover, the non-nodulate plants are numerous enough to be decisive as to how this species of Ceanothus behaves without nodules, and how this behavior is affected by a very fertile soil.

By comparison of non-nodulate plants of the fertile soil with the heavily nodulate plants of the poor habitat soil, a nitrogen function of nodules may be indicated, whose magnitude in terms of soil fertility can be directly read. And this correlation is maintained to a high degree in the fertile soil, where, according to experience with legumes, it would hardly be expected. If nodules exercise a nitrogen effect upon growth then it is apparent, from the habitat soil plant data, that in one season a plant with many large nodules has a six to one advantage over the non-nodulate or sterile plant. The weights of most of these plants are given in Table IV.

The weights of individuals did not vary enough from the averages to cause overlapping of the groups. Unfortunately, some plants were destroyed before the decision to weigh and determine the nitrogen content, was reached.

It is remarkable that plants with many infections were not found in the field studies. It is also remarkable that these infections have the same degree of correlation with growth as is found in the group of medium nodulate plants.

This ratio between non-nodulate and nodulate plants, considered as a handicap, may explain the finding of the small number of dead non-nodulate seedlings in the field which, as noted previously, failed to survive the winter. Since the soils had enough nitrogen to make good growth, it is possible that the parasite in the nodules excretes some hormone-like body which enables the roots to take up amounts or types of nitrogen not otherwise available. That nitrogen is here positively involved is sufficiently apparent from the data of Table V.

Table IV.

Dry Weight of Laboratory Seedlings

Soil and groups of plants	Description of plants	Number of plants	Total weight in grams air-dry.	Average weight of one plant.
Fertile compost:				
Group I	Large nodules	9	7.520	.835
Group II	Medium "	5	2.570	.514
Group III	Small "	1	.445	.445
Group IV	No "	125	54.340	.435
Group V	Many infections (Pl.IX)	2	1.370	.685
Habitat soil :				
Group VI	Large nodules	42	18.195	.433
Group VII	Medium "	40	8.660	.216
Group VIII	Small "	30	3.930	.131
Group IX	No "	71	4.980	.070
Group X	Many infections (Pl.IX)	25	5.200	.208

Note: The decision to determine the dry weight of these plants was not reached until after some plants had spoiled subsequent to the regular nodulation studies of the root systems, hence the numbers of plants are smaller than in Table III.

Table V.

Nitrogen in Laboratory Seedlings and Roots

Part of plant taken, and nitrogen estimation method used.	Oven-dry moisture	Ash per cent. oven-dry	Per cent.* nitrogen oven-dry basis	Total nitrogen oven-dry plant, gms.
Kjeldahl method				
Whole plants Large nodules, Compost soil.	6.60	5.60	2.05	0.0171
Whole plants No nodules, Compost soil.	7.36	6.27	2.14	0.0093
Whole plants Many infections, Compost soil.	7.46	10.38	2.52	0.0172
Whole plants Large nodules, Habitat soil.	8.60	12.60	2.45	0.0107
Whole plants No nodules, Habitat soil.	8.80	11.50	2.54	0.0018
Whole plants Many infections, Habitat soil.	7.33	9.70	2.43	0.0050

* These are averages of closely agreeing duplicate determinations. Blank determinations for nitrogen in reagents were made and deducted, for the above results.

The concentration of nitrogen in the air dry plants of the compost soil averages lower than in the habitat soil, but concentration of nitrogen of the plants with many infections is in the reverse order, in compost soil.

It is remarkable that the non-nodulate plants of habitat soil had the highest nitrogen concentration but less than one-fifth of the total nitrogen found in the nodulate, and about one-third the total nitrogen found in the heavily infected plants of the same soil. In the rich compost these differences are not so great, the total nitrogen of the non-nodulate plants being slightly over one-half that of the nodulate and heavily infected plants. The latter two groups are identical even though the nodulate plants are far heavier, as shown in Table IV. The non-nodulate plants of this soil contain over five times the nitrogen found in the same group of the poor soil and also serve to emphasize the nitrogen relation of nodules to the growth of the plant. But whether this is one of stimulation, enabling the roots to absorb more nitrogen from the poorer soil, so that the nodulate plants of the poor soil were able to get as much nitrogen as the non-nodulate plants got from the richer (three times) soil, or whether the nodules were the seat of nitrogen synthesis, cannot be finally determined by these data alone. Nor do the data of enhanced sprouting given under greenhouse correlations answer this question, which will be further considered under the physiology of the causal organism in the following division.

C. The Causal Organism

According to the descriptions of Atkinson (2) and Arzberger (1), the organism is hyphal and branched. Atkinson referred it to the genus Frankia of which he considered it a new species, F. Ceanothi. Arzberger, as well as Burrill and Hansen

(6), considered this classification more accurate than that of Bottomley (5), who placed the organism among the bacteria, stating that its characters were those of Pseudomonas; he maintained that its morphology (not published) as well as the fact that it showed fixation of atmospheric nitrogen in minute amounts, placed it among the strains of Ps. radicicola. Spratt (16) who studied nodule formation, accepted this classification. Bottomley had not tried the infecting abilities of the organism isolated, and as there was some probability that a pleomorphism might be found which would clear up these conflicting views, the following experiments upon isolation and infection were directed toward this end.

1. Isolation Methods.

Material, consisting of many nodulate plants which were taken from a sandy soil, was potted and allowed to grow in the greenhouse as soon as the resting period was broken. This period lasted in proportion to the lateness in the autumn at which they were dug. Those dug at mid-October remained dormant three weeks, while those dug in mid-November from the same areas remained dormant from six to seven weeks under similar greenhouse conditions.

a. The bacterial media consisted mainly of agars at first, but later several liquid media were also employed. The agars may roughly be divided into strongly nutrient and dilute groups as follows:

Strongly nutrient:

(1) Potato Agar with two per cent. dextrose.

(2) Nutrient Agar with one to two per cent. peptone and

one to two per cent. sugars.

(3) Nutrient Agar with one and one-half per cent. peptone and two per cent. glycerin.

Dilute Media:

(1) Agar with nutrient salts and *Ceanothus* juice.

(2) Agar with nutrient salts and asparagin.

(3) Solutions of salts and asparagin and sugar, (mostly Coons' synthetic medium).

(4) *Ceanothus* juice, sterilized or filtered. Combinations of these; e.g., *Ceanothus* juice boiled or filtered (raw) was also used with slants of the various agars for propagation and for nodule tissue cultures.

The Potato Agar was prepared in the usual way by steeping diced potatoes and straining the broth into the agar, after which dextrose was added.

The Nutrient Agar was prepared from ground beef in the usual way, except that the raw juice was heated to boiling and partly cleared with whites of eggs before being added at 60° to 70° C. to the agar which contained the peptone. In some cases filtration through flannel was necessary. Glycerin and sodium chloride were added to the cold meat juice.

