T H E S I S

on

Some Crown Gall Inoculation Experiments with Special Reference to the Stomata as Possible Ports of Entry

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I. Introduction

Crown gall is a well known disease of plants induced by a particular species of bacterium, Bacterium tumefaciens Smith and Townsend. It has been studied from almost every conceivable angle, and considerable literature is available to anyone who wishes to study this disease. Due chiefly to the efforts of Erwin F. Smith and later of A. J. Riker, I. E. Melhus, and others much interest has been stimulated towards the subject of crown gall in the fields of experimentation and research. There are three important points concerning crown gall which are still in dispute. These points are as follows: (a) the precise method of migration of the bacteria in the plant tissues, (b) the amount of damage that crown gall actually does to the host, especially in producing-orchards and fields, (c) the presence or absence of the pathogene in certain overgrowths which are found on trees, especially those at the union of the stock and scion of nursery stock.

The experiments set forth in this paper do not attempt to solve any of the above major points concerning crown gall. They were begun to determine whether the crown gall organism was able to penetrate the host plant by by means of natural openings--stomata--and what effects, if any, might follow such penetration. Nowhere

in the literature has it been suggested that the organism was capable of entering the plant through natural openings and thus cause infections. In all probability conditions in nature would not be favorable for infection by means of stomata because the bacteria of crown gall are found naturally in the soil. Chances for them to come in contact with the leaves are slight. It is a well known fact, however, that the mode of entrance of many bacterial organisms to the plant is by means of the natural openings, and there is no evident reason to believe that this organism might not be able to enter its host plant by this method and cause infection.

E. F. Smith (36) and Riker (13), on the other hand, have concluded that the presence of wounds is essential before the organism can enter the plant. Riker (13) found that the bacteria were confined mostly to the intercellular spaces and to the wounded cells about the puncture of the needle. When, however, the plant was injured so that the tracheae were punctured by the needle, the bacteria were able to enter them and travel for a distance of several centimeters. Nevertheless, the bacteria in the tracheal tubes were unable to produce galls unless they were allowed to escape by means of mechanical injuries. It is not surprising that infections are not produced by the bacteria in the

tracheal tubes because the bacteria normally occupy an intercellular position and because they can affect only parenchymatous tissues. If, however, bacteria are able to enter the plant by means of the stomata they will then be in direct contact with parenchymatous tissue and ought presumably to produce their characteristic proliferations of some of the cells and their stretching effect of other cells.

The investigation was started in order to find out whether the bacteria could be made to enter the stomata and whether, in case of successful penetration, the expected results would follow.

II. Summary of Present Information Regarding Crown Gall

A. Historical

According to Owens (11) the crown gall disease has been known in Europe for three-quarters of a century, and Cavara in 1897 seems to have been the first one to isolate the organism and make successful inoculations. It is not certain whether the disease was introduced into this country or whether it is endemic. Attention was called in America as early as 1839 to peculiar growths upon beets, which were referred to as warts. These warts many years later were proved to be crown gall according to Townsend (44).

In Europe the disease was studied in Austria, Bohemia, France, Germany, Italy, and Sicily in the latter part of the nineteenth century and the early part of the twentieth century where various causes were ascribed to it. Smith, Brown, and Townsend (29) state: "The disease as it appears on vines is known in France as Broussins, in Italy as Rogna, and in Germany as Krebs and Grind. Viala also gives other names as Exostoses fougiodes, Raude, Kropf, Schorf, Ausschlag, Mauke, Tubercoli, Mallatia dei tubercoli, etc. The gall on the sugar beet in Germany is known as Wurzelkropf."

These early workers attributed the cause of the disease to insects, bacteria, frosts, mechanical injuries, nematodes, and fungi. Other writers in America, Woodworth and Wickson (47), believed that the peculiar condition of the weather, frosts, etc. caused an abnormal stagnation or an excessive flow of sap which produced the galls. Even at this time (1894) these writers were suggesting that bacteria were the possible cause.

Von Schrenk and Hedgcock (45) 1907 suspected bacteria as the cause of the overgrowth on graft unions in young grafted apple stock. They believed that the galls were produced much like those of the olive knot disease. This observation was not surprising because it had been known for a number of years that bacteria caused the over-

growth on olives. Finally in 1907 E. F. Smith and Townsend (26) discovered that a bacterium was the cause of the rapid development of young tissue and the subsequent formation of galls. They were able to isolate the organism from gall tissue occurring in nature, inoculate it into plants, and reisolate the organism again, thus establishing the validity of their deductions. Artificial galls were produced on tobacco, tomato, potato, sugar beet, and peach tree from cultures which were isolated from other plants. They named the organism B. tumefaciens Smith and Townsend.

After working many years with crown gall E. F. Smith and his co-workers (36) demonstrated to their satisfaction that the crown gall bacteria were intracellular. Riker (13) in America and Robinson and Walkden (20) in England demonstrated that the bacteria were intercellular and not intracellular.

E. F. Smith and his co-workers (26-42) have contributed a great deal to our knowledge of crown gall by their intensive research and experimentation. E. F. Smith believed that there was a close relationship between crown gall in plants and tumors in animals. He devoted most of his time when working with crown gall in an attempt to demonstrate this relationship. His contributions on this subject were appreciated by the medical fraternity, and

in 1925 he was elected to the presidency of the American Association for Cancer Research. In 1925 he spent eight months in Europe studying the causes of tumors and trying to determine whether B. tumefaciens had any relationship to the cause of tumors in animals. He was unable to find a definite relationship between the etiology of tumors of plants and those of animals. Probably his chief reason for believing that a relationship existed was that "tumor strands" and "secondary tumors" were produced in plants. The "secondary tumors" were supposed to have arisen from the "primary tumors" and to have migrated into other parts of the host tissue by means of "tumor strands."

Riker (13) and Robinson and Walkden (20), however, are not in accord with E. F. Smith on some of his findings, especially the production of "tumor strands" and "secondary tumors." They found that the "secondary tumors" were produced only when inoculations were made into rapidly elongating tissues. In other words, if a bud were inoculated the dividing cells carried the bacteria along with them and produced the "secondary tumors" and "tumor strands."

Melhus and Haney (7), Townsend (44), Siegler (23), and many others have been engaged in studying methods of control. Riker and Keitt (13), Swingle and Morris (45), Dorsey, et al (4), and others have been carrying on in-

vestigations in an attempt to determine the amount of real damage which is caused by crown gall. So many differences of opinion were evident on the various phases of crown gall that a symposium was held at Cincinnati December 31, 1923, and recommendations for future investigations of crown gall were published. From 1924 to 1930 over thirty articles have appeared relating to the various phases of crown gall.

Patel (12) separated fifteen non-pathogenic organisms from overgrowths on apple stock, which were morphologically similar in cultural and chemical agglutination tests to B. tumefaciens.

Riker, in an unpublished paper, has separated the so called hairy root strain of the organism into a new species. The hairy root strain, as it has been called in the past, will not affect tomato, tobacco, and other plants which the ordinary strain will affect. It is to be remembered that the ordinary strain of crown gall which is found on hops, beets, and other plants will infect many hosts when inoculated into them.

At present there is a keen interest being shown in crown gall investigations, and within the next few years it is predicted that some valuable information will be forthcoming.

B. Economic Importance

Considerable difficulty is being met with in trying

to estimate the damage caused by the crown gall organism because as a rule the host plant is not killed outright but its growth merely reduced. Probably the most serious losses are found in nurseries where it is not uncommon, according to Waite and Siegler (46), to find from twenty-five to fifty per cent of the young grafted trees infected with the disease.

On sugar beets crown gall is considered to be an important disease. Townsend (44) found the quality of sugar which is produced from galled beets is below that produced from normal beets. Riker (13) reports that crown gall is a limiting factor in the cultivation of certain cane fruits in Wisconsin.

The findings of Melhus et al (9) showed that the rate of water flow through galled apple stems was reduced more than thirty per cent below that of normal stems. The subsequent reduction of the flow of water in the stem during periods of drought or high transpiration would undoubtedly have a serious effect on the trees.

Secondary organisms such as wood rotting fungi and other bacteria are able to gain entrance to the plants by means of the galled tissue and create a mechanical weakening.

At the symposium in Cincinnati in 1923 it was brought out that the infection in the southwest is fatal to fruit trees, walnuts and almonds, and destroys the productivity of grapes. The reason for this is that the water balance between intake and loss in that part of the country is quickly affected by the presence of the disease, causing severe damage or death to the trees. It was further brought out that galled tissue may become inactive and disappear from infected trees after planting and that it may also appear on clean trees after setting out.

Some little time will probably elapse before it is actually known just how much damage crown gall is doing to economic plants. This will have to be determined for different varieties and for different sections of the country, for crown gall reacts differently in different climates, soils, and on different hosts.

C. Distribution

Crown gall has been reported from many sections of the world, and apparently it has a world-wide distribution. Owens (11) reports that crown gall is found in South Africa, South America, Australia, Asia, New Zeland, Canada, England, Mexico, and many countries in Europe. The disease has been reported from almost every state in the United States.

Riker (19) in a recent survey on the crown gall situation in England, France, and Holland found that the disease was an important factor in three or four places in England and Holland, but in the northwestern part of France no crown gall was observed. It is regretted that Riker's study was not extended to include Italy and Germany, for a great deal of research has been carried on in these countries on crown gall with beets and grapes. In the grape growing countries of southwestern Europe crown gall has always been considered a serious disease.

D. Host Plants

Probably there are few other plant diseases that are able to attack such a wide variety of hosts as crown gall. E. F. Smith (36) using strains of the organism isolated from daisy was able to produce galls on forty kinds of plants belonging to eighteen different families. These plants are as follows: daisy (sp.), tomato, potato, tobacco (3 sps.), oleander, cabbage, cauliflower, turnip, radish, beet, carrot, grape, clover, peach, almond, raspberry, apple, pear, carnation, hop, Coleus, Citrus, Impatiens (2 sps.), Opuntia, Persea (with difficulty), Juglans, poplar, Pterocarya, Allemanda, mango, stock, Ricinus, cassava, Fuchsia, Reseda, Salvia, Pisum, Calendula, Helianthus, etc.

Brown (2) reported crown gall as occurring naturally on sweet peas in the greenhouse. Cook (3) found crown gall occurring in nature on Arbutus, alfalfa, honeysuckle, pyrethum, pecan, peony, Shasta daisy, and a few other plants which had been previously reported by E. F. Smith and other workers.

On the other hand, there is a great deal of variation in the degree of susceptibility of plants within a given species. Dorsey et al (4) indicates that the Wealthy apple is more susceptible than the Jonathan. E. F. Smith and Quirk (41) found that the leaves of Begonia liecrena were quite resistant to crown gall. They attributed the high degree of resistance to the concentration of oxalic acid in the leaves. The juice of the leaves was almost as acid as N/10 HCl. They found that other plants which were immune to crown gall contained juices considerably more acid than pH [illegible] 5.7, the limit of crown gall toleration in bouillon. However, when a tumor was once started in very acid tissue it made its own conditions favorable for a good growth.

C. O. Smith (25) working with Prunus in California found that Prunus domestica and some of its varieties were quite resistant to crown gall. In nurseries on sandy soil, there was found a rather high infection

of the roots of almonds and plums, and to a less extent on myrobalan plums and apricots. According to Owens (11) the American varieties of grapes show a great deal more resistance to the disease than the European varieties.

It may be concluded from previous investigations that susceptibility depends upon the species or varieties attacked and the environment of the plant, which means ecological conditions.

E. Symptoms

The term crown gall probably originated from the frequent occurrences of gall-like overgrowths near the surface of the ground. This part of the tree is usually referred to as the crown by nurserymen, orchardists, and gardeners. The disease is usually confined to the underground parts of the trunk and the roots on trees, but occurs also on the aerial part of brambles and grapes.

There are two types of the ordinary crown gall, namely the hard and the soft gall. The latter is found to be more common, and in the case of woody stems the gall may be soft at first and later become hard. The terms soft and hard gall as used above are applied only to the malformations which are known as true galls and not to the hairy root or woolly knot type.

Hedgcock (5) recognized two forms of crown gall--the callous-like or typical gall, and the hairy root form. The latter is accompanied by a stunted root system and an excessive production of small fibrous or fascicled roots. According to E. F. Smith (36) the type of gall depends on the individual, the strain of gall organism, species of plants, and the particular cell that receives the stimulus from the organism.

E. F. Smith (37-39, 41, 42) was able to induce the formation of many plant organs, such as leaves, flowers, stems, and roots in places where they do not normally occur by inoculation with crown gall and by using chemical and physical stimuli.

