BACTERIA IN THE ROOTS OF GLEDITSIA TRIACANTHOS L.

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The relation of bacteria to the nodules on roots of leguminous plants has been the subject of almost continuous research since the work of Woronin in 1866. The greater portion of this work, for economic reasons, has been done on crop plants. In 1917 Burrill and Hansen (2) showed that organisms isolated from roots of *Robinia pseudo-acacia L.* would not induce nodule infection on any other species of legume used in their study. Löhnis and Hansen (5) studied organisms causing nodule infection on roots of *R. pseudo-acacia* but were unable to obtain positive results in inoculation tests. Others published researches in which nodules (or the lack of them) on roots of leguminous trees are considered include those of Burrage (1), Frank (3), and Schneider (7) all dealing with *R. pseudo-acacia.* Harrison and Barlow (4) report the absence of nodules on *Gleditsia triacanthos, Gymnocladus dioica,* and *Cercis canadensis.*

This paper is concerned primarily with results obtained from a study of the roots of *Gleditsia triacanthos* L. Work was also begun on *Robinia pseudo-acacia* L., *Cercis canadensis* L., and *Gymnocladus dioica* (L) Koch. Conditions have arisen which necessitate a temporary abandonment of the larger problem and it is thought worth while to make this preliminary report of the work thus far accomplished. It is planned to continue the study of the roots of the other trees mentioned in the near future.

Materials and Methods.—Roots of *Gleditsia triacanthos* were dug in the field and brought to the laboratory. These roots were taken from seedling trees ranging from 30 cm. to 1.5 m. in height and were taken from both poorer clay soils and richer alluvial soils. No structures resembling the spherical or oblong tubercles commonly found on roots of leguminous plants were to be found. It was, however, soon discovered that slightly thickened regions measuring 5-15 mm. in length were disposed at irregular intervals along the smaller root branches. These cylindrical thickenings, as well as the greater portion of the roots, even very old parts, were covered with a very dense covering of root hairs which were first considered to be ectotrophic mycorrhiza. This interpretation was soon shown to be erroneous when cross sections were prepared. This dense mantle of hairs was studied by McDougal (6).

These cylindrical thickenings were cut out and disinfected in 1:1000 mercuric chloride solution for two minutes and then washed through three successive changes of sterile distilled water in as many sterile

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petri dishes. They were then plated out by crushing with a sterile scalpel and adding a tube of Lipman's agar.[1]

These plates were incubated at 35° C. and after four days typical whitish, glistening colonies of a stringy, sticky consistency made their appearance. From these colonies transfers were made to slants of Ashby's agar[2] and Lipman's agar. These cultures served as the starting point for the subsequent cultural and inoculation studies. With these organisms infection was produced on seedlings grown in sterile sand and inoculated. Infection and re-isolation was effected twice.

Infection and isolation experiments. On September 30, 1924, seeds were planted in four-inch flower pots containing soil obtained from about the roots of plants in the field. On November 18 these plants were five inches high at which time they were examined for "nodules" and finally transplated to larger pots of similar soil. These plants had roots with very prominent root-hair zones. Root hair zones were cut, disinfected, and plated out as before. A single colony appeared. This colony was similar in every respect to those obtained from roots dug up in the field. Microscopic examination showed the organisms from both isolations to be identical.

On December 2, 1924, seeds were planted in pots of sterile sand kept moistened with sterile Knop's solution minus nitrates.[3] One lot of the seeds was soaked in mercuric chloride solution for two and one-half minutes. The other lot was untreated. On December 19 two pots from each lot were watered with a soil suspension[4] made from soil taken from about the roots of trees in the field. Ten days later two pots from each lot were inoculated with a suspension of organisms previously isolated from root swellings. Two pots were kept uninoculated for controls. On February 17 examination showed root infection (cylindrical thickenings) in the pots watered with soil suspension and in those

[1] Lipman's agar

Dextrose	10.0	grams
Dipotassium phosphate	0.5	grams
Magnesium sulphate	0.2	grams
Peptone (Bacto)	0.02	grams
Agar	20.0	grams
Distilled water	1000.0	cc.

[2] Ashby's agar

Mannite	20.0	grams
Monopotassium phosphate	0.2	grams
Magnesium sulphate	0.2	grams
Sodium chloride	0.2	grams
Calcium sulphate	0.1	grams
Calcium carbonate	5.0	grams
Agar	20.0	grams
Distilled water	1000.0	cc.

[3] Nitrate-free Knop's solution:

Calcium chloride	4.0	grams
Potassium chloride	1.0	grams
Magnesium sulphate	1.0	grams
Monopotassium phosphate	1.0	grams
Distilled water	2000.0	cc.

