CULTURAL STUDIES

OF

BACTERIUM MALVACEARUM (E. F. S.) E. F. S.

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By

JO ANN PROVINE

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Oklahoma Agricultural and Mechanical College

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APPROVED:

W. Stan Chesher
In Charge of Thesis

W. Stan Chesher
Head of the Department

D. G. McIntosh
Dean of the Graduate School

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CULTURAL STUDIES OF BACTERIUM MALVACEARUM (E. F. S.) E. F. S.

I. Introduction.

Bacterium malvacearum is the causal agent of an important bacterial disease of cotton, known variously as angular leaf-spot, bacterial blight, black arm, bacterial boll-rot, and gummosis. Earlier studies on the physiology of this organism have been largely confined to its bacteriological description, and leave a number of obscure or controversial questions unanswered. In this study I have attempted to find answers to some of these questions: to determine whether the organism can be quickly and readily isolated from seed; whether the bacterium is the cause of a diseased condition in the hypocotyl, which is commonly observed, and if so, whether the hypocotyl test can be used for positive, rapid identification of B. malvacearum; to test the effect of various sugars upon the growth of the organism in connection with the dependence of infection upon photosynthetic activity of the cotton plant; to attempt to isolate the bacteriophage of B. malvacearum and prove its action; and to reach some conclusion as to why cotton is the only host susceptible to the pathogen.

II. Previous findings on the morphology and physiology of Bacterium malvacearum.

Bacterium malvacearum is a yellow, Gram-negative, non-acid-fast, rod-shaped schizomycete[1], motile by one polar flagellum. No endospores, granules, or capsules have been demonstrated.[2] However Stoughton reports that new morphological forms have been observed in this organism. According to him, coccoid bodies are produced, liberated, and germinate to form normal rods. He also describes the formation of densely-staining spherical bodies, apparently arising from the point of fusion of two cells. These bodies seem to be liberated by the degeneration of the parent cell.[3] When grown in gelatin or blood serum, the medium is slowly liquefied. Milk is curdled by a lab ferment. Starch is hydrolized, nitrates are not reduced, and indol is not produced. In litmus milk the reaction is alkaline and the casein is thrown down slowly.

On nutrient agar, surface colonies are pale yellow, round, thin, flat, smooth, and wet-shining. Early in their growth on nutrient agar the colonies are more or less radiate-mottled. The margin is thin and regular except in old colonies.

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1 E. F. Smith, Bacterial diseases of plants, pp. 321-322.

2 R. C. Faulwetter, Angular leaf spot of cotton, S. C. Exp. Sta. Bull. 198, p. 5.

3 R. H. Stoughton, Morphology and cytology of Bacterium malvacearum, E. F. S. Part II. Reproduction and cell-fusion, Ann. of Appl. Biol., 18: 532.

The optimum temperature for growth of this organism is about 27°C. Growth stops almost completely above 35° C.[4] Minimum growth temperature is about 10° C. The thermal death point is between 50° and 51° C.[5]

When exposed to strong sunlight on cover slips for fifteen minutes, a large percentage of the organisms are killed. When exposed for more than fifteen minutes, sunlight proves fatal.[6]

Under conditions of dessication, a few colonies have developed up to 38 days.[7]

Sometimes there are difficulties in the way of isolating this organism, owing to the occurrence on the cotton plant of yellow saprophytes which somewhat resemble it.[8]

III. Experimental.

A. Influence of sugars on the development of B. malvacearum.

When the variegated leaves of a cotton plant are inoculated with B. malvacearum, angular spots appear only in the part of the leaf containing chlorophyll, with the white portion showing minute spots where infection was initiated,

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4 R. H. Stoughton, The influence of environmental conditions on the development of angular leaf-spot of cotton III. The influence of air temperature on infection. Ann. of Appl. Biol. 18: p. 532.

5 Charlotte Elliott, Manual of bacterial plant pathogens, p. 153.

6 C. W. Edgerton, The rots of the cotton boll. La. Agri. Exp. Sta. Bull. 137, p. 13.

7 C. W. Edgerton, Ibid. p. 14.

8 E. F. Smith, op. cit., p. 330.

but failed to continue. In an effort to link this with the photosynthetic products of the plant, the pathogen was grown in media containing six carbohydrates.

The culture of B. malvacearum used in this study was isolated from angular spots on the leaves of a mature cotton plant. Growth of this organism was observed in a 1% solution of each of the following sugars: dextrose, lactose, sucrose, dextrin, maltose, and mannite. Meat extract was added to ten tubes of each of the sugars and ten tubes were left free of the extract. The media were sterilized in free steam for one hour for three successive days, rather than under pressure, in order to minimize hydrolysis of the sugars. Growth was observed over a period of 10 days. During this time no growth took place in the six groups lacking the meat extract. In the series containing the extract, growth was most abundant in dextrose, less so in sucrose, with only slight growth in dextrin, mannite, lactose, and maltose.