Melhus (8) found that certain types of hairy root malformations on apple seedlings previously supposed to be indications of crown gall were discovered in the absence of the organism at the union of the stock and scion. C. O. Smith (24) in California frankly stated that aerial crown gall appears on different kinds of trees in California and the cause has not as yet been demonstrated.

Siegler (21) in some interesting experiments with the apple strain of crown gall on root grafted apple trees found that he was able to make isolations from the

type of malformations commonly known as woolly knot. The bacteria from these isolations were identical with B. tumefaciens. He was able to produce galls on Paris daisies, apple shoots, sugar beets, and Bryophyllum, but not on tomato or tobacco plants.

Riker and Muncie (17) from their experiments believe that many overgrowths have been called crown gall which are only efforts on the part of the plant to heal wounds. This is especially true on nursery stock where a poor union has been made between stock and scion. They concluded that by more careful grafting many of these knots could be eliminated and also that chances for infection would be lessened.

Siegler and Piper (22) in experimenting with the aerial crown galls on apples found that it was possible to produce galls by artificial inoculation, but they were able to isolate the organism from these galls for only a short period of time following inoculation. They were unable to isolate the causal organism from any of the naturally occurring aerial galls, but they found that these galls were morphologically identical with the ones produced by artificial inoculations.

Brown (1) was also able to produce aerial galls on apple trees by artificial inoculation. She concluded that it was theoretically possible for this

type of a gall to be produced naturally in nature, but not at all probable.

Waite and Siegler (46), however, were able to control the size and the frequency of overgrowths at the union of the stock and scion on grafted apple trees by the application of germicides.

Kellerman (6) in making comparisons of the nitrogen fixing symbiotic bacteria and B. tumefaciens found that the outgrowth caused by the former has no more apparent effect on the root than an ordinary branching root or rootlet. The interior of the outgrowths contains flesh colored cells filled with bacteria which are visible with the microscope. On the other hand, crown gall bacteria causes much malformation of the roots, often forcing them to branch profusely. The interior of the tumor is usually white, and the bacteria are intercellular. They are not readily visible to the microscope unless stained, and staining is often a difficult process.

There are many galls on plants which naturally occur in nature. They may be artificially classified as follows:

1. Galls above the ground (on aerial stems)
    a. Burls caused by latent buds.
    b. Twig galls caused by overgrowth of tissue around small stubby limbs.

c. Insect galls (woolly aphis, mites, Cynipids, etc.).

d. Galls caused by fungi (wood rotting).

e. Other bacteria (olive knot).

f. Winter injury.

g. Crown galls (B. tumefaciens)

h. Chemical stimuli (gasses from smelters, spray injuries, etc.).

i. Physical injuries (pruning, from cultivation, rodents, etc.).

2. Galls below the ground (on roots and crown)

a. Insect galls (woolly aphis, etc.).

b. Nematode galls.

c. Mechanical callous (improper union of scion and stock).

d. Bacteria (nitrogen fixing).

e. Fungi (Urophlyctis, etc.).

f. Slime moulds (Plasmodiophora, etc.)

g. Crown gall (B. tumefaciens)

h. Chemical stimuli (fertilizers, etc.).

i. Physical (from cultivation, rodents, etc.).

E. Description of Bacterium tumefaciens

E. F. Smith (36) describes the crown gall organism in the following manner: "This is a small, white, motile,

polar flagellate, non-sporiferous, Gram negative, non-acid fast, non-liquefying, non-nitrate-reducing, aerobic, non-gas-forming, non-starch-destroying, dry-air-sensitive, sunlight-sensitive, chloroform-tolerant, sodium chlorid-tolerant (up to 3.5 per cent), lab-producing, acid-forming (with certain sugars) rod-shaped schizomycete, which grows on agar-poured plates in the form of small circular, somewhat raised, wet-shining, translucent colonies. Streaked on agar from agar the growth is smooth and shining, but when streaked on agar from peptone bouillon the various strains (daisy, hop, etc.) usually give a thin, wrinkled and dull surface. . . . . It grows from 0°C. to ≠ 37°C. The optimum temperature for growth lies between 25° and 30°C. The thermal death-point (10 minutes exposure in the water-bath in test tubes in ≠ 15 peptone beef bouillon) is approximately 51°C. It shows slight toleration for organic acids (malic, citric, acetic) and still less for sodium hydrate. There is more growth in ≠ 15 than in - 15 peptone bouillon. The optimum acidity for this organism in beef bouillon appears to lie between ≠ 12 and ≠ 24 on Fuller's scale (1.2 to 2.4 per cent of N/1 acid).

"The bacterium is sensitive to germicides and slowly loses virulence on culture media. . . ."

III. The Present Investigations

Before beginning these studies it was necessary to obtain pure strains of the organism for use in the inoculation experiments. Further, it was desirable to make a preliminary study of the effects of the organism on the plant tissues in the presence of wounds. In the leaf inoculation experiments it was desirable to know the reaction of the stomata to various factors such as humidity, sunshine, temperature, etc. The experiments were of two types: (a) those where the inoculum was introduced into the plant by means of wounds, and (b) those where it was introduced by means of the stomata or natural openings.

A. Sources of Inoculum

Two strains of the typical crown gall organism were used in these experiments. One was obtained from Dr. A. J. Riker at the University of Wisconsin, Madison, Wisconsin, the other from some diseased hop roots which were sent to the Oregon State College Experiment Station from Independence, Oregon. The former will be designated as A and the latter as B. The isolation of the hope strain was made by cutting off one of the galls near the growing part of the root with a sterile knife, placing it in mercuric chloride (1-1,000) for one minute, and finally washing it thoroughly in sterile distilled water for ten minutes. A small section of

the gall was then sliced away with a sterile knife and a cube about the size of a pea was removed. This cube came from the center of the gall where chances of penetration by the mercuric chloride were very small. The piece of gall tissue was placed in a tube of sterile water and crushed with a sterile scalpel. The contents of the tube were then filtered through a single thickness of coarse cheesecloth to remove most of the material in suspension. Loop dilutions were made in agar tubes held just above the solidification point. These tubes were poured into petri dishes which had previously been sterilized, and examinations were made from day to day.

After four days white colonies appeared on all of the dishes. Transfers from these white colonies were then streaked on potato dextrose agar slants. At the end of forty-eight hours the organisms from these slants were inoculated into tomato, tobacco, geranium, and wax bean plants and subsequently produced the characteristic galls. Reisolations were made from these galls and the characteristic colonies were found, thus proving the identity of the organism.

B. Etiology

A special study of the normal action of the crown

gall organism when introduced through wounds as has been done by previous experimenters was undertaken. In this study puncture inoculations were made into tomato plants growing in the greenhouse, using pure cultures A and B obtained from Wisconsin and from Oregon respectively.

The disease is peculiar in that it does not kill the cells attacked, but stimulates some of them to multiply and others to stretch, the result being a large group of parenchymatous cells poorly supplied with vessels. In contrast to the crown gall organism, most pathogenic bacteria act on plants by plugging the vascular tissues, by dissolving the middle lamella, or by destroying the cells completely, usually resulting in serious injury or death to the plant.

The plants which are affected by crown gall, however, do not die immediately, and most of them manage to live in the presence of the organism to maturity.

When a tomato plant was inoculated by means of needle punctures, no visible swelling was noticed until about five days later. After galls had reached the age of a week, swellings were quite visible, and after three weeks time they were fairly large and

gradually assumed a pale creamish white color which indicated the absence of chlorophyll in the galled tissue. It may be mentioned that there was a certain amount of green color in the early development of galls on the tomato plants.

Tomato plants which were pricked with a sterile needle showed a browning of the tissue around the opening of the wound. The stems were swollen slightly on both sides of the wound, which was quite similar to the effect of inoculations with crown gall in the early stages. In cases where stem punctures were made with a hot needle the wounds failed to heal but dried out, causing the plant to collapse in many instances. After one has examined a number of inoculated and uninoculated wounds of plants, it is very easy to determine the type of overgrowth which is caused by crown gall.

The galls produced by these artificial inoculations increased in size on tomato plants for a period of about six weeks, and as long as the plants were kept in a healthy growing condition they remained alive. Plants which were inoculated with the organism at intervals of 5 cm. on the stem on November 15 were still alive April 15.

Riker (18) found that when a cell began to divide under the stimulus of the organism, the resultant daughter cells became smaller and smaller with each division and the nuclei were correspondently reduced in size. He further found that the greatest number of mitotic figures appeared at 10:00 o'clock at night. This is about the same time that mitosis appears to be most active in onion root tips. In the same group of experiments Riker also found that the smaller daughter cells appeared on the side next to the bacteria. In this case a similarity in structure existed between crown gall tissue and wound tissue in the early stages of gall formation. Examinations which were made of the needle punctures showed that abnormal cell division had taken place adjacent to the wounds. This division was similar to that which occurred in the presence of the crown gall bacteria. In this case the smaller cells appeared near the stimulus which is probably the air. Riker claims that all living cells not lignified respond to the stimulus of the crown gall organism in the presence of an injury, and that cell division occurs only following mitotic nuclear division.

C. Methods of Treating Plants Before and After Inoculation

In the leaf infection experiments the bacteria were sprayed on the plants in sterile water. It was desirable to have leaves in which the stomata were open for a maximum period of time. It was presumed that the motile bacteria would need a continuous film of water from the surface of the leaf, through the pores of the stomata, in the sub-stomatal air chambers, and in contact with the spongy parenchyma and palisade layers of cells. Under these conditions the bacteria on the surface of the leaf would very likely swim to the pore of a stoma, enter it, and then swim to the parenchyma cells which were supposed to be susceptible to the effects of the organism. To obtain the condition of wide open stomata on the leaves a specially constructed chamber and bell jars were used. The former, however, proved to be the better when a large number of plants were being treated.

The moist chamber. In constructing the moist chamber a pan of galvanized iron 37" wide, 37" long, and 4" deep was filled with water. A box was made of tongue and grooved flooring which was 35" wide, 35" long, and 35" deep with a tight glass lid for a top. The box was placed in the galvanzied pan of water and a raised floor put in the bottom of the box. The floor

was far enough above the surface of the water so that the potted plants would not be saturated with moisture.

This sort of an arrangement, however, did not produce sufficient humidity to keep the heavy leafed plants (Geraniums, Bryophyllums, etc.) moist at all times. In order to produce added humidity a twenty-five watt bulb 5" long was placed in the box and used as described below.

The upper part of the bulb and all of the connections and cord were wrapped with heavy friction tape to prevent any possible short circuits from the presence of so much moisture. The bulb was then wrapped in moist cotton, the ends of which were placed in the water. The heat of the exposed part of the bulb evaporated the water from the moist cotton, thus giving off sufficient moisture into the chamber to keep the surfaces of the leaves covered with water and the humidity of the atmosphere close to 100%. The plants remained in a very succulent condition during the time they were in the moist chamber.

During the day when the greenhouse was warm, the light was usually turned off to keep the temperature of the moist chamber down as low as possible.

The temperatures in the moist chamber ranged from 50° to 85° F. depending upon the amount of sun-

shine during the day. The water in the galvanzied pan was renewed frequently, thus aiding in keeping the temperature down to a minimum. The glass over the top of the chamber was shaded during the day to keep the sun from increasing the temperature inside the box.

Stomatal examinations with a microscope showed that the treatment was successful in keeping the stomates open during most of the day.

Bell jars as moist chambers. Bell jars inserted over the tops of plants were found to work equ lly as well as the large moist chamber when only a few individual plants were being treated, but they were not adequate in capacity when it was necessary to treat a large number of plants.

The cooling chamber. After the plants were taken out of the moist chamber, they were placed under a gauze roof which had water dripping on it. The drip was obtained by drawing out one end of a curved glass tube to a very fine diameter and then placing the opposite end in a pan of water which was set above the gauze inclosed chamber. The water was thus siphoned over and dripped on the gauze. The evaporation of the water on the gauze kept the plants inside the chamber cool. This arrangement helped to keep the

plants in a succulent condition after removing them from the moist chamber. It also gave the plants a chance to become acclimated to the temperature of the greenhouse in a more gradual manner after being removed from the warmer and more humid chamber in which the inoculations were made.

D. A Study of the Stomatal Movement

Object. This study was begun to note the time of day that the stomata were open and to further determine the factor or factors that tend to keep them open or closed. The knowledge of these factors was essential in order to apply the bacterial sprays used in the inoculation work at the most opportune time, that is, when the stomata were wide open, for if the stomata were closed when the bacteria were applied to the surface of the leaves, there was a possible chance for the bacteria to die or be washed off before another period of opening occurred. It turned out that this investigation revealed interesting variations in the stomatal action of different kinds of plants.