The *Ceanothus* juice was prepared from roots and subterranean sprouts in about equal proportions, ten grams of this material being ground in a mortar and 50 cc. water being added later. The nutrient salts were mostly sodium phosphate (NaH_2PO_4) and Magnesium sulfate in the proportions used by Coons(7).

In general, the relative values of these media are in proportion to their density and nitrogen content; the Nutrient agar with glycerin being the best, Potato agar and Asparagin agars or solutions being intermediate, and the nutrient salt and Ceanothus juices being weakest in their ability to bring out many and large colonies. At the beginning, a nutrient agar with tannin in low concentrations was used but a comparison with nutrient agar growth showed the advisability of discontinuing its use.

The two media finally used continuously were Nutrient glycerin agar, as above described, and the Asparagin agar of Coons. The latter tends to remain clearer after growth of colonies, permitting a better view of what happens under the colonies. It is also free from the tyrosine coloration produced by several organisms in the Nutrient glycerin agar, although this was reduced some by reducing the amount of peptone.

b. Procedure in Isolation.

Nodules which were unbranched and several years old were washed and in some cases sterilized by chlorazine or by mercury bichloride, for from one-half to one minute, then dried under sterile conditions.

At first these were crushed before plating, but this brought out too many soil organisms from the unsterilized nodules, while the sterilized nodules resulted in blank plates. Peeling of nodules was then resorted to, under a large reading glass. This still brought forth a wide variety of organisms with no characteristic colonies. Then the tips of dry nodules were broken off and the nodules were methodically peeled in a revolving pair of tweezers, mounted vertically on a pinion. This resulted in sterile plates and tissue slants. Few species were obtained and these varied greatly. They were all too large and too unlike the nodule organism in vivo, to be tried out in the production of nodules. Nevertheless, ~~twelve~~ fifteen of the most likely ones were tried in sterile culture,, described later, but without producing nodules.

Finally, the nodules were lightly scraped under a stream of sterile nutrient salt solution and only the tips were used. This brought out multitudes of similar colonies, about the first of April 1925. These were immediately multiplied on slants from which in a few days a heavy growth of inoculum was produced.

The inoculum was prepared by softening the growth on agar slants with a few cubic centimeters of sterile nutrient salt solution (M/133 concentration) described later. This was stirred carefully with a transfer needle, then decanted into 100 cc. of similar solution which was poured upon the roots of the sterile seedlings, using special precautions to prevent contamination. In the earliest tests, the roots of the seedlings were placed into such inoculum for several minutes previous to planting.

In plating, the nodules were first crushed in a sterile mortar, under a glass cover using about 10 cc. of sterile salt solution for from two to five scraped nodule tips. One dilution only was needed, and this was usually made by pouring a drop or two of the mixed first tube, which itself had only a few drops of crushed nodule juice, from the mortar, into a second tube. Contaminations were few in the second dilution which usually contained from fifty to two hundred colonies of the causal organism whose description from preliminary study follows.

2. Some Characters of the Causal Organism.

Morphologically, the organism in culture, is a minute rod 0.15 micron to 0.20 micron in diameter at first. This may increase to 0.50 micron or more at full growth. Its length is from two to five or more microns, depending on age and medium of growth. It shows motility, but flagella are difficult to demonstrate. In fact, it is doubtful whether true flagella occur, as the polar projections may be formed as the rods break apart. Threads are formed, which seem to branch in age, thus corresponding with fresh threads in the growing nodule tip where septa or disjunctions of cells are hard to demonstrate, and where the diameter may be greater and more variable,-- the ends especially becoming thickened or club-shaped at maturity. In vitro, these clubbed ends have rarely been seen, and branching is also not common. From agar colonies the younger cells are somewhat gram positive, but in vivo they are strongly so.

The colonies are roughly circular and slightly raised at the edges, especially on dilute media; while on strong media they become almost hemispherical, and change from the first opalescent bluish tinge to a dark brown color in eight to ten days. Occasionally they are associated intimately with a filamentous fungus, whose threads they follow and with which grumous root-like processes are formed, looking like gnarled roots. These penetrate the agar or they radiate out on the surface, forming star-like surface colonies. Once it was found associated with a yeast, but this combination could not successfully be transferred.*

Some physiological characters were examined in the following experiments:

a. Actions on Starches.

The materials used in the starch reaction tests were: Potato Starch, Corn Starch, and Ceanothus Nodule Starch. These were placed in 50 cc. Erlenmeyer flasks and after the addition of 15 cc. of heavy inoculum and three per cent. toluol, were incubated at 28° C. for ten days with the following results:

(1). Flask with potato starch and boiled inoculum, no action.

(2) Flask with potato starch and inoculum, almost complete solution of all grains.

(3) Flask with corn starch and sterile inoculum, no action.

(4) Flask with corn starch and inoculum, very decided disintegration of most grains, due to a centripetal action resembling the drilling of many holes toward the center of the grain.

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* It was felt that more extensive bacteriological studies of the causal organism than are here given, should not be permitted to delay the completion of this thesis. Such studies are, however, being made for future publication.

(5) <u>Ceanothus</u> nodule starch with sterile inoculum gave no result.

(6) <u>Ceanothus</u> nodule starch with inoculum showed two types of solution; one a hollowing-out of the grains from one point which was also noted in the potato starch experiment, the other action was one of equal solution over the whole surface of the grain.

(7) Raw filtered nodule juice alone seemed to act somewhat less on the nodule starch than when the inoculum was added.

b. Relation to Atmospheric Nitrogen.

Since Bottomley (4) had claimed nitrogen fixation with a limited series of tests on liquid media and since the organism here described seemed to thrive far better on solid media, fourteen different cultures of known composition (the controls gave almost exactly the sums of ingredients) were set up as follows:

Nutrient Glycerin Agar (surface inoculated):

(1) Three flasks of the pure organism.

(2) Three flasks of the organism inoculated with a combination of the organism and a fungus, the commoner of the two previously mentioned.

Coons' Synthetic Agar (surface inoculated):

(3) Two flasks of the pure organism.

(4) Two flasks of the above-mentioned combination.

(5) Two checks of each combination (i.e., two of Nutrient agar and two of Coons' synthetic) were also run.

These had an equal amount (5 cc.) of sterilized inoculum added.

The 150 cc. agar used in each flask was solidified against the sides of 600 cc. Kjeldahl flasks, by spinning them in a vertical position in a centrifuge. This greatly increased the surface area for the aerobic growth of the organism. After fifteen days' growth they were subjected to the modified Kjeldahl analysis as used by Bristol and Page (6) with the following results (Table VI):

Here somewhat larger gains are shown in the nutrient glycerin than were found by Bottomley (4) who used 100 cc. per culture flask, ~~while here~~ as against 150 cc. here used, the surface being much greater, and probably constituting a factor in synthetic activity. These gains are also larger than those found by Löhnis and Pillai (14) for Azotobacter in a glycerol medium.