Another series was prepared in which only sucrose and dextrose were used. The media contained meat extract, and the sugars in 1/3% concentrations. Growth was recorded for ten days. At the end of that time the sucrose nutrient broth showed very abundant growth. Although the dextrose nutrient broth showed good growth, it did not equal the growth in the sucrose. Uninoculated controls in both of the above experiments showed no growth.

Miller states that sucrose is the most abundant and the most widely distributed sugar in green plants, and in the variegated leaf, hexoses are, in most cases, also found in the green portion, while sucrose is found principally in the yellow portion.[9] This suggests that the pathogen is able to produce normal infection in the green portion of the variegated cotton leaf because of the presence of both dextrose and sucrose which support its growth, while in the yellow portion, infection is only initiated because of the presence of only one of the supporting sugars, sucrose.

B. Attempts at isolation of B. malvacearum from hypocotyls of infected seedlings.

All workers with bacterial blight agree that primary infection of the cotton plant arises from the attack of B. malvacearum present in or on the cotton seed. Thirteen trays of fuzzy cotton seed were germinated in an attempt to quickly isolate the organism from infected hypocotyls. A large percentage of the germinating seeds showed a water-soaked condition of the hypocotyl. Yellow bacteria were obtained in pure culture from this region, and two methods of distinguishing the pathogen from other yellow bacteria were used. Sterile seedlings grown in water agar were inoculated and potato cylinders were streaked. Since the minimum time for infection to appear in seedlings is ten days, the

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9 E. C. Miller, Plant physiology, pp. 410 and 440.

latter method was used in most cases. Smith states that B. malvacearum gives a copious, pale-yellow growth on potato and that this may be used as a means of separating that organism from other yellow organisms.[10] None of the cotton plants showed infection and no copious, yellow growth developed on the potato cylinders. The abundance of the two bacteria always found in conjunction with B. malvacearum probably accounts for the difficulty met with in isolating the latter organism.[11]

C. Attempts to isolate the bacteriophage of B. malvacearum.

Matsumoto and Huzioka have reported the isolation of a bacteriophage from diseased cotton plants and its action in dissolving bacterial cells of B. malvacearum in a liquid medium.[12] During the isolation and culturing of the pathogen from diseased cotton leaves, one strain consistently produced "moth-eaten" or "windowed" colonies, terms often used in describing colonies containing bacteriophage. (See Plate II.) Complete dissolution of a colony was never observed. After repeatedly pouring plates from this strain two distinct types of colonies were obtained, one of the "moth-eaten" type and one of the homogeneous type. Twenty-five tubes of sterile nutrient broth were inoculated

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10 E. F. Smith, op cit., p. 331.

11 C. W. Edgerton, op cit., p. 12.

12 T. Matsumoto and Y. Huzioka, Bacteriophage in relation to Bacterium malvacearum E. F. S. 1. Preliminary study, Ann. of Phytopath. Soc. of Japan VII, 3.4: 194.

with organisms of the homogeneous type. Five tubes of sterile nutrient broth were inoculated with organisms of the "moth-eaten" type. Three days later, the bacterial culture containing the latter type, was filtered through a Seitz bacterial filter. The filtrate was diluted with sterile broth, in a logarithmic dilution series from 1/1 to 1/2048. One cubic centimeter of each dilution was added to a tube of the bacterial growth of the homogeneous type. Thirteen tubes were left as checks.

Three days later, no lysis in the bacterial cultures had apparently taken place. This does not necessarily indicate the absence of bacteriophage, instead, the bacteriophage may have been present, but the technique may have been inadequate for demonstrating bacteriophage in B. malvacearum or its virulence may have needed to be built up by further culturing.

D. Infection Tests.

The black arm phase, the leaf phase, and the boll phase of bacterial blight have been studied thoroughly. The present study was concerned with the development of the root system of infected cotton seedlings.

Sterile cotton seedlings which were grown in water agar in test tubes were inoculated with a virulent strain of B. malvacearum soon after the cotyledon leaves had unfolded. Plugs were left in the tubes for 48 hours to maintain high humidity. First infections appeared in about ten days in

the form of water-soaked spots on the cotyledon leaves. Continued observation of the inoculated seedlings showed in most cases less extensive root systems with fewer secondary roots than the check plants. Difference between the root systems of inoculated and check plants is shown in Plate I. The plants were observed for a month and during that time the water agar became dehydrated and the plants died due to lack of moisture before lesions were observed on the hypocotyl. Attempts were made to add sterile water to the tubes, but these failed to prolong the life of the seedlings.