This study was made with week old tomato and wax bean plants, twelve week old castor bean and tobacco plants, and Geranium plants of an unknown age. An

attempt was made to select the leaves on the upper part of the plant which were about two-thirds grown.

Technic. A review of the literature on stomatal behavior failed to reveal any studies which had been made with the plants which were under investigation.

One set of plants was placed under bell jars, and another was left uncovered for comparison beside the covered plants. This study was conducted in the greenhouse. All of the plants were kept well watered by placing them in saucers of water.

The examinations of stomatal condition were made by stripping off small pieces of the epidermis with a pair of tweezers and immediately plunging them into absolute alcohol. The specimens so taken were examined under the 4 mm. and 16 mm. objectives. The covered and uncovered plants of the same species were examined at the same time so that a valid comparison could be made.

The degrees of openness for the stomata were classified as follows: 0. closed, 1. slits, 2. one-fourth open, 3. one-half open, 4. three-fourths open, and 5. wide open. The numbers 0, 1, 2, 3, 4, and 5 respectively were used as symbols for these different degrees of openness. Twenty stomata were counted for each leaf, and when 80% or more of

the openings were found to correspond to one of the degrees of openness the symbol was recorded.

To check the accuracy of stripping the epidermis and immersing it in absolute alcohol, the collodion imprint method was used. This method had been used by the experimenter to make stomatal counts on fresh and dried Ribes leave in connection with another study. It had been found to be very satisfactory for that purpose because the exact position of the guard cells was obtained at the time the collodion was applied. With dried leaves satisfactory results had been especially easy to obtain because the hairs, debris, etc. could be rubbed off with a stiff brush beforehand.

Before applying the collodion, a little alcoholic safranin was added to increase the visibility of the imprint under the field of the microscope. The collodion was then applied to the surface of the leaf in a very thin solution with a camel's hair brush, allowed to dry for five minutes, pealed off with a pair of tweezers, and examined under the microscope on a slide. When the contour lines of the epidermis were found to be very close together, it was necessary to use a cover slip in order to flatten out the imprint.

The collodion method was again tried out in the present investigation but was found to require too much time in making stomatal counts on fresh material, so that it was used here merely as a check on the accuracy of the other method.

Using the absolute alcohol method described above the stomata of the leaves of the covered and uncovered plants were examined during the entire day on March 2 and 8, and during the afternoon on March 5 and 18.

Stomatal examinations, March 2, 1930. On this day the stomata were examined hourly from 8:00 a.m. to 6:00 p.m. The ventilators of the greenhouse were open about six inches, and the temperature remained above 70° F. during the period when the observations were made. Very little of the heat came from the sun's rays, but mostly from the artificial heating in the greenhouse. The clouds obscured the sun for most of the day, and the longest continuous period of sunshine was one hour in length. See Table I.

Stomatal examinations on the afternoon of March 5, 1930. Hourly stomatal examinations were made on the afternoon of this date from 1:00 p.m. until 6:00 p.m.

This was a very cold, cloudy day with a cold wind blowing from the north. The ventilators in the greenhouse were wide open, and the temperature ranged from 62° to 66° F. The sun was completely hidden behind the clouds during the entire day. See Table I-A.

Stomatal examinations, March 8, 1930. On this date further hourly observations were made on the stomata of the covered and uncovered plants from 6:30 a.m. to 5:30 p.m. The sun was very warm, and the plants were exposed to its rays six or seven hours. The ventilators of the greenhouse were open about six inches, and the temperature ranged between 50° and 85° F. See Table II.

Stomatal examinations on the afternoon of March 18, 1930. Again observations on the stomatal movements were made hourly from 1:30 p.m. to 6:30 p.m. using the covered and uncovered plants. The day was very warm, and the temperature of the greenhouse ranged from 70° to 90° F. The ventilators of the greenhouse were kept open about six inches.

Results. In the comparative trials made with the collodion imprint method and the absolute alcohol method for determining the position of the guard cells of the

stomata, no difference was observed on the plants which were used.

The stomata on all of the plants under observation were found to be opening at 6:30 a.m. See Table II-A. No observations were made as to the time the stomata were beginning to open in the morning. The stomata on the plants which were uncovered reached their maximum aperture for the days observed between 9:00 a.m. and 11:30 a.m. This same group of plants were beginning to close their stomata about noon, and most of them were closed or nearly closed by 6:30 p.m.

On the other hand, the stomata of the plants which were covered were found to have a greater aperture at 6:30 a.m. than the uncovered ones. The maximum period of openness ranged from about 9:00 a.m. until as late as 3:30 p.m. in some of the plants in the covered group. This period, however, varied a great deal with the species of plants used.

The stomata on most of the covered plants were wide open to half open at 12:00 noon, and some of them remained in these positions until 4:30 p.m. The stomata appeared to remain open for a longer period of time and close more slowly on the covered plants than on the uncovered ones.

When the temperature of the greenhouse was low and the sky was cloudy, there was less activity of the stomata in most cases than when the temperature was higher and the sky was cloudy.

The combination of high temperature, sun, and humidity kept the stomata open for a long period of time, while the factors of sunshine and temperature caused the stomata to remain open for only a short period during the day.

The wax bean plants showed a tendency to close regardless of the humidity, especially in the presence of high temperature and sunshine.

The influence of humidity on the stomata of tomato plants was more striking than on the other plants. Humidity seemed to be the controlling factor in keeping the stomata open.

These studies showed that in the presence of sunshine, high temperature, and humidity, the stomata of the plants under investigation were kept open for a longer period of time than the ones under the influence of these factors. The two most important factors influencing the stomatal movement were probably humidity and temperature.

The information obtained from these stomatal studies was valuable in that it suggested a definite time of day

in which to make inoculations on the different kinds of plants used.

The applications of the bacteria in sterile water to the surface of the leaves were made at 9:00 a.m. because it was found that this was the time when most of the stomata were opening, and in every case but one, they were half open. It was believed that if the bacteria were to enter the stomata their chances to do so would be increased if they were on the leaf during the period when the apertures of the stomata were the greatest.

* Table I

Time	Cloudy or Sunny	Temperature #		March 2, 1930									
				Tobacco		Geranium		Wax Bean		Tomato		Castor Bean	
			&	C	U	C	U	C	U	C	U	C	U
8:00 A.M.	Cloudy	73	%	3	3	3	3	4	4	2	2	3	3
9:00 A.M.	Cloudy	75		3	3	3	3	4	4	2	2	3	3
10:00 A.M.	Sunny	80		4	4	4	4	4	4	3	3	4	4
11:00 A.M.	Cloudy	80		3	3	3	3	3	3	4	4	4	4
12:00 M.	Cloudy	83		3	3	3	3	3	3	4	4	4	4
1:00 P.M.	Sunny	83		3	3	3	3	3	3	3	3	4	4
2:00 P.M.	Cloudy	85		3	2	3	2	3	2	3	2	4	4
3:00 P.M.	Sunny	77		3	2	2	2	2	2	3	2	3	3
4:00 P.M.	Cloudy	75		2	2	2	2	2	2	2	2	3	2
5:00 P.M.	Dusk	72		1	1	2	2	1	1	2	2	3	2
6:00 P.M.	Dark	72		0	0	2	1	0	0	1	0	2	2

* This table gives the results of hourly observations made on the stomatal movement of plants in the greenhouse. One group was covered with bell jars, and the other group was left uncovered. Observations were made on the plants between 8:00 A.M. and 6:00 P.M., March 2, 1930, on a cloudy day. The range of the temperature in the greenhouse was between 72° and 85° F.

Represents degrees F. & C Represents plants covered; U Represents plants left uncovered. % Numbers indicate degree of openness of stomata as follows: 0, closed; 1, slits; 2, one-fourth open; 3, half open; 4, three-fourths open; 5, wide open.

* Table I-A

Time	Cloudy or Sunny	Tempera-ture	March 5, 1930--P.M.									
			Tobacco		Geranium		Wax Bean		Tomato		Castor Bean	
			C	U	C	U	C	U	C	U	C	U
1:00 P.M.	Cloudy	65	2	1	3	2	3	1	3	2	4	2
2:00 P.M.	Cloudy	66	2	2	3	2	2	1	3	1	3	2
3:00 P.M.	Cloudy	66	2	1	3	1	2	0	2	0	3	2
4:00 P.M.	Cloudy	65	2	2	2	1	2	0	1	0	3	2
5:00 P.M.	Dusk	62	2	2	2	1	2	0	1	0	3	2
6:00 P.M.	Dark	64	1	1	2	1	1	0	1	0	2	0

* This table is similar to Table I except that the observations were made on the afternoon of March 5, 1930.

* Table II

Time	Cloudy or Sunny	Tempera-ture	March 8, 1930									
			Tobacco		Geranium		Wax Bean		Tomato		Castor Bean	
			C	U	C	U	C	U	C	U	C	U
6:30 A.M.	Dawn	50	3	1	3	1	2	1	3	1	4	4
7:30 A.M.	Sunny	52	3	2	3	2	2	1	4	1	5	4
8:30 A.M.	Sunny	60	3	2	4	2	3	1	5	1	5	5
9:30 A.M.	Sunny	72	5	5	5	5	5	2	5	3	5	5
10:30 A.M.	Sunny	82	4	4	5	5	4	1	5	2	5	5
11:30 A.M.	Sunny	84	4	2	5	4	3	0	5	2	4	5
12:30 P.M.	Sunny	84	4	2	5	4	3	0	5	1	4	4
1:30 P.M.	Sunny	85	4	1	5	4	1	0	5	0	4	4
2:30 P.M.	Sunny	81	2	1	4	2	0	0	4	0	4	2
3:30 P.M.	Sunny	84	2	1	4	2	0	0	4	0	4	2
4:30 P.M.	Dusk	76	2	1	3	1	0	0	3	0	4	2
5:30 P.M.	Dusk	72	1	0	2	1	0	0	3	0	3	1

* This table is similar to Tables I and I-A except that the observations were made on March 8, 1930.

* Table II-A

Time	Cloudy or Sunny	Tempera-ture	March 18, 1930--P.M.									
			Tobacco		Geranium		Wax Bean		Tomato		Castor Bean	
			C	U	C	U	C	U	C	U	C	U
1:30 P.M.	Sunny	90	4	2	5	4	4	1	5	1	5	4
2:30 P.M.	Sunny	90	4	2	5	4	3	0	5	0	4	3
3:30 P.M.	Sunny	84	2	1	5	2	3	0	5	0	4	3
4:30 P.M.	Sunny	84	2	1	4	2	2	1	4	0	4	1
5:30 P.M.	Dusk	76	2	0	4	1	0	0	3	0	3	1
6:30 P.M.	Dark	70	2	0	3	1	0	0	2	0	3	1

* This table is similar to Tables I, I-A, and II except that the observations were made on the afternoon of March 18, 1930.

E. Leaf Inoculation Experiment--1

Object. This experiment was begun to determine whether the crown gall organism was present on or in the leaves of Geranium, tomato, tobacco, castor bean, and wax bean plants not artificially inoculated.

Methods and materials. Four wax bean, four castor bean, four tomato, four tobacco, and four Geranium plants were placed in the moist chamber described on page 23 and allowed to remain there for a period of sixty hours at a temperature ranging from 60° to 80° F. in a high humidity. At the end of the sixty hour period the plants were removed from the moist chamber. The age of these plants was not recorded, but they were healthy, rapid growing specimens. Two leaves near the top of each plant were picked off after the plants were removed from the moist chamber. These leaves were then washed separately in a 500 cc. beaker of sterile water for five minutes, removed from the beaker, and rinsed in sterile water again. Then, both surfaces of the leaves were scrubbed with sterile, moist cotton and returned to the beaker for another washing for five minutes. The leaves were then rinsed again in sterile water, allowed to dry for an hour, and passed quickly through a gas flame. They were then crushed in test tubes containing 5 cc.

of sterile water.

Loop dilutions were made from the resultant liquids and poured into petri dishes of nutrient agar. The petri dishes were examined after six days for white bacterial colonies which resembled the crown gall bacteria.

Results. After six days numerous white colonies of a white bacterium appeared on all of the dilution plates which were made from the crushed Geranium leaves. Two dilution plates which had been poured from one of the tobacco plants also showed colonies of white bacteria, but they were not very numerous on the surface of the plates. One dilution plate each from a tobacco, a castor bean, and a tomato leaf showed a few colonies of yellow bacteria, while on another plate from a tomato leaf a few colonies of pink bacteria appeared.