The associated fungus seems either to reduce the action of the bacterium on gaseous nitrogen or else to possess some activity which liberates nitrogen in some gaseous form from the organic nitrogen present. No provision was made in these experiments to retrieve any combined nitrogen lost in this manner, nor to detect the reduction of organic nitrogen, hence it may not be known from these experiments alone whether one or both of these actions took place.

In the Coons' Agar which had only about one-fourth as much nitrogen, the gains lie too near the experimental error to be significant. The acidity of the media was between 6.7 and 6.8 pH at the beginning of the experiment and had changed only slightly at the end.

Table VI.

Nitrogen Fixation by the _Ceanothus_ Organism

Medium and Culture	Controls	Nitrogen in Culture, Grams	Gain over Average of Controls.
Nutrient Glycerin Agar:			
Flask 1. Pure culture		0.0596	0.0043
Flask 2. Pure culture		0.0596	0.0043
Flask 3. Pure culture		0.0603	0.0050
Flask 4. Two organisms		0.0587	0.0034
Flask 5. Two organisms		0.0588	0.0035
Flask 6. Two organisms		0.0590	0.0037
Flask 7.	0.0532		
Flask 8.	0.0574		
Average	0.0553		
Coons' Synthetic Agar			
Flask 1. Pure culture		0.0159	0.0006
Flask 2. Pure culture		0.0158	0.0005
Flask 3. Two organisms		0.0159	0.0006
Flask 4. Two organisms		0.0159	0.0006
Flask 5.	0.0152		
Flask 6.	0.0154		
Average	0.0153		

In digesting these cultures it was noted that the controls were much more resistant to combustion than the cultures of either the organisms alone or of two organisms together, the digestion of the controls requiring about two hours longer, and about 25 cc. more of the digesting acid. This seems to indicate considerable disintegrating power of the causal organism as it grows on agar.

3. Infection of Roots of Ceanothus and Production of Nodules.

a. Methods.

Plants whose roots are to be tested by infection must be sterile throughout. Such plants were produced as follows:

Tall culture dishes were prepared as germinators, by adding a layer of sand, previously digested with hydrochloric acid and thoroughly washed (free of chlorides). To this was added a sterile salt solution originally composed of sodium acid phosphate, calcium chloride, and magnesium sulphate in equimolecular proportions, the total having an M/135 concentration with no iron present. These were sterilized at fifteen pounds' pressure in the autoclave. Seeds which had been carbonized with strong sulfuric acid were then, under aseptic precautions, planted in these germinators at distances of one-half inch or more from each other. Some seeds were internally infected with fungi by an insect which deposits its eggs within the seed, but while these seeds would not sprout, yet, since the carbonized seed coat furnished a good medium for growth, the fungi would emerge, sporulate and become a menace to the sterile plantlets. The latter were tested out on nutrient agar till the sterile ones could be certainly judged by appearance. The sterile seedlings were then planted into sterile culture tubes or dishes having a nutrient solution of the same composition as that of the germinators except that each culture (except in the earliest tests) had added to it enough calcium carbonate of M/100 solution to bring the total calcium concentration to M/400 strength, and enough ferrous sulphate to bring its own final concentration to M/5000 or M/10,000. At first greater dilutions of iron were used but seedlings became etiolated after four or five weeks' growth. The calcium which was at first added in the form of carbonate, although present in fine suspension did not enable the plants to thrive due, perhaps, to the toxic effect of the magnesium which apparently could not be balanced by the ample amount of calcium present. Later, when the calcium was introduced, mostly as chloride, the difficulty disappeared.

The seedlings were at first transplanted with a strong transfer needle, but this was too hazardous. A special transplanting forceps was designed and made of nichrome wire in such a way that its jaws could not crush the tender plantlets, but would still hold them firmly. No plants were lost by dropping or breaking of roots after these forceps were used, and the element of time was greatly shortened thus making aseptic conditions more certain.

After transplanting, the roots were flooded with 5 to 10 cc. of the inoculum, prepared in sterile solution as previously described under the head of isolation methods, but during the testings of the first isolations the rootlets were submerged in the inoculum several minutes before transplanting.

The numbers of inoculation cultures with the causal organism, and the results obtained are as follows:

b. Inoculation Cultures.

About fifteen different organisms which were isolated during the year 1924-25 were tested out in tube cultures without results. That these organisms did not possess the proper morphology was of course known at the beginning. They also presented no constant picture on the poured plates, but it was felt that the morphology of the causal organism might be different in vitro from that in vivo, and that it might appear in isolation plates only sporadically due to the possibility that the media used might not be suitable to its growth, or that it might have a dormant stage. It is not here considered feasible either to describe these non-infecting organisms or their cultures (usually consisting of six tubes with two checks each).

The cultures in which infection took place and in which some nodules were produced were the following:

In large test tubes (37 mm. wide and 300 mm. long) nine cultures (two controls) were made of the pure culture and nine (two controls) of the organism associated with a fungus (as previously described under the physiology of the organism). These tubes had 90 grams of acid-washed sand and 35 cc. of the non-nitrogenous solutions. Growth was always good in the checks. A similar set was also furnished with calcium nitrate instead of chloride and carbonate as described above. These made especially good growth and as compared with the growth of wild plants under competition, their growth was phenomenal, indicating that these salts with the sand are not a handicap.

In capsules which had been used mostly as checks in the special testing of previous organisms, the same proportions of sand and non-nitrate solution tests were also carried out. These contained older plants which were growing slowly and consisted of the following numbers:

12 nodulate cultures (all but three finally sterile; i.e., no other organisms present), of which six had the pure culture inoculation and six the combination inoculation of fungus and causal organism;

10 non-nodulate controls (eight originally and finally sterile), which had sterilized inoculum added, to equalize the nitrogen content of the culture;

Finally, thirty-four pot cultures were set up having three plants each. The tube cultures all had one plant each, while the capsule cultures had from one to three plants each. The soil in the pots was not sterilized but was carefully taken from sandy subsoil several miles from any Ceanothus habitat. Of these cultures, eighteen were inoculated and sixteen were used as controls. Half of the inoculated pots were supplied with pure culture inoculum and half with the combination inoculum, as previously described.

The results (to June 10th) are as follows:

Two of the pure culture tubes produced nodules while several others have infections. Not all have been completely examined.

All inoculated capsule cultures were infected and of the twelve, nine produced large nodules, five of which were in pure culture (Pl. IV) and finally free from other organisms, while of the four which had had the combination inoculum introduced, two were finally free from other organisms and two were not free, having become contaminated by an alga.

Ten uninoculated checks were all sterile; i.e., they had no infections and no nodules.

It seems that nodule growth in sand cultures is very slow, and that the seedlings in tube cultures are too young to have developed nodules in the four weeks' growth which they have made. This is borne out by the fact that in a special series not above detailed, nodules were formed in four weeks in every case (fifteen in number) where soil infusion from heavily inoculated habitat soil was used upon sterile garden soil. All controls of raw or sterilized soils examined (twenty in number) have been found free of infections and nodules.