Delinted cotton seed which had been surface sterilized, was planted in sterile soil in flats. One flat was left as a control. Another flat was inoculated soon after the cotyledon leaves had unfolded. A burlap hood was placed over both flats for 48 hours following inoculation to insure high humidity. First infections appeared in nine days. The plants were watered regularly with tap water at 24 hour intervals, but on the eighth day the plants were not watered until 36 hours had elapsed. At this time the inoculated plants were noticeably temporarily wilted evidently because of their reduced root system. The check plants showed no signs of wilting.

Ten days after inoculation, the soil was removed from several plants, both of the inoculated and the checks. The

root systems of both groups were examined with a low-power magnification. Both groups showed a slight infection of Rhizoctonia sp. but this infection was not severe enough to be fatal to the seedlings. The inoculated plants also had a second type of lesion on the primary root. These lesions were characterized by a slight yellow discoloration and a constricted, water-soaked region on the tap root with this condition extending into any branch roots arising from that region. The lesion was of the type one would expect to be caused by this pathogen, but from the plates poured, using the infected tissue as the inoculum, no colonies typical of B. malvacearum were produced.

E. Growth of B. malvacearum in media containing cotton and hollyhock extracts.

The only hosts listed for B. malvacearum are Gossypium sp. and Thurberia thespesioides.[13] Cotton (Gossypium hirsutum) and hollyhock (Althea rosea) are both members of the Malvaceae family and in an effort to determine why hollyhock is resistant to this pathogen, four extractions were made from the leaf tissue of these two plants.

The leaf tissue of both plants was macerated and divided into approximately four equal parts, and parallel extractions made on the four parts. The solvents used were absolute alcohol, acetone, hot water and cold water. These extrac-

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13 C. Elliott, op cit., p. 154.

tions were filtered, and set aside in order that the solvent could evaporate. When evaporated to dryness the residue was resuspended in a physiological salt solution (.85%), and 1 cubic centimeter of each suspension was added to three tubes of nutrient broth. This was then sterilized in the autoclave in free steam, rather than under pressure to minimize hydrolysis of the dextrose in the broth. Four days following inoculation, a slight turbidity was observed in the culture containing the acetone extract of the cotton leaf but not in that of the hollyhock. Microscopic inspection showed that this turbidity was due to bacterial growth. This appears to indicate that the resistance of hollyhock to B. malvacearum is due in part to an acetone soluble substance possibly lipoidal in nature.

Conclusions

B. malvacearum is constantly found associated with chlorophyll-bearing parenchyma, never being isolated from roots or hypocotyls, or non-chlorophyll bearing parts. According to literature, the green parts contain sucrose, and in some cases dextrose. This organism grown in dextrose and sucrose containing media, and is able to infect when these sugars are present. It grows poorly or not at all in media containing other sugars. Therefore infection appears to be dependent on the photosynthetic activity directly by way of the dextrose.

Work with extracts appears to indicate the resistance of the hollyhock to B. malvacearum is due in part to a chemical factor that is acetone soluble, non-water soluble.

With the inability to use the hypocotyl infections for rapid determination of B. malvacearum, the most effective method at present is the inoculation of sterile cotton seedlings on agar, and infection appears as water-soaked spots on the cotyledon leaves, in about ten days.

Summary

1. Bacterium malvacearum is the causal agent of an important bacterial disease of cotton, known variously as angular leaf-spot, bacterial blight, black arm, bacterial boll-rot, and gummosis.

2. Previous findings on the morphology and physiology of the organism was given.

3. Tests on various sugars show that B. malvacearum grows best in media containing dextrose and sucrose.

4. Isolation of B. malvacearum was never made from chlorophyll-free parts of the plant (hypocotyls or roots), and its presence in only the chlorophyll-bearing parts of the plant indicates that infection is dependent upon the photosynthetic activity of the plant.

5. In attempting to prove the action of the bacteriophage of B. malvacearum from "moth-eaten" colonies, no apparent lysis of bacterial cultures had taken place at the end of three days. This does not necessarily indicate the

absence of the bacteriophage, instead, the bacteriophage may have been present, but that the technique may have been inadequate for demonstrating bacteriophage in B. malvacearum or its virulence may have needed to be built up by further culturing.