[4] Soil suspension made by shaking 500 grams of soil in 1000 cc. of sterile distilled water.

watered with bacterial suspension but none were found in the uninoculated pots of the control. Seedlings from seeds that were disinfected with the mercuric chloride solution seemed to have better developed swellings than those that were not disinfected. Unfortunately, these roots were destroyed before an attempt could be made to photograph them. Others, however, were immediately planted.

On February 25, 1925, seeds were planted in four six-inch pots of sterile sand. These were watered with sterile nitrate-free Knop's solution.[3] On April 13, after the cotyledons had dropped from the seedling the sand was inoculated with a suspension of organisms previously isolated from roots and grown on beef agar. These roots were examined on May 19. Plants from the inoculated pots showed root systems much better developed than those from uninoculated pots. Inoculated plants again showed swellings on their roots while none were to be found on those uninoculated. Photographs of these roots were taken after which all of the well-marked swellings were severed for isolation studies and microtome sections.

Colonies appearing on plates made from these root swellings were similar in every way to those previously obtained from swellings on field and laboratory roots. Subsequent cultural and microscopic characters showed them to be identical.

Plants from the inoculated soil were replanted in the same soil in which they had previously grown. Additional seeds were planted in sand first sterilized and then inoculated with a suspension of the organisms. On July 20 both the replanted seedlings and those from fresh planting were dug, washed free from sand, and their roots ex-

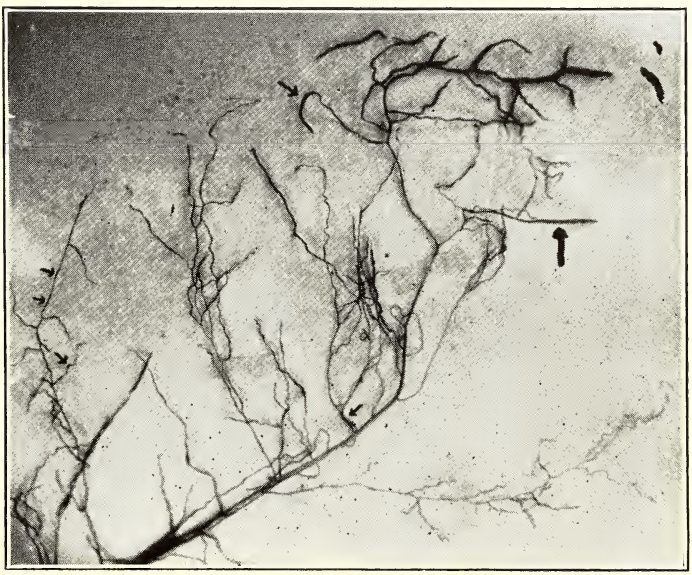

Fig. 1—Roots from seedling grown in sterile sand inoculated with organisms previously isolated from roots. Seedlings planted May 19 and photographs taken July 20. Arrows indicate some of the characteristic swellings in which the bacteria occur.

amined and photographed. Figure 1 shows roots from seedlings obtained from the seeds planted on May 19. Note the numerous well developed cylindrical swellings indicated in each case by arrows.

Growth characters of isolated organisms. Growth characters of the organisms isolated from the roots of Gleditsia were determined and compared with the characters of *Bacillus radiobacter* as determined by Löhnis and Hansen (5). This comparison was deemed necessary since *B. radiobacter* is a spore producing soil organism likely to be obtained when attempting to isolate nodule organisms in case the external disinfection is not thorough.

From a study of these results it is seen that the organisms first isolated from roots grown in the field have the same growth reactions as those subsequently isolated from roots grown in the laboratory and that the growth characters of both of these cultures are very different from those exhibited by *B. radiobacter* as determined by Löhnis and Hansen (2). Figure 3 shows cultures of the honey locust organism obtained from roots in the field.