Two of the white colonies from each plate were streaked upon nutrient agar slants in test tubes. At the end of forty-eight hours the bacteria were scraped off of the slants and inoculated into healthy tomato plants by means of needle punctures. Four inoculations were made from each of the agar slants into tomato stems.

The plants were kept under observation for a

period of three or four weeks, but no gall developed where the stem inoculations had been made. The yellow and the pink organisms were not tested because they did not resemble the appearance of the crown gall organism on this type of medium.

This experiment was repeated, using only the tobacco and Geranium plants, the ones yielding the white colonies of bacteria, but negative results were obtained for the second time. In this case numerous white bacteria were obtained from the crushed leaves of the Geranium plants, but none were obtained from the leaves of the tobacco plants.

The Geranium plants were being attacked by a bacterial disease under the humid conditions of the moist chamber. It was supposed that the white bacteria which were isolated from the tobacco the first time were the ones that were causing the rot of the Geranium plants. The presence of the white bacteria in the tobacco leaves could have been due to the leaves of the tobacco plant coming in contact with some of the infected leaves of the Geranium while in the moist chamber. The above suppositions, however, were not verified by experimental evidence.

Conclusions. Under the conditions of this experi-

ment the crown gall bacteria were not found in the leaves of tobacco, tomato, castor bean, wax bean, or Geranium plants.

F. Leaf Inoculation Experiment--2

Object. This experiment was undertaken to demonstrate the possibility of the crown gall bacteria entering the leaves through the stomata when they were sprayed on their surfaces in a suspension.

Methods and materials. The Wisconsin strain A of the crown gall bacteria was used in this experiment. Six wax bean, six Geranium, and six tomato plants were used in this test. They were divided into three lots, numbered 1, 2, and 3. Each lot contained two wax bean, two Geranium, and two tomato plants. Lots were treated as follows:

Lot 1. This lot was placed in the moist chamber and kept there for a period of sixty hours at a temperature ranging from 60° to 80° F. with a high humidity. They were then sprayed with a suspension of bacteria in sterile water by means of a small atomizer. The bacterial suspension was prepared by scraping a forty-eight hour growth of strain A from the surface of nutrient agar test tube slant culture. The scrapings were stirred into about 20 cc. of sterile, distilled

water. When smaller amounts of water were used, the mixture clogged the nozzle of the atomizer.

The plants were turned upside down, and the under side of all the leaves were sprayed thoroughly. The plants were returned to the moist chamber immediately after spraying, and the upper surfaces were then sprayed until the leaves dripped with moisture. The weight of the moisture caused the leaves to droop a little, and as a result there was a considerable accumulation of water near the tips of the leaves. There was, however, a good film of moisture distributed over most of the surfaces of the leaves.

The plants were kept in the moist chamber for forty-eight hours after spraying, during which time the leaves remained covered with a film of moisture. At the end of the forty-eight hour period the leaves were picked off for examination, and tests were made for bacterial penetration.

Lot 2. These plants were not placed in the moist chamber either before or after inoculation. They had been growing on a bench in the greenhouse where the temperature varied from about 50° to 90° F. During the period of investigation, the relative humidity was rather low due to the large size of the house

and the small number of plants growing there at the time.

This lot of plants was sprayed on the same day as lot 1 with a similar suspension of strain A of the crown gall organism. After spraying, they were allowed to remain on the greenhouse bench for forty-eight hours, after which time leaves were removed for examination, and tests were made for bacterial penetration. It was noted that the film of moisture on the leaves evaporated completely within two hours after spraying, leaving the leaf surfaces dry in appearance from then on.

Lot 3. These plants were the checks, and they were treated like those in lot 1 except that they were not sprayed with the bacterial suspension. The leaves were removed from the plants at the same time the ones were removed from lots 1 and 2.

Methods of testing for bacterial leaf invasion by cultures and reinoculation. Two leaves from each plant of lots 1, 2, and 3 were picked off. Each leaf was washed separately in a 500 cc. beaker of sterile water for five minutes, removed from the beaker, and rinsed in sterile water. Both surfaces were then scrubbed with sterile, moist cotton. The leaves were

then returned to the beaker for another five minutes of washing, rinsed in sterile water again, allowed to dry for one hour, and passed quickly through a gas flame. They were then crushed in test tubes containing 5 cc. of sterile water.

Loop dilutions were made from the resultant liquids and poured into petri dishes of nutrient agar. The petri dishes were examined after six days for white colonies of bacteria which resembled the crown gall organism. From the petri dishes which produced white colonies, one typical colony was streaked on nutrient agar slants in test tubes. At the end of forty-eight hours the resultant growths were inoculated into healthy tomato plants by means of needle punctures. Four inoculations from each nutrient agar slant were made. The plants were kept under observations for a period of three weeks.

Results of cultures and reinoculation. From lot 1, inoculated plants kept in the moist chamber, four colonies were isolated from Geranium plants, three colonies from tomato plants, and two colonies from wax bean plants. All of these colonies produced typical galls by puncture inoculations on tomato stems. In all, 75% of those tested proved to be crown gall

bacteria. See Table III.

From lot 2, inoculated plants not kept in the moist chamber, one colony was isolated from a Geranium plant and another colony from a tomato plant. These colonies produced typical galls when inoculated into stems of healthy tomato plants. No colonies isolated from the wax bean plants produced galls when inoculated into tomato plants.

From lot 3, the unsprayed plants, no white bacteria appeared on any of the plates.

After two of the leaves of each plant had been removed, the rest of the leaves were examined from day to day for evidence of gall formations on the leaves. The examinations, however, after four week's time showed no signs of galls on the leaves in lots 1, 2, and 3.

Small pieces of the leaves were removed in all three of the lots just before the specimens were crushed in the test tubes of sterile water as described on page 43. The pieces of leaves were killed and fixed in formal-acetic-alcohol, dehydrated, and embeded in paraffin by the usual method. They were then cut between 8 or 12 microns in thickness on a rotary microtome. The sections were stained by the method which

was suggested by Riker (13). This method in brief was as follows: The sections were stained for one minute in carbol-fuchsin which had been greatly diluted (1 part by volume of carbol-fuchsin to 100 parts of water). Then, after very rapid treatment with absolute alcohol, the sections were cleared and stained in a saturated solution of light green in clove oil, rinsed in xylol, and mounted in balsam. Slides made in this manner failed to show any bodies or groups of bodies which resembled the short rod-shaped crown gall bacteria with their rounded ends.

Numerous other dilutions were made with the carbol-fuchsin and used in conjunction with the light green in an attempt to demonstrate the presence of the crown gall bacteria in the leaves of lots 1, 2, and 3. The results, however, were all negative.

Conclusions. Under the conditions of this experiment it was possible to demonstrate the presence of the crown gall organism in the leaves of wax bean, tomato, and Geranium plants.

The failure of the galls to form on the leaves indicated that conditions for infection were not favorable within the leaf tissues of these plants.

It was not possible to demonstrate the actual presence of the bacteria within the leaf tissues

by means of the stains which Riker (13) used in his experiments.

This experiment was repeated, but only the culture and reisolation method was used. The white organisms which were isolated from the crushed leaves were inoculated into sunflower plants instead of tomato. The following is a summary of the results from lot 1 which was inoculated and kept in the moist chamber, lot 2 which was inoculated and kept on the greenhouse bench, and lot 3 which was not inoculated, but kept on the greenhouse bench. From lot 1 crown gall bacteria were isolated from 50% of the leaves tested; from lot 2, from 16 2/3% of the leaves tested; lot 3 failed to give a single isolation.

Table III-a gives a summary of the results which were obtained when the culture and reisolation part of the experiment was repeated.

Table III-a

	Lot 1	Lot 2	Lot 3
No. of leaves ✓	6	1	0
No. of leaves -	6	11	12

✓ Represents leaves from which B. tumefaciens were isolated.
- Represents leaves which failed to show B. tumefaciens.

* Table III

Treatment	Geranium			Tomato			Wax Bean		
	Plant No.	Colony No.	Reinoculation	Plant No.	Colony No.	Reinoculation	Plant No.	Colony No.	Reinoculation
Treated in moist chamber and sprayed with crown gall strain A	1	1	≠	5	9	≠	9	17	-
		2	≠		10	-		18	≠
	2	3	≠	6	11	≠	10	19	-
		4	≠		12	≠		20	≠
Not treated in moist chamber. Sprayed with crown gall strain A	3	5	-	7	13	-	11	21	-
		6	≠		14	-		22	-
	4	7	-	8	15	≠	12	23	-
		8	-		16	-		24	-

≠ Represents positive results of inoculations into tomato plants.
- Represents negative results of inoculations into tomato plants.

* Shows the results of spraying bacterial suspension of strain A on Geranium, tomato, and wax bean plants; also the results of keeping one lot of these plants in a warm, humid chamber and another lot in a warm, dry greenhouse. The check plants which were treated in the moist chamber and left uninoculated gave all negative results.

G. Leaf Inoculation Experiment--3

Object. This test served as a check on the previous culture method of determining bacterial penetration into the leaf. There was an objection to the method used in the previous experiment. Possibly the washing, scrubbing, and flaming did not remove or kill all of the bacteria on the surfaces of the leaves. Thus, it was possible that the crown gall bacteria which appeared in the cultures came partly or entirely from the outside instead of from within the leaf. This experiment was therefore designed to find out in a different manner if the presence of the crown gall bacteria could be demonstrated underneath the epidermis of the leaf.

Methods and materials. The Wisconsin strain A of the crown gall bacteria was used in this experiment.

Six wax bean, six castor bean, six tomato, six tobacco, and six Geranium plants were used in this experiment. They were all young, healthy plants. The plants were divided equally into three groups, numbers 1, 2, and 3.

Group 1 was placed in the moist chamber and given the same treatment as lot 1 in the previous experiment. Group 2 was placed on the greenhouse bench and given the same treatment as lot 2 above. Group 3 was

left on the greenhouse bench but was not inoculated with the crown gall bacteria.

Isolations were attempted forty-eight hours after inoculations were made. Instead of placing the leaves in a test tube of sterile water and crushing them as described in the previous experiment, the epidermis was stripped from the surface of the leaves. A sterile needle was used to lightly scrape up the underlying mesophyll tissues and transfer the materials thus gathered to sterile water blanks to be used for isolation cultures. Extreme care was used in order not to break through the other epidermis of the leaf. Whenever the opposite epidermis was broken through, the materials which were taken were not used.

The removal of the epidermis proved to be a tedious task until sufficient practice brought a little skill. The material from the mesophyll tissues was placed in a tube containing 2 cc. of sterile water. Loop dilutions were made from these tubes, using sterile potato dextrose agar which was kept just above the melting point. The agar dilutions were poured into sterile petri dishes and watched for the development of bacterial colonies. Very little trouble was experienced with contamination by fungi. Bacterial

contaminations, however, occurred in a number of cases.

In removing the epidermis of the leaf every precaution was taken to prevent any outside contamination. The instruments were sterilized by dripping them into alcohol and flaming, the surface of the table on which the work was done was washed with a solution of mercuric chloride, 1-1,000, and the fingers were dipped in 95% alcohol before each leaf was handled.

The colonies of white bacteria which appeared on the surface of the petri dishes one week after pouring were inoculated into young, rapidly growing tomato plants. These inoculations were made by piercing the stem of the plant with a hot needle and then applying the bacteria into the needle punctures.

Check plants were punctured with a hot needle to make comparisons with the ones which had been inoculated with the bacteria taken from the petri dishes.

All of the plants were kept under observations for a period of four weeks to note whether gall formations occurred on the leaves. Small sections of the leaves were killed and fixed in formal-acetic-alcohol. Thereafter, these sections were treated and stained by the method which was used by Riker (13). This method was described in the previous experiment.

Results. The observations on the leaves of the plants which were made from day to day failed to show any gall formations at the end of four weeks.

It was not possible to demonstrate the presence of the bacteria in the leaves by staining with carbol-fuchsin and light green.

Culture and reisolation tests. From group 1 which was treated in the moist chamber and sprayed with crown gall strain A, two Geranium, three tomato, three wax bean, one castor bean, and four tobacco leaves gave white organisms which proved to be the crown gall bacteria. From group 2 which was sprayed with the crown gall strain A and left on the greenhouse bench, one tobacco, one tomato, one castor bean, and two Geranium leaves gave white organisms which proved to be the crown gall bacteria. No bacteria were isolated from group 3 which uninoculated and left on the greenhouse bench.

The culture and reisolation part of this experiment was repeated, using the B strain of the crown gall bacteria. The following is a brief summary of the number of leaves which gave white organisms and later were proven to be the crown gall bacteria.