Platings made from the inoculated sand cultures showed that the organism was present in very small numbers, indicating that it does not thrive under these conditions. Since it is of importance to re-isolate from the nodules apparently produced by pure culture inoculation, the following trials were made:

4. Re-isolation of the Organism.

a. Four large nodules (Pl. IV) were carefully scraped and crushed as previously described under isolation of the organism. Twelve plates were poured including six dilutions. Both nutrient glycerin and Coons' synthetic agar were used.

b. These platings in two days resulted in many small colonies having the peculiar blue color of the original pure cultures. Their morphology and staining reactions are identical with the original from which the inoculum was prepared. One nodule from the combination inoculum was separately plated, but it showed only the characters of those derived from pure inoculum nodules, thus indicating that this fungus does not survive, and that it is not, in such experiments, essential in infection.

Whether the previous passage through agar media had any hastening effect on the growth of these colonies, or whether the media were better adapted than the original media of isolation of the same formula, is not known; but they developed in about half the time, or at twice the rate of the original colonies.

Thus it is clear that the organism here isolated, whatever may be its final classification, has the ability to infect Ceanothus americanus under suitable conditions, and that it can be re-isolated from uncontaminated cultures, and from the nodules produced by infection with a pure culture.

General Discussion.

As was stated in the historical part, no correlative ecological data on growth, as related to nodulation, had been given by a previous writer. The determination of the age, and if possible, the annual growths of the nodules had to be made before accurate correlation with general growth could be made. Year by year, age determination was also necessary, inasmuch as the amount of nodule tissue is not always proportional to the age of the plant for a given number of nodules and in a given soil. A plant at first lightly nodulate may become heavily nodulate after it is several years old and yet not make up in growth sufficiently to correlate accurately with its numbers of nodules and their amount of nodule tissue. When such exceptional cases are properly excluded by the accurate determination of annual growths and age of nodules, the tabulations show great regularity and strong correlation of general growth with nodule growth, as presented in Tables I, II, and III. The more accurate dry weights of greenhouse seedlings bear out the relationship found in the field, and in addition the total nitrogen of greenhouse plants gives some indication that a nitrogen assimilating function in the nodules is concerned. The uniformly small size and low nitrogen of non-nodulate plants rules out heredity, and makes a special nitrogen avidity of roots for soil-nitrogen less probable.

The sprouting of nodulate versus denodulate plants reinforces this indication, but inasmuch as the root systems in some pairs were heavily pruned and did not again become normal in extent, either a stimulating action upon the roots is indicated, whereby greater ability to remove nitrogen from the soil is acquired, or, what is more probable, the youth of roots makes them more efficient in absorption. These considerations make a definite decision as to the exact total action of nodulation upon general growth impossible.

The preliminary studies on the morphology and physiology of the causal organism, make further discussion of its taxonomy unprofitable till more details are available, but the tendency to accumulate nitrogen in vitro lends very strong if not final support to the theory that the nodules are the seat of some nitrogen accumulation. However, even if this should be proven by the later analysis of nodulate versus non-nodulate plants as grown in sand cultures (where no combined nitrogen is present in nutrient solution), the special stimulation to general growth, by small amounts of nitrogenous or non-nitrogenous bodies, made only in the nodules or in enlarged infections, is not excluded.

The following principal conclusions may be drawn from the observational and experimental data presented:

Conclusions.

1. A strong correlation between growth and amount of root nodulation is shown in Ceanothus americanus by field (wild) plants of different ages and by greenhouse seedlings of one season's growth.

2. The poorest growth of non-nodulate seedlings was made in the poorest soil while good growth was made in the nitrogen rich soil. The growth of the latter was as heavy as that made by very nodulate plants in poor soil and conversely, indicates that there is either a nitrogen accumulation or a root stimulation, or both, due to the nodules, which is equivalent to the advantage accruing from a nitrogen fertile soil.

3. Dormant plants brought into the greenhouse and forced into sprouting showed earlier sprouting where nodules were abundant; somewhat later sprouting, where few in number; and retarded sprouting where nodules were removed.

4. The causal organism isolated and preliminarily described is able to dissolve nodule and other starches. It is also able to cause the fixation of very small amounts of nitrogen in vitro, which supports the theory that the nodules are the seat of nitrogen accumulation.

5. The causal organism was re-isolated and its morphological characters were found to have remained constant in vitro. These characters varied somewhat from its morphology within the nodules.

6. Ceanothus seedlings made very good growth in sterile conditions, if supplied with certain concentrations of salts. Those supplied with nitrate and sufficient iron made the most rapid growth.

7. The general conclusion follows that a nitrogen assimilation function is rendered far more probable by these observations, but another special stimulation to growth by the root nodules is not thereby ruled out, and both functions may exist within the nodules of Ceanothus species.

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EXPLANATION OF PLATES

Plate I. Nodules of different ages, showing branching and positions relative to roots.

Figs. 1 and 27 represent normal roots.
Figs. 2, 3, 6, and 7, and 34 represent common nodules one year old or less.
Figs. 4, 5, 9, 10, 12, 14, 19, 20, 26, and 28 represent nodules from 2 to 3 years old.
Fig. 8 represents a tetrachotomous nodule.
Fig. 9 represents a branched nodule, one branch presenting a pentachotomous branching.
Figs. 11, 13, 19, 31, 32, and 33 represent nodules in positions which are unusual for roots.
Fig. 11 shows a fasciated nodule extending over a whole infection.
Figs. 15, 16, 18, 21, and 25 represent very young nodules,
Figs. 17, 21, 22, 23, 24, 29, and 31 represent infections which can develop into visible nodules within a short time.
Figs. 20, 28, and 35 represent trichotomies.

Plate II. Upper part of Plate represents fruiting tops of two plants, 8 years old from habitat 1.

Fig. 1 represents a heavily nodulate plant which had 4 large nodules near the crown and several other smaller ones near the upper part of the primary root.
Fig. 2 represents a very large plant with 11 very large nodules on the primary root and its large branches near the crown. Over a score of medium-sized nodules were attached to smaller roots near the larger nodules. The wood of this plant is over twice that of plant in Fig. 1. Lower part of Plate represents part of sub-series b, top and root pruned and forced to shoot in the greenhouse. Members in each of these 5 pairs were equal in size, nodulation, and age. The minus member of each pair had its nodules removed when potted. Sprouts shown were developed in three weeks. Pairs 2 and 7 were very heavily nodulate. These plants range from 5 to 6 years old.

Plate III. Seedlings 1 and 2 years old, as dug from habitat 2, plot 3, fertile soil (Phot. 1 x).