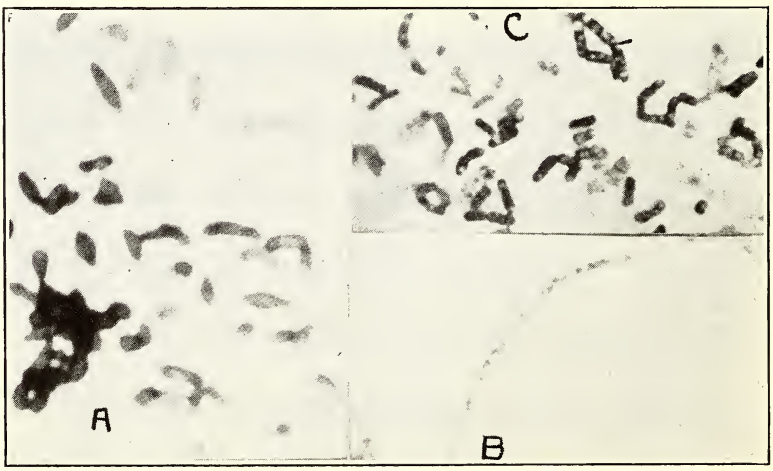

Fig. 2—Photomicrographs of stained bacterial cells from cultures isolated from roots of Gleditsia. A, cells from Ashby's agar. Note irregular shapes of involution forms and compare with figures 4, 5, and 6. B, cells from potato, rods with rounded ends, in long chains, and containing much highly refractive, non-staining globules. C, bacterial cells from beef agar, rods of variable length, single and in short chains; containing much highly refractive, non-staining globules.

Growth characters of organisms isolated from roots grown in the laboratory were identical in every regard. The growth on beef agar was very striking; the figure shows the very prominent longitudinal ridges and the unridged margins with slightly echinulate contour. The growth on potato was very abundant but not ridged.

In the description of cultural characters on the various media listed below, the honey locust organism isolated from roots grown in the field is designated as A, the same from roots grown in the laboratory as B, and *B. radiobacter* as C., from Löhnis and Hansen (5).

Lipman's agar. A. Five days old: growth good, center shiny, white, edges more transparent, echinulate. Involution forms of various shapes. Unstained cells full of dark globules. Took gentian violet stain lightly.

B. Forty-one hours old; growth very good, shiny, glistening white, edges transparent echinulate, center white opaque. Numerous involutions, rods with rounded ends, full of globules, single and short chains, numerous ovals.

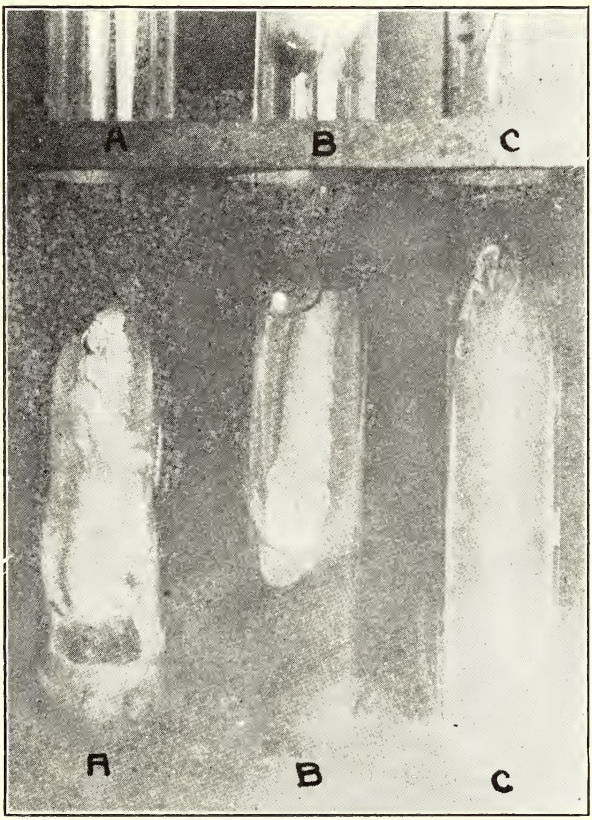

Fig. 3—Growth reactions of honey locust organism on potato (A), beef agar (B), and Ashby's agar (C). Note the prominent longitudinal ridges on beef agar.

Ashby's agar. A. Five days old: growth good, center nearly transparent, edges milky white, echinulate. Involution forms of various shapes. Full of dark globules. 14 days old: involutions and rod forms.

B. Forty-one hours old: growth good, nearly uniform, glistening white, edges echinulate. Seven days old: same. Seven days old: involution forms of various shapes, some rod forms. Cells full of dark globules.

Beef agar. A. Two days old: growth abundant, light cream in color, prominent parallel longitudinal ridges, margins echinulate. Nine

days old: ridges beginning to disappear. Two days old: Chains of short rods with rounded ends. Chains very irregular. Cells with refractive globules. Five day old: chains more regular. Fourteen days old: resembling chains of rounded beads with refractive globules near center.

B. Forty-one hours old: growth abundant, light cream in color, prominent parallel ridges throughout length of growth, margins slightly echinulate. Seven days old: same as above. Seven days old: irregular chains, ovals, rods, and involutions of various shapes. Cells filled with refractive globules.