Table IV-a

	Group 1	Group 2	Group 3
No. of leaves ✓	8	2	0
No. of leaves -	12	18	20

✓ Represents leaves from which B. tumefaciens were isolated.
- Represents leaves which failed to show B. tumefaciens.

Conclusions. The second set of inoculations gave results in all essential respects similar to those of the first set, and the second method of isolation used in this experiment gave results like those obtained in Leaf Inoculation Experiment--2.

Table IV

Treatment	Geranium			Tomato		
	Plant No.	Leaf No.	Reinoculation	Plant No.	Leaf No.	Reinoculation
Treated in moist chamber and sprayed with crown gall strain A	1	1	+	5	9	+
		2	-		10	-
	2	3	+	6	11	+
		4	-		12	+
Not treated in moist chamber. Sprayed with crown gall strain A	3	5	+	7	13	+
		6	+		14	-
	4	7	-	8	15	-
		8	-		16	-

p.55 not used.

H. Leaf Inoculation Experiment--4

Object. This experiment was performed to note if a vacuum would have any influence in forcing bacteria through the stomata of leaves. Stomata are generally cutinized which inhibits the ready entrance of liquid moisture. It was believed that if a plant which had been sprayed with a suspension of crown gall bacteria were subjected to a partial vacuum and the vacuum suddenly released, perhaps the sudden changes of atmospheric pressure would force the bacteria into the leaf through the stomata.

Methods and materials. Eight wax bean, eight Geranium, and eight tomato plants were used in this experiment. The crown gall bacteria of the Oregon strain B were used to make the inoculations. The plants were divided into four lots, 1, 2, 3, and 4, with two plants of each kind in a lot.

Lot 1. Lot 1 was given a sixy hour treatment in the moist chamber. The bacteria on the surface of an agar slant were scraped off and stirred into 20 cc. of sterile, distilled water. This mixture was sprayed on the plants by the following method:

The plants were turned upside down, and the lower

surfaces of the leaves were sprayed by means of an atomizer until the excess moisture dripped from them. They were placed in the vacuum chamber where the tops were sprayed. A suction pump was attached to the vacuum chamber and allowed to run for a period of ten minutes, at the end of which time a partial vacuum was obtained. After the suction pump was turned off, the stop cock was opened suddenly, allowing the air from the outside to rush into the chamber. After this treatment the plants were returned to the moist chamber for forty-eight hours. One leaf was picked off of each plant at twenty-four intervals for one week, and then samples were taken at the end of the second and third weeks.

Part of the epidermis of each leaf was removed and the same steps followed as outline in the previous experiment in which the crown gall organism was isolated from beneath the epidermis of the leaf.

Lot 2. Lot 2 was given the same treatment as lot 1 except that the plants were not sprayed with a suspension of the crown gall bacteria but with sterile water.

Lot 3. Lot 3 was given the same treatment as lot 1 but was not placed in the moist chamber or given the vacuum chamber.

Lot 4. Lot 4 was placed on the greenhouse bench to be used as a check.

Results. No galls were observed on any of the plants which were kept under observation for a period of four weeks. There were only a few leaves left on the plants at the conclusion of this experiment as most of them had been picked off to make isolation tests.

Culture and reisolation tests. In lot 1 the crown gall bacteria were recovered from beneath the epidermis of the leaves of the Geranium, tomato, and wax bean plants at the end of three weeks. They were also isolated from the leaves at twenty-four intervals in most cases. Lot 2 gave a few white bacteria, but in all cases they failed to develop galls when inoculated into healthy tomato plants. No crown gall bacteria were isolated from plants of lot 3 after the expiration of ninety-six hours. The results obtained from lot 4 were all negative.

Conclusions. Under the conditions of this experiment the crown gall organism, when sprayed on the leaves of Geranium, tomato, and wax bean plants, was able to enter the leaves, but it did not produce galls. The humid condition of the moist chamber probably was one of the factors which assisted the crown gall bacteria

in intering the stomata. The vacuum apparently had no marked influence upon the entrance of the bacteria by means of the stomata. See Table V.

* Table V

Treatment	Plant No.	Intervals of Sampling In Hours							In Weeks	
		24	48	72	96	120	144	168	2	3
		Geranium								
Lot 1	1	≠	≠	-	≠	≠	-	≠	-	≠
	2	≠	-	≠	≠	≠	≠	-	≠	≠
Lot 2	3	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-
Lot 3	5	≠	≠	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-
		Tomato								
Lot 1	7	≠	≠	≠	-	≠	-	≠	≠	≠
	8	≠	≠	≠	≠	-	-	≠	-	≠
Lot 2	9	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-
Lot 3	11	≠	≠	-	-	-	-	-	-	-
	12	≠	≠	-	≠	-	-	-	-	-
		Wax Bean								
Lot 1	13	≠	≠	-	≠	-	-	-	-	≠
	14	≠	-	≠	≠	≠	-	-	≠	≠
Lot 2	15	-	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-	-
Lot 3	17	-	≠	≠	≠	-	-	-	-	-
	18	≠	-	-	≠	-	-	-	-	-

≠ Represents positive results.
\- Represents negative results.

* Shows the results of spraying bacterial suspension of strain B on Geranium, Tomato, and wax bean; also results of keeping lot 1, inoculated, in moist chamber and then vacuum chamber for ten minutes; lot 2, uninoculated, in moist chamber and then vacuum chamber for ten minutes; lot 3, inoculated, not in moist chamber or vacuum. The check lot left on the greenhouse bench failed to yield any bacteria at all.

I. Leaf Inoculation Experiment--5

Object. Riker (14) found that the sap of tomato plants exerted a positive chemotactic stimulus upon the crown gall bacteria. It was therefore thought possible that in the presence of juices of the plant on the surface of the leaves there might be a better chance for bacteria to enter the plant by means of the stomata. Furthermore, the question arose as to whether the juices extracted from the host could gain entrance to the intercellular spaces of the leaves by means of the stomata. If the bacteria were introduced at this time they might possibly increase in number sufficiently to be readily observed, at least when stained.

Methods and materials. Four Geranium, four wax bean, and four Bryophyllum plants were used in this experiment. They were divided equally into two lots and treated as follows:

Lot 1. This lot was placed in the moist chamber for sixty hours. At the expiration of this period the plants were sprayed with the expressed juices taken from their respective tissues. They were then returned to the moist chamber for twenty-four hours.

The leaves and stems from which the juices were extracted for this experiment were scrubbed with a brush and washed several times in 500 cc. of sterile water. The materials were then crushed in a mortar. The resultant extract was filtered through sterile cotton to take out most of the coarse particles.

Twenty-four hours after the plants had been placed in the moist chamber, they were removed and sprayed with a suspension of the A strain of the crown gall bacteria in the extracted juices of their respective plants. After the application of the spray was made, the plants were moved to the cooling chamber, which has previously been described, where they were observed for three weeks for the development of galls on the leaves.

Samples of the leaves were taken, killed and fixed in formal-acetic-alcohol, and dehydrated. They were then embedded in paraffin and sectioned on a rotary microtome to 10 microns in thickness. Some of the sections were stained in a very dilute solution of carbol-fuchsin (1 part of carbol-fuchsin to 100 parts of water), given a very rapid treatment in absolute alcohol, stained in a saturated solution of light green in clove oil, rinsed in xylol, and mounted in balsam. Riker (13) used this method in staining the

crown gall bacteria in plant tissues. Some other dilutions of carbol-fuchsin which were made with water were tried in combination with the light green in clove oil. These dilutions were as follows: 1 part of carbol-fuchsin to 150, 200, 250, and 300 parts by volume of water.

Lot 2. Lot 2 was given the same treatment as lot 1, but the former was not sprayed with the juices of the plants or the crown gall bacteria.

Results. The presence of the organism within the leaf was successfully demonstrated by the culture and reisolation method which was used in previous experiments. The presence of the organism was not, however, demonstrated in the leaves of the Bryophyllum plants by any method used.

The staining method failed to demonstrate the presence of the short, rod-shaped, small and rounded end bacteria within the tissue of the leaves.

The plants which were kept under observation did not develop galls on the leaves of either of the two lots of plants.

Conclusions. It was not actually demonstrated that the juices of the plants obtained entrance to the intercellular spaces by means of the stomata. If the

juices were present they failed to stimulate the bacteria to multiply in the leaf tissues.

J. Experiment on Tracheal Migration of Bacteria

Object. Riker (13) found that when the tracheal tubes of a tomato plant were injured and the crown gall bacteria were introduced into the wounds, galls could be produced as far as 8 cm. above the wounds when a sterile needle was used to puncture the stem and the tracheal tubes. He concluded, "While one may not be fully confident of the exact course followed by the bacteria, these experiments suggest that, under favorable conditions, the crown gall bacteria can pass through the tracheae and induce galls at some distance from the point of entry if allowed to escape from the vessels and suitable circumstances for development." In the light of Riker's findings this experiment was begun to determine, if possible, whether or not the bacteria were able to enter the stomata of the leaf, make their way into the tracheae of the vein, and travel down into the stem. It was thought that their presence in the stem could be demonstrated by means of needle punctures and the subsequent production of galls.

Methods and materials used. Four Geranium, four wax bean, four castor bean, four tomato, and four

tobacco plants were used in this experiment. The plants were divided equ lly into two lots, 1 and 2. In lot 1 the stems of the plants were cut off near the surface of the ground, inserted through small holes in corks which were placed in bottles containing sterile water so that the cut ends were submerged. A hole was made by means of a sterile needle clear through the stem of the plant just above the surface of the liquid. The openings in the tops of the bottle and around the stems were then sealed with paraffin to prevent any outside contamination of the liquid within the jars.

After sealing the jars with paraffin, the plants were placed in the moist chamber for sixty hours and then sprayed with strain A of the crown gall bacteria suspended in 20 cc. of sterile water. They were returned to the moist chamber for twenty-four hours after spraying and then placed in the cooling chamber for two weeks under observation. After two weeks in the cooling chamber, they were kept under observation for an additional two weeks. The plants were then removed from the jars, and the wounds which had been made were examined.

In lot 2 the same treatment was given except that a suspension spray of the crown gall bacteria was not

applied to the leaves of the plants.

Results. One wax bean and one Geranium plant in lot 1 showed positive results, but a careful examination revealed that these two plants had not been tightly sealed. The bacteria had probably run down the stems and entered the wounds when the leaves were inoculated in spraying. The rest of the plants in both lots 1 and 2 showed no signs of gall formations on the wounded areas.

Conclusions. This experiment, therefore, gave negative results, and did not demonstrate that bacteria could enter the leaf by means of stomata, find their way into the tracheal tubes of the plant, move down into the stem, and produce galls in the presence of wounds under the conditions which were present in this study.

K. Experiment on Bacterial Migration from Stem to Leaf

Object. If the crown gall bacteria were able to travel in the tracheae, then there was a possibility that they would migrate into the mesophyll tissues of the leaf by the way of the petiole, the midrib, and the branching veins. If it were possible for the bacteria to move about the plant in such a manner, it was thought that an injury to the mesophyll tissue would probably produce galls in their presence.

Methods and materials. Six tomato, six tobacco, six wax bean, six castor bean, and six Geranium plants were used in this experiment. The plants were equally divided into three lots, 1, 2, and 3.

Lot 1. The stems of the plants in this lot were cut under water and placed in separate jars which contained the crown gall bacteria in sterile water. The plants were placed in the cooling chamber for one week after which time the leaves were punctured with a sterile needle. In some cases extreme care was used not to break through the opposite epidermis of the leaf. On the other hand, some of the leaves were punctured through with the needle.

They were then returned to the cooling chamber for 10 days and observations were made on them for the

development of galls.

Lot 2. This lot was treated in the same manner as lot 1 except no crown gall bacteria were placed in the jars of water in which the cut end of the stems were submerged.

Lot 3. It was treated the same as lot 2 except the crown gall bacteria were sprayed on the leaves immediately after the wounds were made.

The Wisconsin strain A of the crown gall bacteria was used in this experiment in lots 1 and 3.

Results. The leaves in lot 1 failed to show any galls forming on any of the plants at the end of the ten-day period.

The leaves in lot 2, the checks, also gave negative results.

In lot 3 almost all of the wounds on the Geranium leaves produced galls. The tobacco, wax bean, castor bean, and tomato plants failed to produce galls on the leaves even in the presence of the organism on wounded leaf tissue. Small galls were produced by wound inoculations on the veins and midribs of all the plants used. This part of the experiment was repeated and the same results were gotten, namely, the positive results with the Geranium and negative with the tobacco wax

bean, castor bean and tomato.