6. In the cotton and hollyhock extractions, increased bacterial growth appeared only in the acetone fraction of the cotton extract. This appears to indicate that the absence of an acetone soluble factor or the presence of an acetone-soluble inhibitor in the hollyhock is responsible for the resistance of that plant to B. malvacearum.

Literature Cited

1912 Edgerton, C. W. The rots of the cotton boll. La. Argi. Exp. Sta. Bull. 137.

1919 Faulwetter, R. C. Angular leaf spot of cotton. So. Car. Exp. Sta. Bull. 198.

1920 Smith, E. F. Bacterial diseases of plants. W. B. Saunders Co., Philadelphia, Pa.

1930 Elliott, C. Manual of bacterial plant pathogens. The Williams and Wilkins Company, Baltimore, Md.

1931 Miller, C. E. Plant physiology. McGraw-Hill Book Co., New York.

1931 Stoughton, R. H. The influence of environmental conditions on the development of angular leaf-spot of cotton. III. The influence of air temperature on infection. Ann. of Appl. Biol. 18: 524-533.

1932 Stoughton, R. H. The Morphology and cytology of Bacterium malvacearum, E. F. S. Part II. Reproduction and cell-fusion. Pro. Royal Soc., B, 3: 46-52.

1938 Matsumoto, T. and Y. Huzioka. Bacteriophage in relation to Bacterium malvacearum E. F. S. 1. Preliminary study. Ann. of Phytopath. Soc. of Japan VII, 3.4: 193-202.

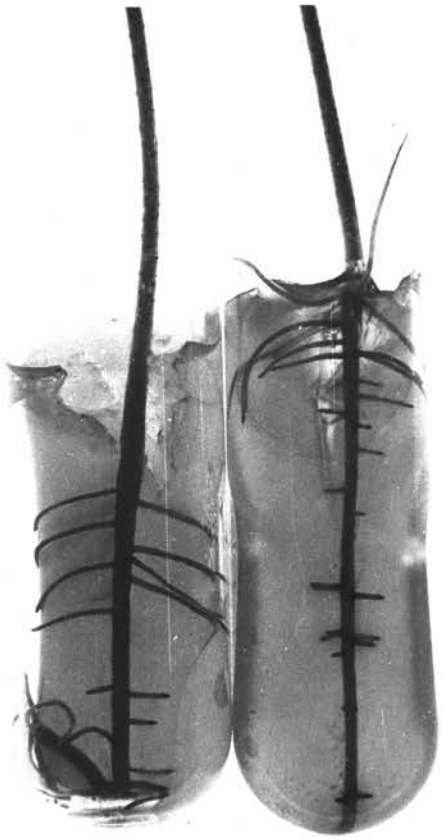

Plate I. Left: Uninoculated plant
Right: Plant inoculated with
B. malvacearum showing secondary
inhibition of root development.

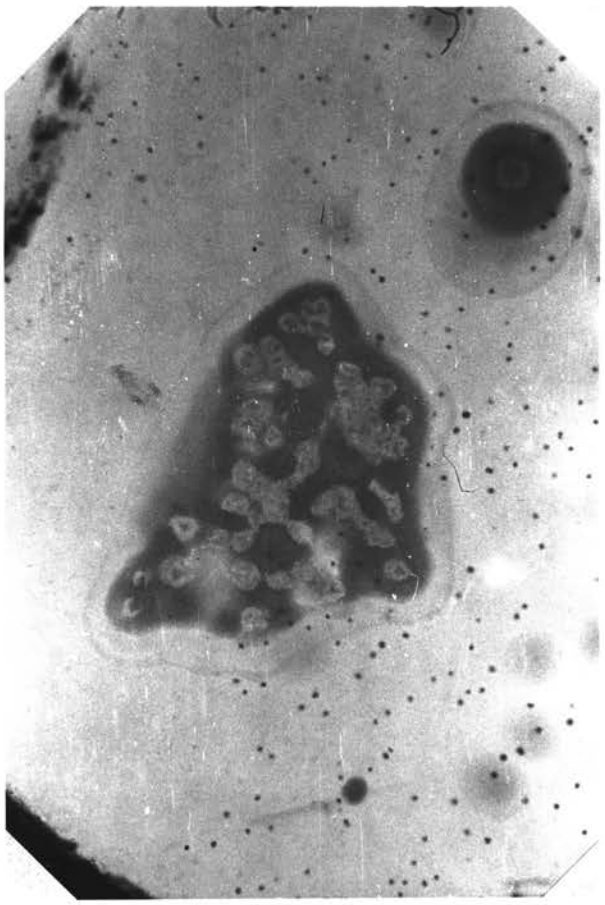

Plate II. "Moth-eaten" colony of
B. malvacearum.