Figs. 1 and 3 are poorly nodulate plants two years old.
Fig. 2, a two-year old heavily nodulate plant which grew within a few feet of those in Figs. 1 and 3.
Fig. 4, moderately heavily nodulate one-year old plant, nearly as large as two-year old plants in Figs. 1 and 2.
Figs. 5 and 6, practically non-nodulate plants 1 year old.

Figs. 5 and 6, practically non-nodulate plants one year old. Fig. 6 had two minute nodules near the distal end of the primary root.

Plate IV. Seedlings grown in sterile sand culture.

Middle plant from two organism inoculum. Small plant has nodule on dead tap root. (Circular spots on stem of right hand plant and among leaves are bubbles. Glass rods were necessary to keep some roots below the surface of the water in the dish). Some of these nodules are more branched than those naturally produced.

Plate V. Whole, one- and two-year seedlings of moderate nodulation forced 8 weeks in greenhouse (Sub-series a). Members of pairs were almost identical in size, shape, and amount of nodulation, but pairs varied in size and shape. The "minus" plant of each pair was denodulated. The differences were slight, but easily measurable and they were consistent. (Phot. 1/3 x).

Plate VI. A series of greenhouse seedlings including nodulate and non-nodulate plants within the same pots. Large plants in pots 4,5,7,8,9,10,11, and 12 are heavily nodulate. Pot 6 has greenhouse compost; the large plants had few nodules. Smallest plants in pots 3,4,6,7, and 10 had practically no nodules after 5 months' growth. Intermediate plants had, as a rule, intermediate nodulation. (Phot. 1/3 x).

Plate VII. Three plants from pot 9 shown in Plate VI. These illustrate 3 of the weighed groups of Table IV,-- Fig. 1 representing Group VIII; Fig. 2, Group VII; and Fig. 3 Group VI. (Phot. 2/3 x).

Plate VIII. Root systems of plants shown in pot 2, Plate VI, (2/3 x). Plant 1 at left has several young but large nodules indicating late infection. Plant 2 has a few small young nodules. Plant 3 has no easily visible nodules, but it has many old infections, shown in detail (2x) in Plate IX. This anomalous condition was found in a relatively small number of plants (Group X, Table IV). This group of plants made as good growth as the young heavily nodulate plants.

Plate IX. Part of root system of plant 3, Plate VIII, showing many infections and incipient nodules (magnified 2 x). Infections and nodules are indicated at I. At Io a young root has penetrated an infection.

Plate X. More detailed (4x) photograph of nodules shown (2x) in Plate IV.

Plate XI. Winter (top figure) and Spring (bottom figure) placement of sterile sand cultures.

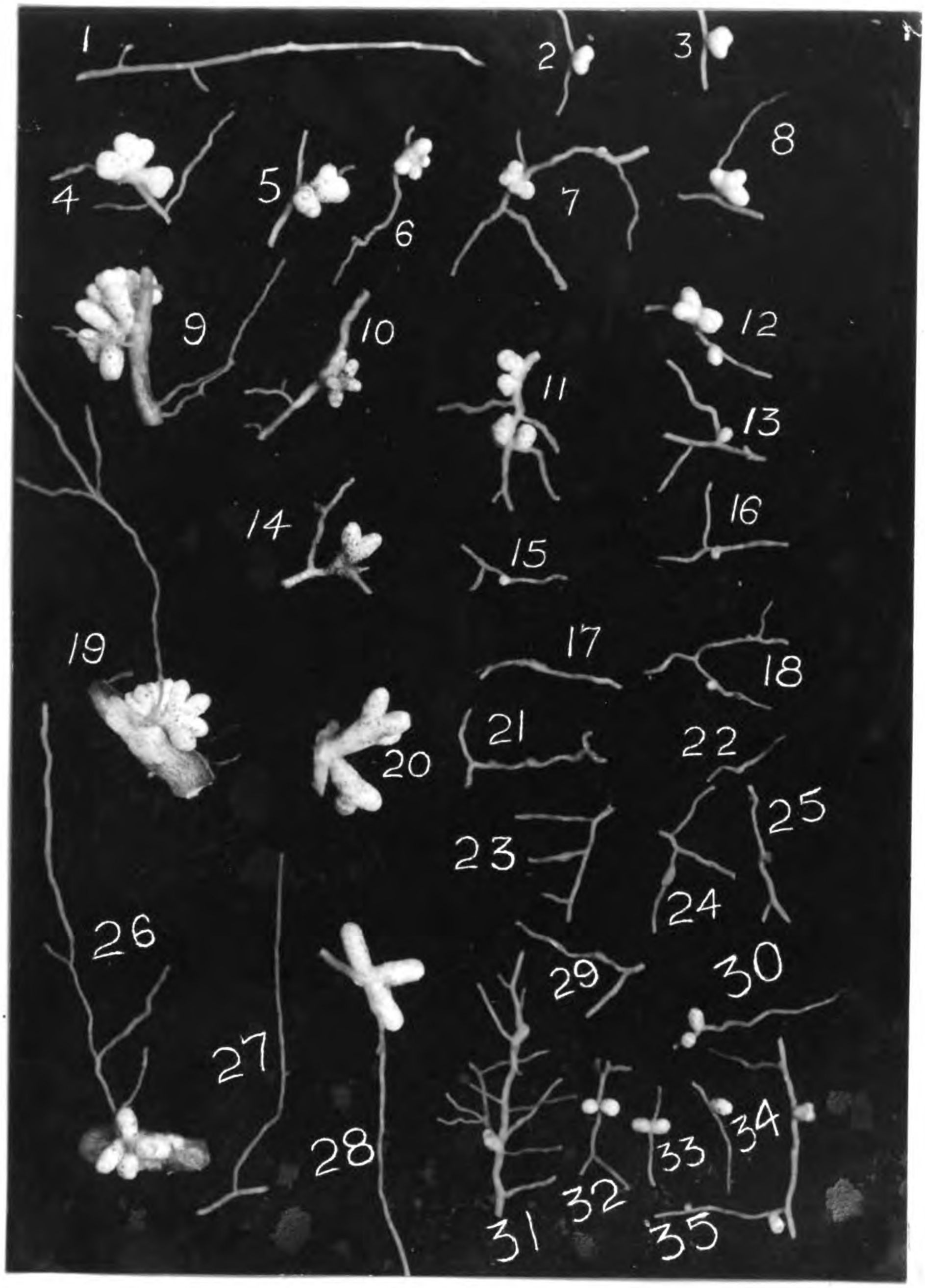
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Plate II.

Plate III.

Plate IV.