Conclusions. Under the conditions of this experiment it was not possible to demonstrate that the crown gall bacteria migrated from the tracheae to the mesophyll tissues of the leaf. Further artificial wounds produced galls on the leaves of Geraniums but not on tobacco, wax bean, castor bean and tomato plants in the presence of the bacteria.

L. Experiment on Bacterial Migration from Stem to Branch

Object. This experiment was begun to determine whether or not the crown gall organism would enter the cut end of the top of a tomato stem, travel its length towards the roots and enter another stem.

Methods and Materials. A potted tomato plant 23 inches high was bent over to a horizontal position and kept that way. Three inches of the top were cut off, and the cut end was placed in a flask containing crown gall bacteria of the strain A suspended in sterile water. The stem was allowed to grow in this position for the duration of this experiment. It was punctured at intervals of one inch with a hot sterile needle six times near the top end of the stem. Three days after the shoot was bent over another one sprouted and grew perpendicular to the horizontal stem. This stem which sprouted was at the base of the plant. Three weeks from the time a second stem sprouted, it was punctured with a hot sterile needle.

Results. Typical galls were in evidence on the horizontal shoot at all points where the punctures were made after three weeks time. The younger perpendicular shoot which was punctured with a sterile

needle also produced typical galls over its full length. This shoot was seven inches high.

A check plant, which was treated the same way as the one above except that the stem was not exposed to crown gall bacteria, failed to produce galls on either shoot.

Conclusions. The results of this experiment indicate that the crown gall organism was able to travel backwards in the horizontal stem for approximately 9 inches, enter another shoot and produce galls on this branch in the presence of artificial wounds. The needed punctures probably allowed the bacteria in the tracheal tubes to escape and then produce galls. It is supposed that the bacteria entered the cut ends of the tracheal tubes with the water and passed through them to the branch. No gall-like growths occurred anywhere else than at the punctures.

M. Experiment on the Length of Time the Bacteria Remain Infective on the Surface of the Host.

Object. This experiment was designed to demonstrate the number of days the crown gall bacteria would live on the outside of the host and produce galls when wounds were created.

Methods and Materials. A set of ten tomato plants was placed in the usual moist chamber. Another set of ten plants was left on the greenhouse bench. The stems of the plants in both sets were covered with strain B of the crown gall organism. The bacteria from a forty-eight hour old culture were used to make the inoculations. The bacteria were diluted with 10 cc of sterile water. The application was made by means of a camels hair brush. On the next day and every day thereafter for 10 days one of the plants in each set was punctured 10 times with a sterile, hot needle. The punctures were distributed at equal intervals from the top to within one inch of the surface of the soil. The set of plants which were kept in the moist chamber remained moist during the entire experiment. The moisture on the plants not in the moist chamber was not in evidence four hours after the application of the gall organism. During the first four days of the experiment the temperature of the greenhouse

did not exceed 76° F but later rose to 90° during the afternoon. The humidity during the day time was about 20% . The temperature in the greenhouse after the fourth day reached a maximum of 92° F. The temperature in the moist chamber ran from about 60° to 80° F. while the humidity was close to 100%.

Results. The set of plants in the moist chamber produced galls in the presence of artificial wounds ten days after the bacteria had been applied to the stem but the set of plants kept in the open air of the greenhouse, where the air was warmer and drier for most of the period of the experiment, failed to produce as many galls. The bacteria were apparently dead, on most of the plants, after remaining on the stems for a period of four days in the open greenhouse.

Conclusions. From this experiment it would seem that the bacteria remain alive on the stem of tomato plants for a longer period of time in the moist chamber than on the stems in the open greenhouse. See Table V.

* Table VI

Number of Punctures	Number of days elapsing between date of application of culture and date of puncturing	Number of infections evident after four weeks incubation	
		Plants kept in open air of greenhouse	Plants kept in moist chamber
10	1 day	10	10
10	2 days	8	10
10	3 days	3	9
10	4 days	2	10
10	5 days	0	5
10	6 days	0	3
10	7 days	0	4
10	8 days	1	5
10	9 days	0	3
10	10 days	0	2

* Table VI shows length of time the crown gall bacteria remained alive on the stems of tomato plants as indicated by gall formation following puncturing at daily intervals after the application of culture to surface of stem.

IV. Discussion and Conclusions

The primary purpose of the studies reported in this paper was to discover whether or not crown gall bacteria could be made to enter the stomata of the leaf of susceptible host plants and produce the characterisitc crown gall malformation. The leaf inoculation experiments which were conducted with this question in mind have shown consistently by isolation and reinoculation methods that B. tumefaciens can be made to enter the leaves through the natural openings. In no case, however, did the invading bacteria produce galls on the leaves or give any other indications of pathogenic activity.

A secondary purpose developed after the leaf inoculation experiments were begun. This purpose was to determine whether the bacteria would enter the vascular system from the tissue of the leaves or whether they would ender the leaves from the vascular system.

Plant pathologists who have worked with bacteria know that it is often a very difficult problem to differentiate them in plant tissues. E. F. Smith and his co-workers tried many combinations of stains over a period of about fifteen years but failed to locate the crown gall bacteria. However, they finally concluded that the organism was located within the cells,

but later it was demonstrated by means of improved staining by Riker (13) and Robinson and Walkden (20) that the organism normally occurred in the intercellular spaces.

It is not surprizing that the crown gall bacteria were not located until 1923 because anyone who has examined plant materials with a microscope would realize what a host of bacterium-like objects may be found within the tissues of plants. It was found that some of these bacterium-like bodies took the stain as did the bacteria.

It was also possible that if the crown gall bacteria were to enter the leaf and not cause infection, an increase in numbers probably would not result. To differentiate a few bacteria in a leaf to any degree of certainty would be practically an impossibility. On the other hand, if the bacteria had entered the leaf through the stomata there would probably have been a multiplication and massing of them in the intercellular spaces, and in such a case differential staining would perhaps have been possible.

In the light of the evidence which has just been presented, it would seem that the bacteria under the

conditions of these experiments entered the leaves of the host plants, but conditions within the leaf were not suitable for their growth and development. There was an exception to this in that the presence of the crown gall bacteria was not demonstrated in the leaves of Bryophyllum plants. Riker (16) found that comparatively small galls developed in the presence of an abundance of moisture. In an uninjured leaf there may be considerable moisture, and the absence of oxygen. In an injured plant there is an abundance of moisture and also considerable oxygen present.

The crown gall bacteria were enter some cells of the cut stem, presumably the tracheae, and travel some distance. They remained in these tubes without producing injury to the plant unless they were liberated by breaking the tracheal tubes. In the experiments conducted, the experimenter was not able to demonstrate that the bacteria would enter the tracheae by means of the leaf tissue, nor was it demonstrated that they would enter the leaf tissue by way of the tracheae.

Under different conditions than the ones which were used in these experiments, it might be possible to induce the bacteria to enter the stomata and produce malformations in the mesophyll tissues.

V. Summary

1. It was found that when tomato, wax bean, Geranium, tobacco, and castor bean plants were given a moist chamber treatment, B. tumefaciens would enter the uninjured leaves when sprayed on their surfaces. In one experiment with Bryophyllum, the bacteria did not enter the stomata.

2. The crown gall bacteria did not produce intumescences or other visible effects on the leaves which they entered in this manner.

3. The writer was unsuccessful in his attempt to demonstrate the presence of bacteria in leaves by staining methods, but their presence was successfully demonstrated by two different isolation methods followed by positive reinoculation tests using stem punctures.

4. B. tumefaciens were recovered from the mesophyll tissues of the leaves of wax bean, tomato, and Geranium plants three weeks after the application of the bacteria to the surfaces of the leaves.

5. Crown gall bacteria were made to enter the cut ends of vascular bundles, travel for a distance of approximately 25 cm. in a horizontal stem, thence into a branch, and cause galls at injured places.

6. In none of the tests conducted did invading crown gall bacteria cause proliferation of the cells of the host except in the presence of wounds. It was found in the experiments conducted that galls could be produced on the leaves of Geranium plants by inoculation, but not on tomato, wax bean, castor bean, or tobacco leaves.

7. In a study of stomatal behavior as a basis for timing leaf inoculations, it was found that the stomata of the bean, tomato, castor bean, and tobacco plants were widest open between 9:00 and 11:00 o'clock in the morning and that they were open wider and remained open longest on plants kept in a moist chamber than on plants held in the open air of the greenhouse where the tests were conducted.

Literature Cited

(1) Brown, Hellie A.
1924. Apple stem tumors not crown-gall. Jour. Agr. Res. 27: 695-698.

(2) ----.
1927. Sweet pea fasciations of crown-gall. Phytopathology 17: 29-30.

(3) Cook, M. T.
1911. Crown-gall hairy root. N. J. Agr. Exp. Sta. Circ. 34.

(4) Dorsey, M. J. et. al.
1924. The crown-gall resolution. Am. Ass. of nurserymen. 39 unnumbered pages.

(5) Hedgcock, G. G.
1910. Field studies of the crown-gall and hairy root of apple. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 186.

(6) Kellerman, K. F.
1911. The relation of crown-gall to legume inoculation. U. S. Dept. Agr., Bur. Pl. Ind. Circ. 76.

(7) Melhus, I. E., and Haney, T. J.
1921. A study of the control of crown-gall on apple grafts in the nursery. Iowa Agr. Exp. Sta. Res. Bul. 69.

(8) ----. Muncie, J. H., and Ho, Wm. T. H.
1924. Measuring water flow interference in certain gall and vascular diseases. Phytopathology 14: 580-584.

(9) ----.
1926. Crown-gall of apple nursery stock. Jour. Economic Ent. 19: 356-365.

(10) Moss, H.
1917. Field experiments with crown-gall. Tex. Agr. Exp. Sta. Bul. 211.

(11) Owens, C. E.
1928. Principles of plant pathology. John Wiley & Sons, Inc. pp. 182-197.

(12) Patel, M. K.
1929. Biological studies of Pseudomonas tumefaciens. Smith and Townsend. and 15 related non-pathogenic organisms. Bul. Series 21. Crop Protection Digest, Crop Protection Institute. Wash. D. C.

(13) Riker, A. J.
1923. Some relations of the crown-gall organism to its host tissue. Jour. Agr. Res. 25: 119-132.

(14) ----.
1923. Some morphological responses of the host tissue to the crown-gall organism. Jour. Agr. Res. 26: 425-435.

(15) ----. and Keitt, G. W.
1926. Studies of crown-gall and wound overgrowth on apple nursery stock. Phytopathology 16: 765-804.

(16) ----.
1926. Studies on the influence of some environmental factors on the development of crown-gall. Jour. Agr. Res. 32: 83-96.

(17) ----. and Muncie, J. H.
1926. Suggestions on the preparation of apple grafts. Bul. 9 Crop Protection Institute. Wash. D. C.

(18) ----.
1927. Cytological studies of crown-gall tissue. Amer. Jour. Bot. 14: 25-37.

(19) ----.
1928. Notes on the crown-gall situation in England, France and Holland. Phytopathology 18: 289-294.

(20) Robinson, W., and Walkden, H.
1923. A critical study of crown-gall. Ann. Bot. 37: 299-324.

(21) Siegler, E. A.
1928. Studies on the etiology of apple crown-gall. Jour. Agr. Res. 37: 301-313.

(22) ----. and Piper, R. B.
1929. Aerial crown-gall of the apple. Jour. Agr. Res. 39: 249-262.

(23) ----.
1929. The woolly knot type of crown-gall. Jour. Agr. Res. 39: 427-450.

(24) Smith, C. O.
1912. Further proof of the cause and infectiousness of crown-gall. Bul. 235 Cal. Agr. Exp. Sta. pp. 531-557.

(25) ----.
1917. Comparative resistance of prunes to crown-gall. Am. Nat. 51: 47-60.

(26) Smith, E. F. and Townsend, C. O.
1907. A plant tumor of bacterial origin. Science 25: 671-673.

(27) ----.
1909. The etiology of plant tumors. Science 30: 223.

(28) ----.
1911. Crown-gall and sarcoma. U. S. Dept. Plant Indus. Circ. 85.

(29) ----., Brown, N. A. and Townsend, C. O.
1911. Crown-gall of plants: its cause and remedy. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 213.

(30) ----.
1912. Etiology of crown-gall on sugar beets. Phytopathology 2: 270.

(31) ----., Brown, N. A. and McCulloch, L.
1912. The structure and development of crown-gall: a plant cancer. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 255.

(32) ----.
1916. Crown-gall studies showing change in plant structure due to a changed stimulus. (preliminary paper) Jour. Agr. Res. 6: 179-182.