C. "Flat whitish slimy layer, thick sediment below. After 7 days: small ovals and short rods imbedded in slime. * * * * Some rods with thick unstained capsules forming symplasm. Three to four weeks: normal cells, stars, and large globules and clubs from symplasm."

Nutrient Broth. A. Three days old: very thick pellicle on top, sediment on bottom, otherwise very clear. Eight days old: small rod in irregular chains. No refractive globules.

B. Three days old: very heavy creamy pellicle on top. Flaky all through tube. No turbidity. Four days old: involutions long and short rods in chains. Refractive globules abundant.

C. "Broth turbid, * * * * whitish film on surface, much whitish sediment. Small ovals, short rods budding and branching * * * * globular regenerative bodies. Threads and fine stars from symplasm."

Potato. A. Two days old: growth very abundant, glistening, edges echinulate, light cream color. Five days old: growth rather slimy. Two days old: long rods (2-8x1 micron) with many refractive globules. 13 days old: same.

B. Three days old: growth very heavy, crust-like over top, edges echinulate, color dull rich cream. Seven days old: small slightly raised, glistening blisters over growth, somewhat slimy. 14 days old: growth very abundant all over slant. Four days old: rods and ovals in chains, cells filled with reractive globules, some involutions.

C. "First gray, later coli-brown, no slimy layer, potato frequently turns brown. First small ovals and short rods, budding and branching, later also oval and globular gonidangia and symplasm with stars."

Litmus milk. A. Two days: color disappearing, consistency of junket. Three days: litmus decomposed except for very slight watery layer on top. Acid reaction. 10 days: litmus decomposed, milk digested, muddy brown color. 10 days: short rods, 1-2 microns long, in chains, no refractive globules.

B. Two days: consistency of junket. Four days: nearly all digested. Seven days: completely digested: litmus entirely decomposed, muddy tan color, acid reaction. 10 days: same as last. Eight days: short rods 1-2 microns long, in chains, no refractive globules.

C. "First slime ring and serum zone on top. Later whole milk turning brown. Seven days: ovals and rods, later small and large cells from symplasm."

Gelatin 20-22° C. B. Organism last isolated only one studied. Inoculated by spreading organism over surface. Liquefaction: three days, 3 mm; seven days, 9 mm; ten days, entire.

Microscopic characters of organism. The organisms exhibited considerable variation in shape and internal structure, varying from long or short rods held together in long or short chains to variously shaped involution forms. The irregular involution forms seemed to be much more common on synthetic media lacking nitrogen than on media containing these compounds. Both the long and the short rods contained abundant highly refractive bodies which show up as clear regions in stained cells (fig. 2). The involution forms more often stained uniformly though cells were abundant with non-staining centers and deeply stained peripheries. Refractive globules (non-staining) were also present in some involution forms.

Attempts to stain flagella were unsuccessful. Occasional organisms in poor preparations had what seemed to be one or two polar flagella. These were often displaced slightly from the exact pole of the cell.

Microscopic examination of root swellings. Some of the root swellings were cut from plants grown and inoculated in the laboratory. These were killed and fixed in medium chromo-acetic acid, dehydrated, imbedded in paraffin, sectioned 12 microns thick and stained in iodine and Flemming's triple stain. The iodine (Gram's formula) was used after the safranin and before the gentian violet. The gentian violet was used in 1 per cent aqueous solution and the orange G was used in 1 per cent clove oil solution.

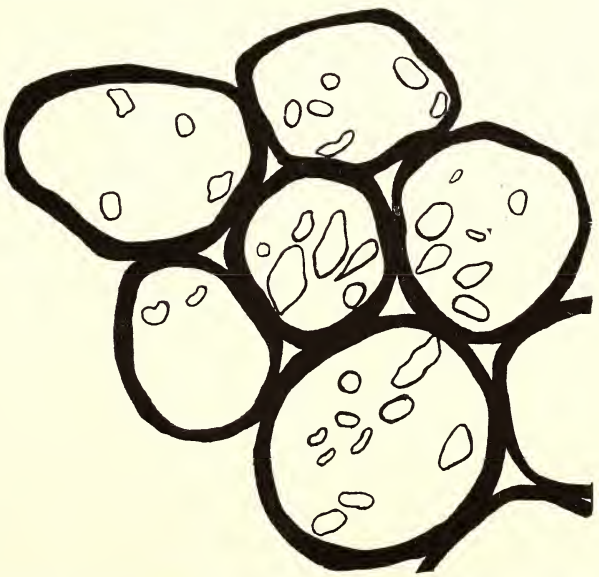

Fig. 4—Cells from the central cylinder of the root of *Gleditsia triacanthos* showing bacteria in situ. Drawn from microtome section with camera lucida. Compare shape of bacterial cells with those shown in figure 6, a. Magnification, 1700X.