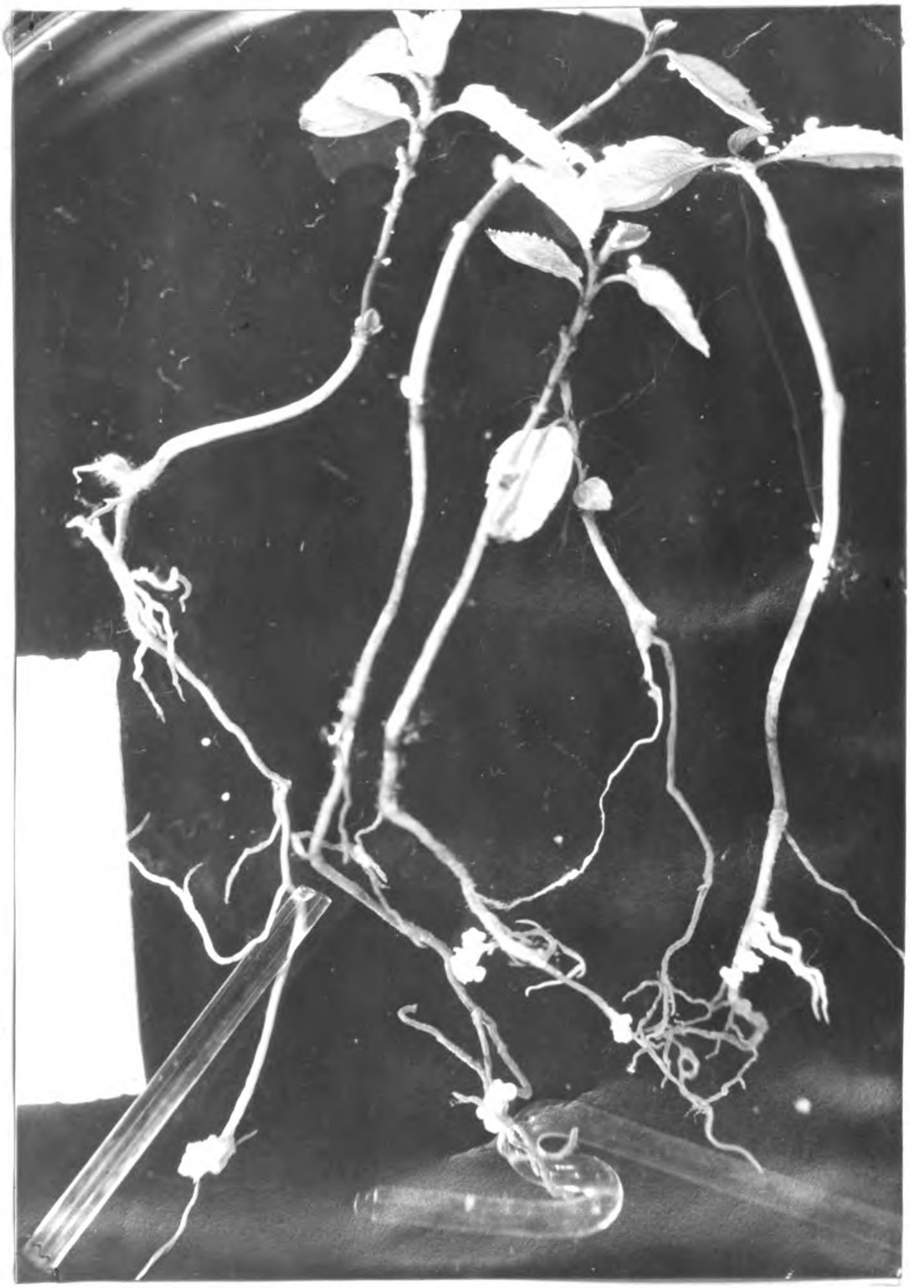

PLATE V


43 -N
44 -N
34 +N
34 -N
43 +N
44 +N
39 -N
40 -N
41 -N
42 -N
39 +N
40 +N
41 +N
42 +N
35 -N
36 -N
37 -N
38 -N
35 +N
36 +N
37 +N
38 +N


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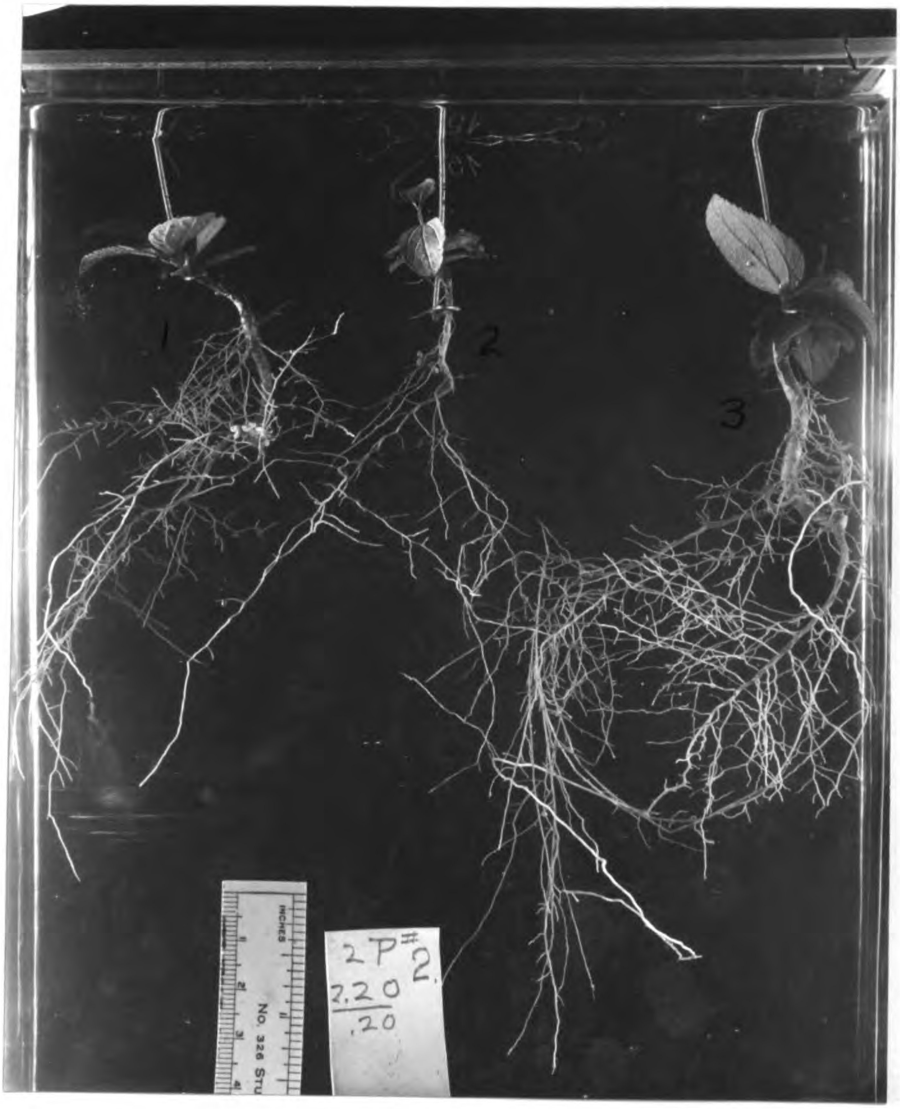
12
3
7
11
2
6
10
1
5
9