(33) ----.
1916. Studies on the crown-gall of plants: its relation to human cancer. Jour. Cancer Res. 1: 231-258.

(34) ----.
1916. Tumors in plants. Science 44: 611-612.

(35) ----.
1917. Mechanism of tumor growth in crown-gall. Jour. Agr. Res. 8: 165-188.

(36) ----.
1920. An introduction to bacterial diseases of plants. W. B. Saunders & Co. pp. 413-472.

(37) ----.
1920. Production of tumors in absence of parasites. Reprinted from Archive of Dermatology and Syphilology 2: pp. 176-180.

(38) ----.
1921. Effect of crown-gall inoculations on Bryophyllum. Jour. Agr. Res. 21: 593-598.

(39) ----.
1922. Fasciations and prolepsis due to crown-gall. Jour. Agr. Res. 12: 265-270.

(40) ----.
1925. Cancers in plants and in man. Science 61: 419-420.

(41) ----., and Quirk, A. J.
1926. Begonia immune to crown-gall with observations on other immune or semi-immune plants. Phytopathology 16: 491-508.

(42) ----.
1927. Tumors, cysts, pith-bundles and floral proliferations in Helianthus. Nat. Acad. of Science. 22: 1-50. Wash. D. C.

(43) Swingle, D. B. and Morris, H. E.
1919. Crown-gall injury in the orchard. Montana Agr. Exp. Sta. Bul. 121.

(44) Townsend, C. O.
1915. Field studies of crown-gall of sugar beets. (prof. paper) U. S. Dept. Agr., Bur. Pl. Ind. Bul. 203.

(45) Von Schrenk, H. and Hedgcock, G. A.
1907. The wrapping of apple grafts and its relation to the crown-gall disease. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 100.

(46) Waite, M. B. and Siegler, E. A.
1926. A method for the control of crown-gall in the apple nursery. U. S. Dept. Circ. 376.

(47) Woodworth, C. W. and Wickson, E. J.
1892-4. Root knot on vines. Report of Cal. Agr. Exp. Sta. pp. 436-440.

Plate I

Fig. A. Galls on tomato stem five weeks after puncture inoculations with the *B. tumefaciens*. (Strain B)

Fig. B. Galls on tomato stem four months after puncture inoculations with *B. tumefaciens*. (Strain A)

Figures approximately natural size.

Plate I

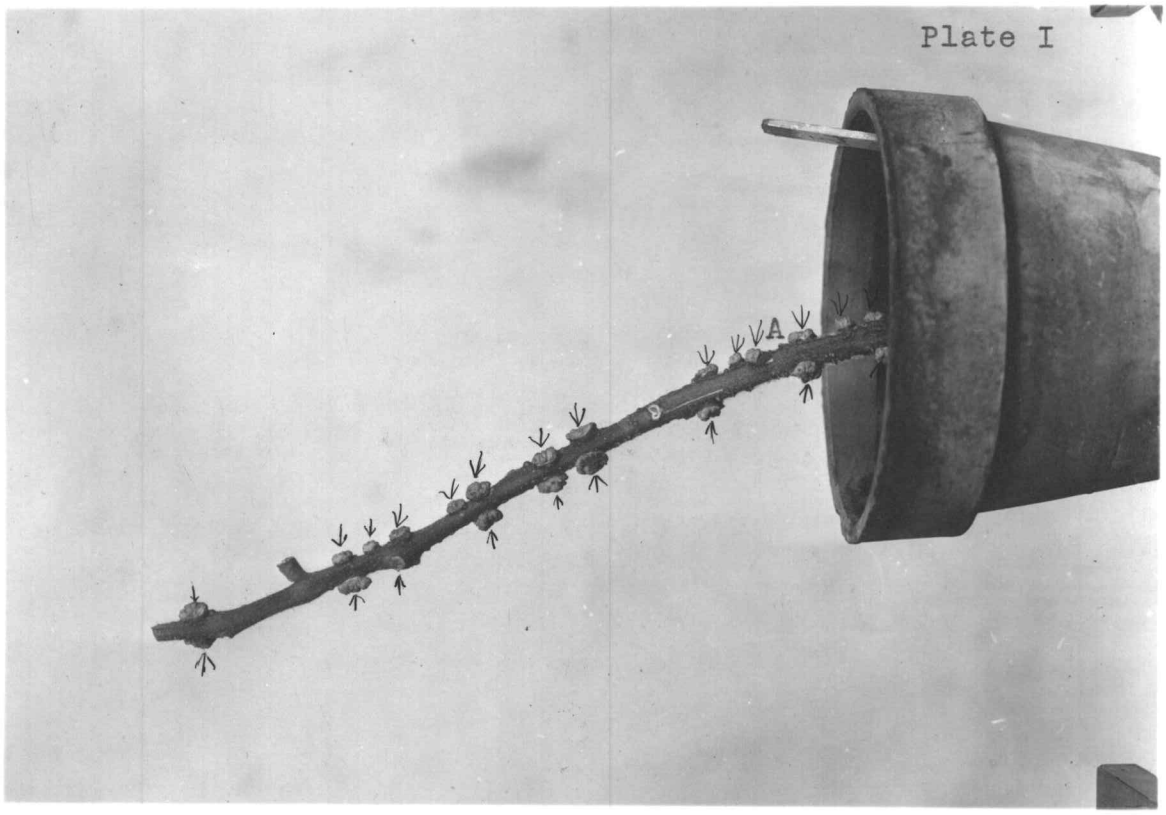


Plate II

Figs. A, C, E, G, I. Galls on plants four weeks after puncture inoculations with B. tumefaciens. (Strain A)

B, D, F, H, J. The respective checks for above figures, made with needle punctures.

A, B. Petioles of Geranium leaf.
C, D. Stems of wax bean.
E, F. Stems of castor bean.
G, H. Flower stalks of Geranium.
I, J. Stems of tobacco.

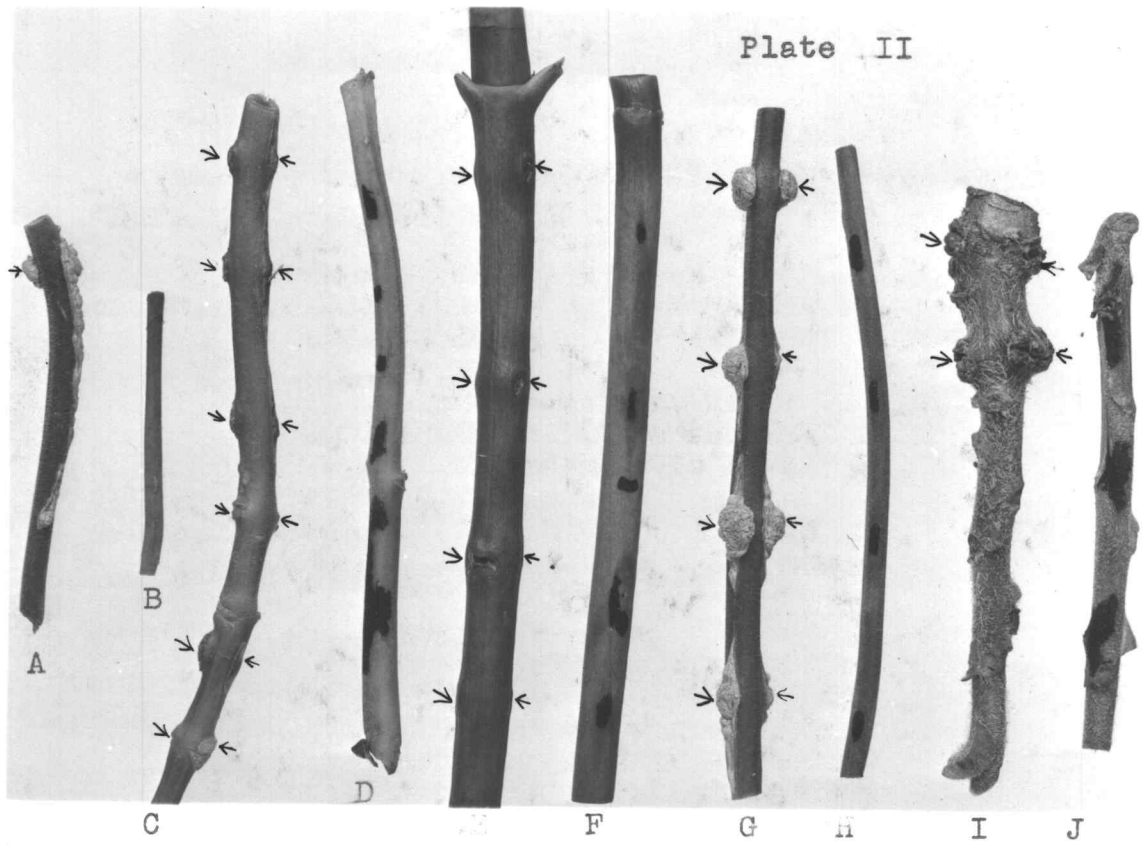
Figs. L, M, N, O, P. Galls on tomato plants ten weeks after inoculations with B. tumefaciens. (Strain A)

L. Inoculation on petiole with large, hot needle.
M. Inoculation by pin pricks on stem.
N. Inoculation by means of large needle. Epidermis of stem barely broken.
O. Inoculation on stem after piece had been sliced off with knife. Note the small galls along the edge of the wound.
P. Inoculation on stem made by hypodermic needle injections into wounds previously made with a hot needle.

Fig. K. Check. Punctures made on stem with a sterile needle.

Black places on stems and other parts of plant were made by using India ink to contrast wounds on check plants. Arrows indicate position of galls. All figures approximately natural size.

Plate II



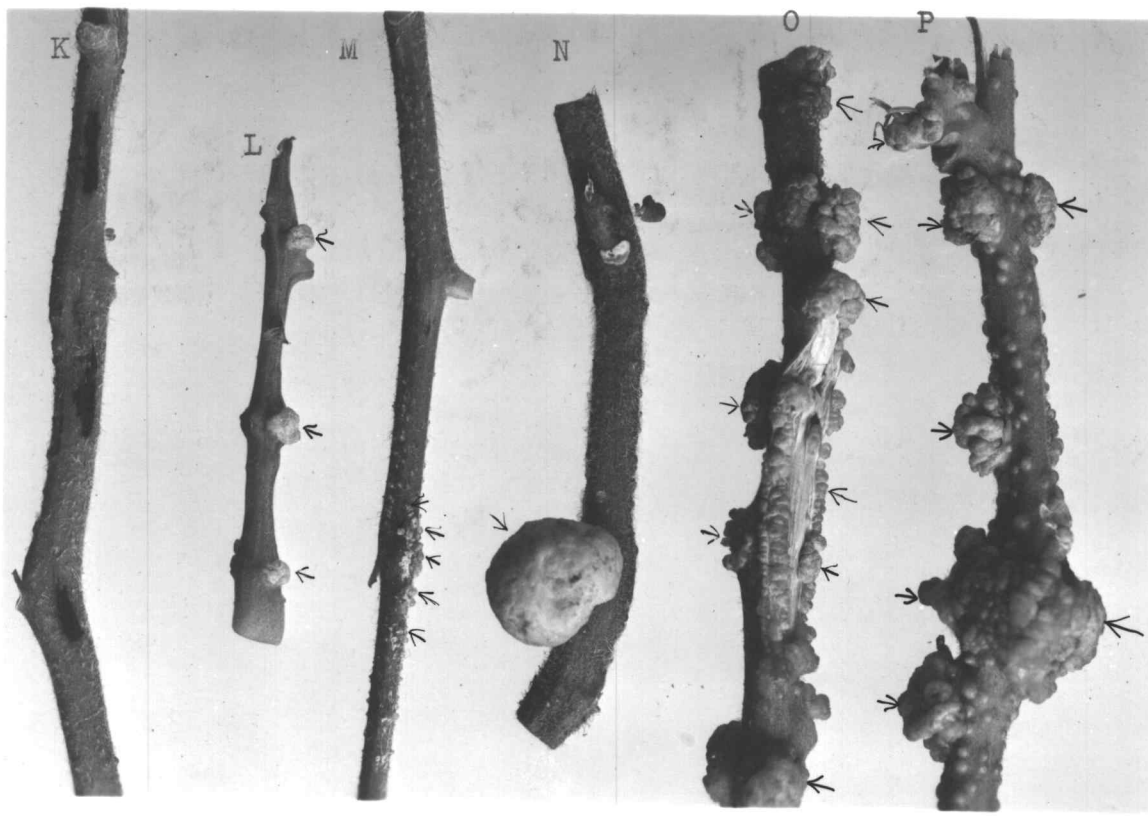


Plate III

Fig. A. Galls on Geranium stem ten weeks after puncture inoculations with B. tumefaciens. (Strain B)

B. Galls on Geranium stem ten weeks after puncture inoculations with B. tumefaciens. (Strain A)

C. Check. Wound punctures made with sterile needle at same time A and B were made.

Fig. D. Same as A. About twice natural size.