Microscopic study of these stained sections revealed the presence of numerous bodies of various shapes in the cells of the central cylinder. These cells stained violet with the gentian violet. Figure 4 is a camera lucida drawing of some of the cells of the central cylinder containing these deeply staining bodies. Figure 5 is a photomicrograph of similar cells. Many of these bodies are of such shapes that one might well doubt whether they are bacteria at all if it were not for the fact that they gave no starch reaction and for the fact that every unusual shape found in root cells was readily duplicated from smears made from cultures grown on laboratory media and stained with the same stain. Comparison of cells in figures 4 and 5 with those of figure 2 shows the

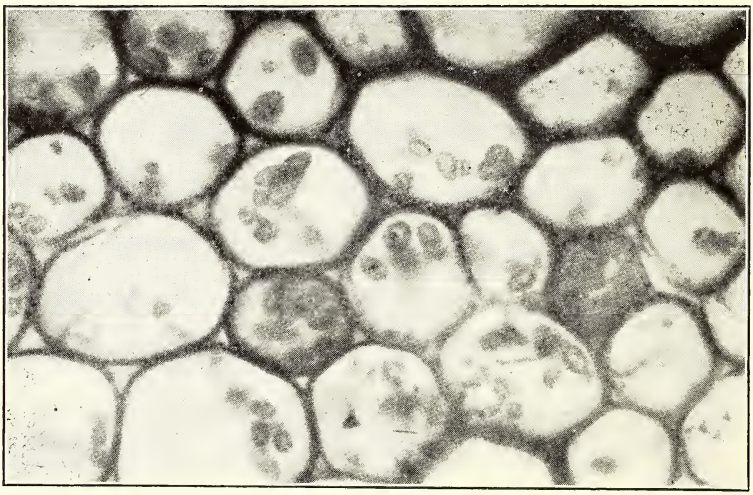

Fig. 5. Photomicrograph of root cells showing bacteria in situ. Compare shape of bacterial cells with those shown in figures 4 and 6.

similarity of cells from roots and from cultures. Figure 6 shows a series of camera lucida drawings of organisms from laboratory media (a) and cells from root sections (b). It will be noted that there is a very close similarity between the bacterial shapes in root cells and on culture media. Every shape of involution form can be found from both sources. True rod-shaped bacterial cells were not abundant in the root cells.

Harrison and Barlow (4) consider the absence of root nodules on *Gleditsia triacanthos* to be due to the presence of ectotrophic mycorrhiza. McDougal (6), has shown that the roots of Gleditsia are covered with a dense coat of persistent root hairs which appears superficially to be mycorrhiza. McDougal suggests that the absence of root nodules may be due to the impossibility of root infection because of the dense mantle of root hairs. From the present study it seems certain that bacteria do occur within the root cells. There they cause very slight hypertrophy of the root at irregular intervals. These swollen places are in no sense to be considered comparable, from a morphological standpoint, to the exogenous root nodules commonly found on leguminous plants.

Fig. 6. Outlines of bacterial cells drawn with camera lucida: from laboratory cultures (a), and from microtome sections (b). Magnification, 2400 X in each case. Note that the various unusual shapes of bacterial cells in the host cells can be duplicated from cultures isolated from the roots and grown on laboratory media.

Root infection probably occurs from the soil. Infection probably is not carried in the seed. These points will need further study to make certain, but in no case were root swellings to be found on uninoculated seedlings. Infection probably occurs in the very young part of the root. Bacteria were isolated from zones cut only a few millimeters from the tip.

SUMMARY.

1. *Gleditsia triacanthos* L. produces cylindrical swellings 10-15 mm. in length on the main axis of the smaller roots instead of the exogenous nodules ordinarily found on leguminous plants.

2. Bodies resembling involution forms of bacteria are found within the central cylinder of the root in the region of these swellings.

3. These involution forms may be isolated and grown on laboratory media from which stained smears yield again the same variety of shapes as found in microtome sections of root swellings.

4. Laboratory inoculation of seedlings grown in sterile sand and watered with nitrate-free Knop's solution yields typical root swellings from which the organisms may again be isolated.

5. Root systems of inoculated plants were much more vigorous than those of uninoculated plants when both were watered with nitrate-free Knop's solution.

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