2 P
#9
20
20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2
3
INCHES
NO. 326 STU
2P #2
2.20
.20

PLATE IX

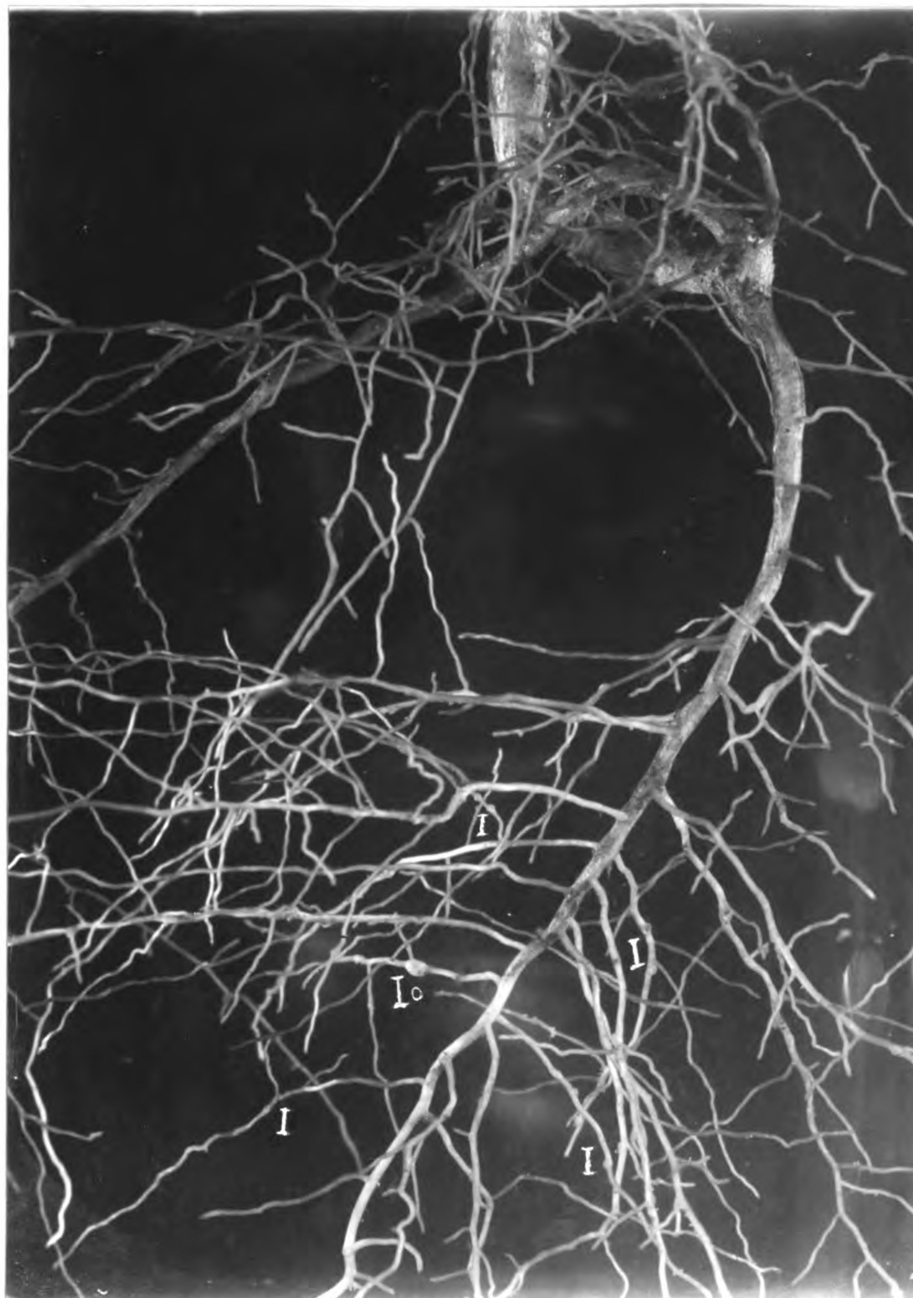


PLATE X.