E. Same as B. About twice natural size.

Black places on stem of Fig. C were made by using India ink to contrast wounds on check plants.

Plate. III

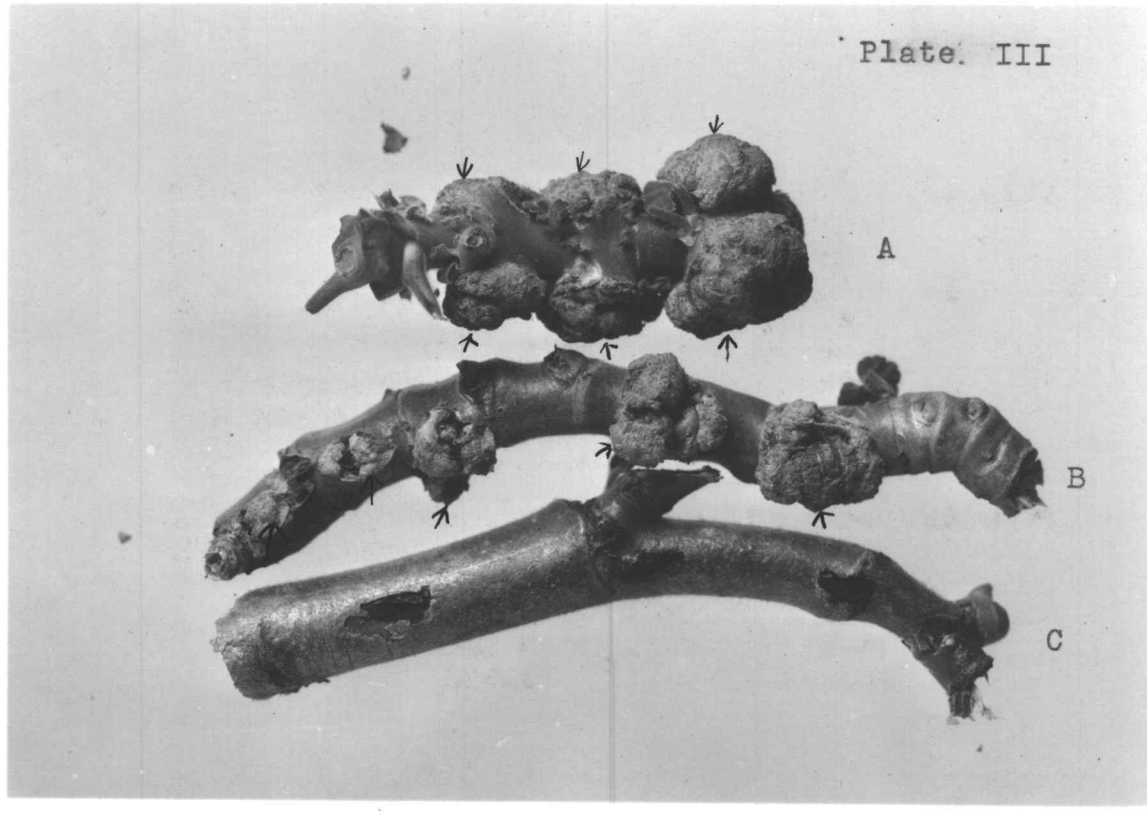


Plate IV

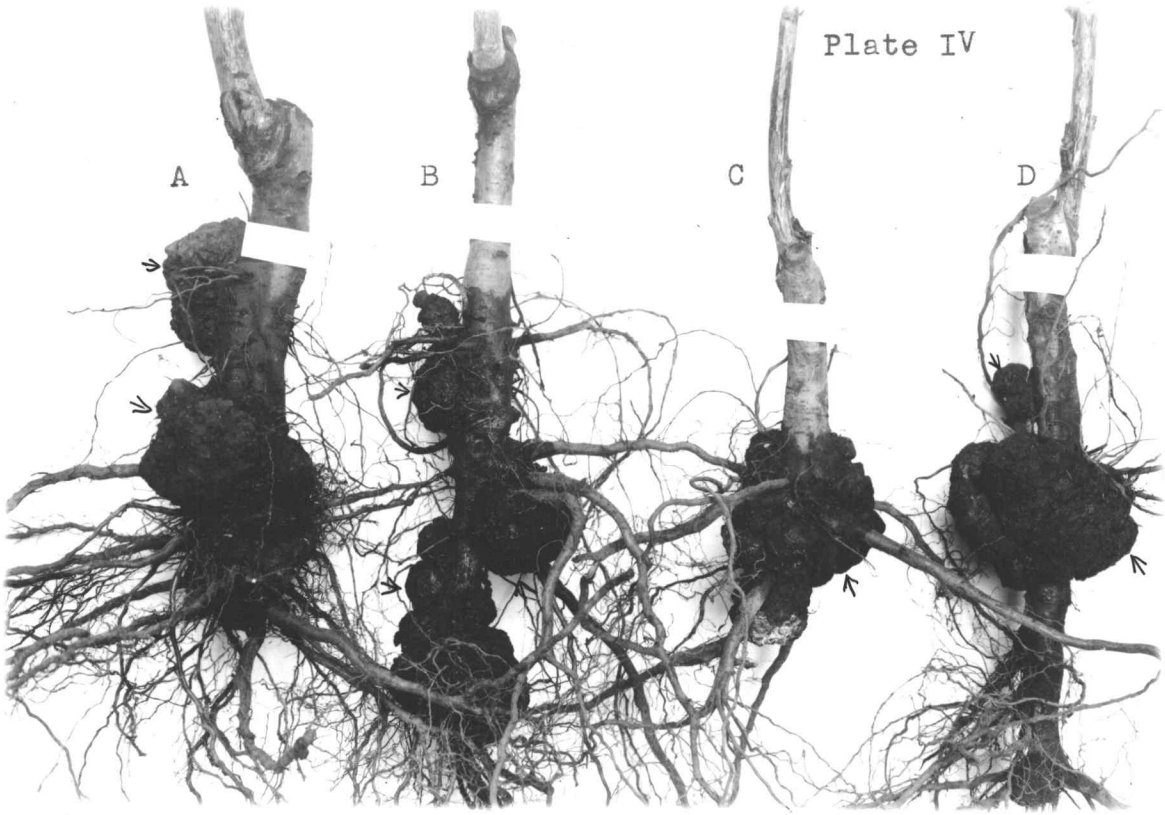
Figs. A, B, C, D. Specimens of crown gall on grafted cherry seedlings taken from a nursery. One-fifth normal size. Photo by H. P. Barss.

Fig. E. Natural occurring growths on Geranium leaf.

G. Check leaf. Scraped and punctured with sterile needle.

F, H, I, J. Galls on Geranium leaves four weeks after inoculations with B. tumefaciens. (Strain A) Inoculations made by scraping surface of leaf and puncturing with needle. Twice natural size. Arrows indicate positions of galls.

Plate IV



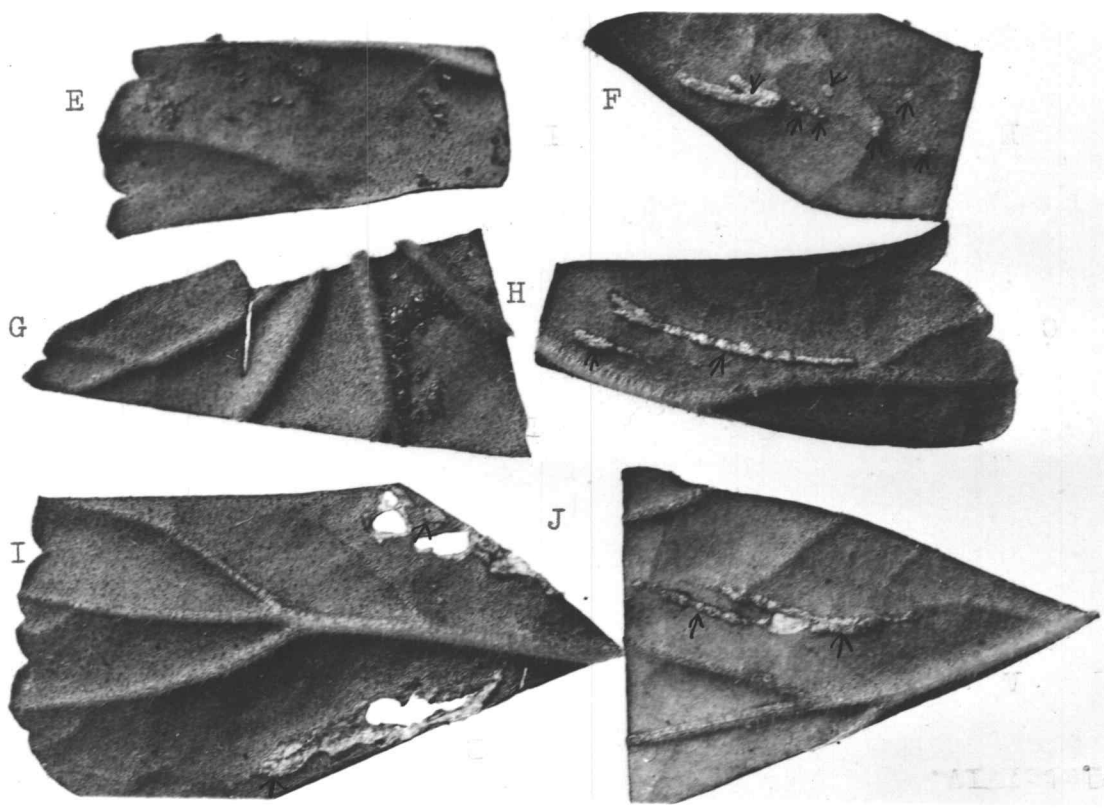


Plate V

Fig. A. Moist chamber described in experiment C on page 23.

Fig. B. Cooling chamber described in experiment C on page 25.

Plate V

A

B

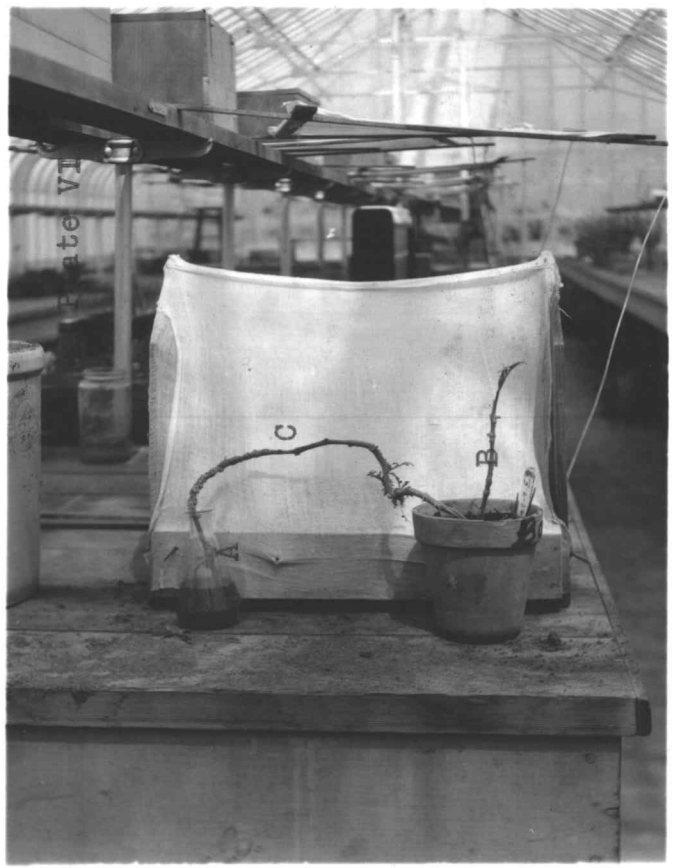
Plate VI

Fig. A. Set-up of experiment L on Bacterial Migration from Stem to Branch. page 70.

B. Perpendicular stem.

C. Horizontal stem.
Between A and C, points of inoculations on horizontal stem. About one-half natural size.

Fig. E. Crown gall bacteria about four days old on nutrient agar. The semi-transparent colonies are the crown gall bacteria, and the white ones are a contamination of the plate. Figure about natural size. Photo by H. B. Barss.


C
B
A