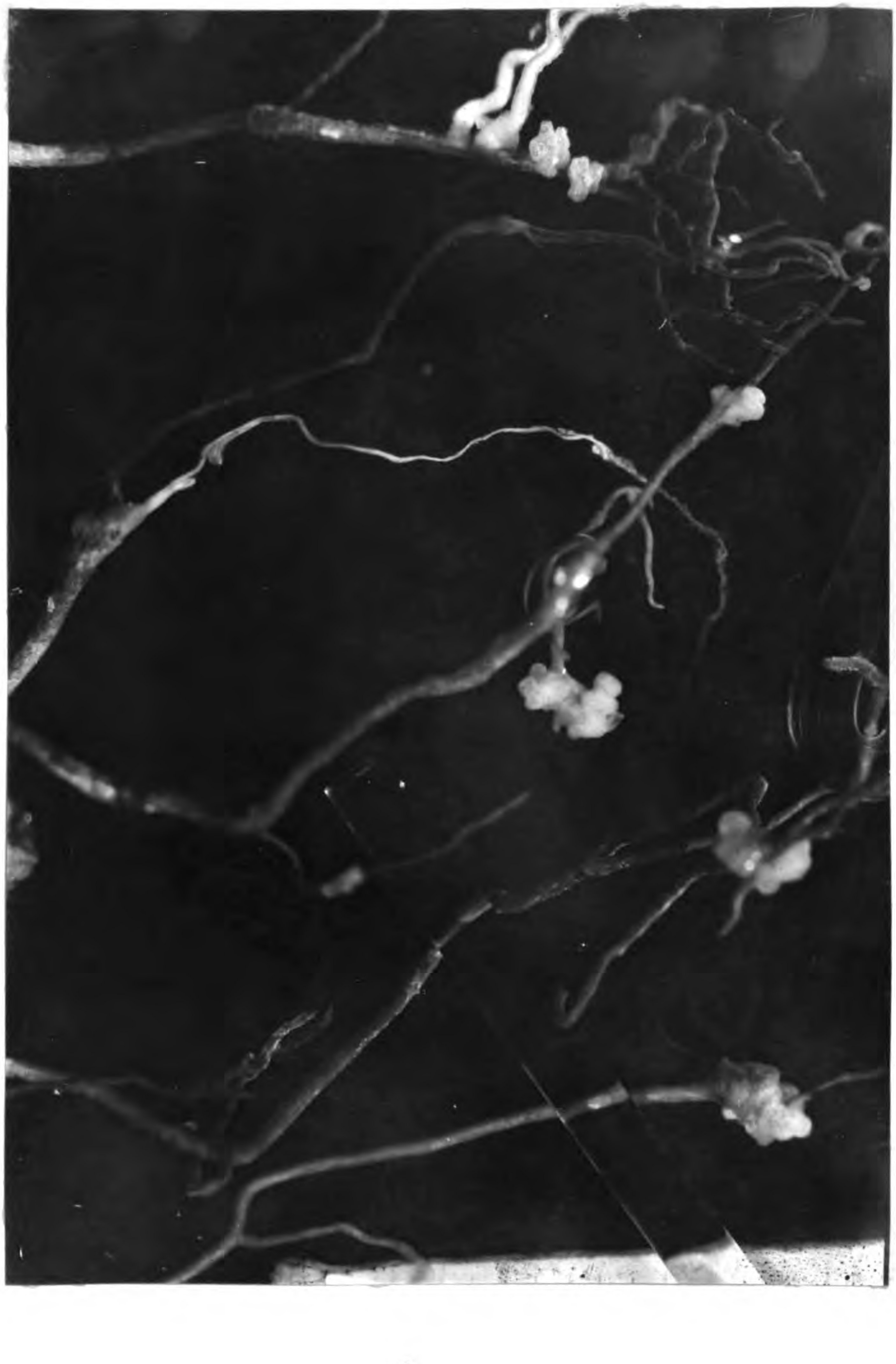
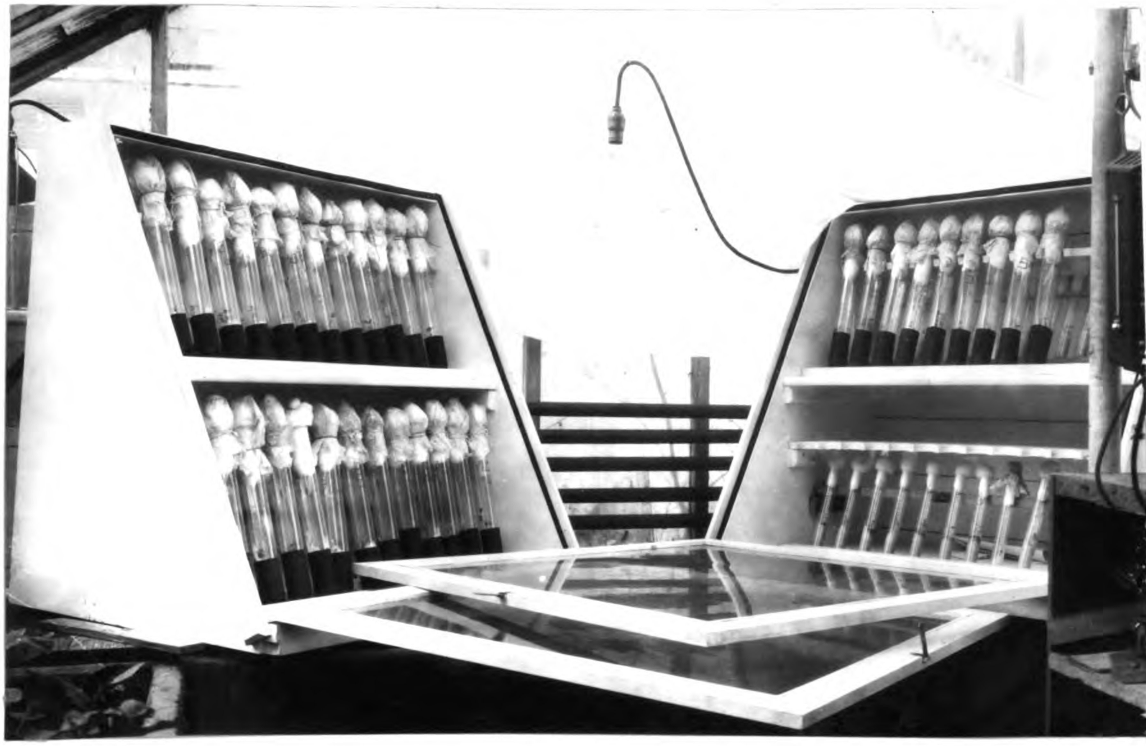
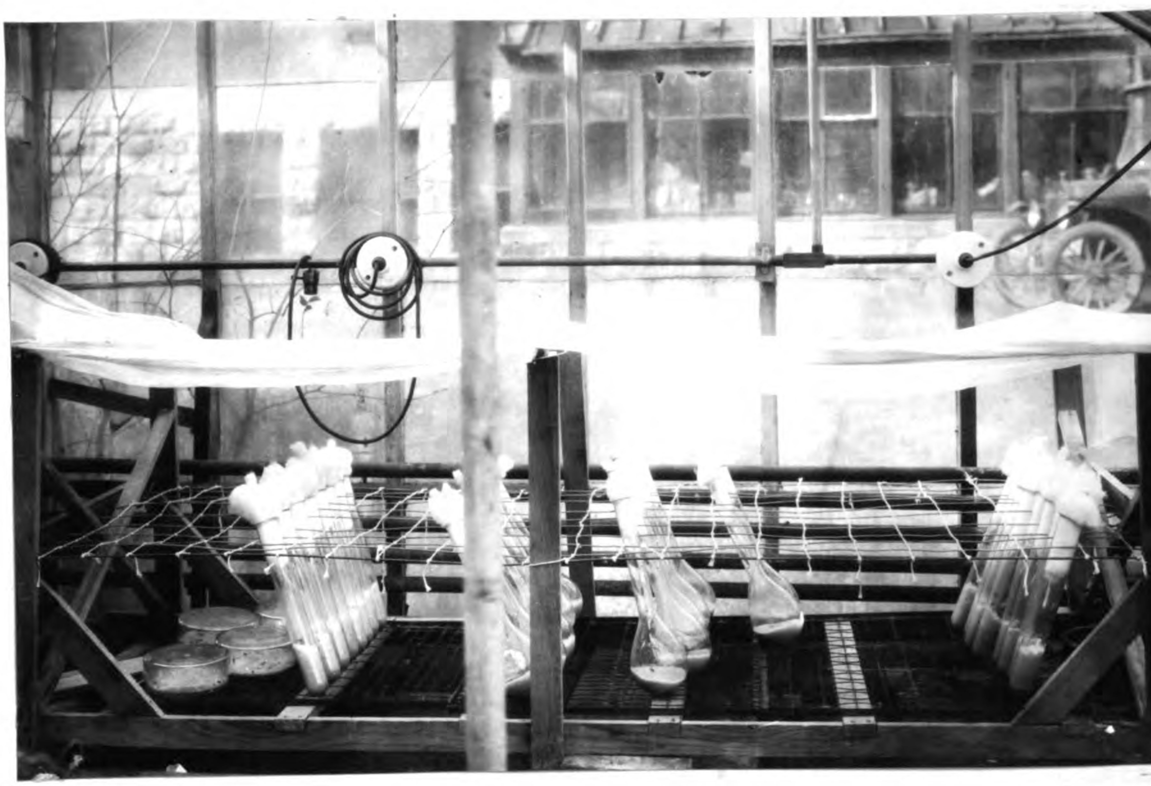


Plate XI





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