MUTATION IN *XANTHOMONAS MALVACEARUM* AS RELATED TO STABLE PLANT DISEASE CONTROL

By

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MUTATION IN XANTHOMONAS MALVACEARUM AS RELATED TO STABLE PLANT DISEASE CONTROL

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CHAPTER I

INTRODUCTION

Bacterial blight of cotton is found throughout the Cotton Belt of the United States and in other areas of the world where cotton is grown. The causal organism, Xanthomonas malvacearum (E.F. Smith)Dowson, is a bacterium capable of infecting all above ground parts of the cotton plant. The source of inoculum for initial infection may come from overwintering bacteria on the surface of the seed, from within the seed coat, or on residue left from the previous crop. Dissemination of the pathogen is primarily due to wind driven rain and sprinkler irrigation.

Disease symptoms appear on the leaves as water-soaked angular lesions which later turn brown or black when dry. Boll infection results in a round water-soaked sunken lesion which in time turns black. Stems and petioles may become black after infection in highly susceptible varieties. Because of the various symptoms, the disease is often referred to as "angular leaf spot," "boll rot," or "blackarm."

Destruction of photosynthetic area and partial defoliation cause a lowering of yield, but probably the greatest economic loss is from boll infection where the bacterial slime may stain the fiber thus reducing its grade. The boll lesion may also provide a port of entry for secondary invasion by other microorganisms. Severe overall infection of the plant may hasten maturity but seldom results in death.

Control has been achieved in some parts of the world through programs of quarantine and sanitation. Seed treatment has also been

employed, but the most recent emphasis has been on development of resistant varieties.

Unfortunately, almost as soon as resistant varieties were released and grown on a large scale under conditions that favored the disease, new virulence was recognized in the pathogen population and resistance was lost. Probably the best explanation for this phenomenon is that mutation in the pathogen population brings about a multiplicity of variants for virulence. Natural selection in the bacterial population while in the host medium favors the variant that has the best chance for survival, i.e., virulence on the host. If a virulent bacterium is thus selected, a compatible host-pathogen relationship is established and pathogenesis ensues.

Genotypes of host plants most vulnerable to this phenomenon are those whose level of resistance is conditioned by what Van der Plank (54) has labeled "oligogenes." These are genes of major effect that confer a certain level of "specific" resistance. Early resistant varieties relied heavily on this source of resistance. Another source of resistance that has more recently been given emphasis is the "non-specific" resistance conferred by genes of minor effect (Van der Plank's polygenes). A contrast in definition between specific and non-specific resistance is that specific resistance is effective only against certain strains of the pathogen, whereas non-specific resistance reacts the same against all strains.

Within the past ten to 15 years, immunity to bacterial blight has been gained by combining these two kinds of resistance in a single host genotype. This resistance has been stable and extremely effective. If the basic mechanism for this combined resistance were known, more stable

and higher levels of resistance might be obtained for other diseases of cotton, and also other crops and diseases.

The objective of this study was to gather data to help characterize stable plant disease resistance and to develop antibiotic resistant cultures of X. malvacearum for further investigations. Experimentation reported here-in was performed during the summer and fall of 1974.

CHAPTER II

LITERATURE REVIEW

Currently there are four species of Gossypium being used in cotton production around the world. G. hirsutum L., a New World tetraploid, accounts for the vast majority of all cotton grown, especially in the United States. G. barbadense L., G. arboreum L., and G. herbaceum L. are grown to a lesser extent (25). G. barbadense is generally highly susceptible to bacterial blight (5); the other three species vary with susceptibility and different degrees of tolerance and resistance.

R.L. Knight is probably the foremost pioneer in the research done in developing genetic resistance to bacterial blight in cotton. Beginning in 1935, Knight and Clouston (36,37) and Knight (31,32,33,35) surveyed cultivated and wild species of cotton in the Sudan. Knight and Hutchinson (38) between 1944 and 1948 extended the previous survey to include a world collection. Knight (34) postulated that blight resistance was due to one or a few genes of large effect based on evidence of resistance found in species originally grown where the disease was absent. He designated these genes of large effect as "B" genes, each of which was large enough in effect that its segregation could be followed in his inheritance studies (34). He also recognized genes of lesser effect, usually associated with each source of resistance, whose segregation could not be followed. These genes were designated as minor genes. Using Van der Plank's definitions (54), the "B" genes would be known as

oligogenes and the minor genes as polygenes. To date, 16 oligogenes have been described that confer blight resistance (6).

Although Knight worked with the G. barbadense genetic background in his inheritance studies, most of Knight's oligogenes have been transferred by Hunter and Brinkerhoff (23) from G. barbadense to the susceptible upland cultivar Acala 44 (G. hirsutum).

Burkholder and Starr (11) have described the genus Xanthomonas as being comprised of plant pathogens that produce abundant slimy, yellow growth on sugar-containing media and as being aerobic, gram negative, non-spore forming rods with a single polar flagellum. X. malvacearum is closely related to other plant pathogenic xanthomonads (4,11,56), but is taxonomically distinct on the basis of its pathogenicity on cotton (17,42,56), and on the basis of enzyme patterns determined by acrylamide gel disc electrophoresis (18). Dye (16) in 1958 reported that beans were attacked by X. malvacearum and a number of other xanthomonads and concluded that host specificity was not a valid character in differentiating species of Xanthomonas. Logan (42) was unable to corroborate Dye's findings in 1960 when he was unable to "adapt" X. malvacearum to bean or to break the blight resistance of a current resistant variety. Schnathorst (46) too, took exception to Dye's report, however, later evidence (15,48) has shown X. malvacearum and X. phaseoli (E.F. Smith)Dowson, a bacterial pathogen of beans, more closely related than other xanthomonads studied. Taxonomic descriptions of X. malvacearum at levels below species have been a problem for workers since the mid-1940's, although other xanthomonads were known to be variable in pathogenicity as early as 1919 (3). In the period 1947-1949 when here-to-fore resistant varieties were attacked by X. malvacearum, it was postulated that different races of the

pathogen were responsible (2). Since then it has become a well established fact that X. malvacearum exists with a wide range of virulence (5,6,22).

In 1968, Hunter et al. (24) developed a set of upland cotton differential varieties for use in designating races of the pathogen based on the different disease reactions. To date, 17 races have been identified. Work done by Arnold and Brown (1) in Uganda led them to conclude that the continuous variation in virulence of their isolates did not lend itself well to race designation, and that an attempt to define races would be of little value. However, Brinkerhoff (6) has suggested that the variation in virulence was possibly just a reflection of similar variation in the resistance of the hosts on which their isolates had evolved and stated that the chief value of designation of races lies in its use as a tool in screening for more stable resistance in the host.

Historically, blight resistant cultivars with single oligogene resistance have been readily attacked. The predominant race on susceptible cultivars in the United States is race 1 (5). In New Mexico and West Texas where blight resistant cultivars were first grown, Hunter and Blank (22) in 1954 reported that resistance had been lost and a new race was present. Work done by Chew et al. (12) published in 1969 showed that during the time Acalas with single gene resistance to race 1 were grown, 40-60% of all isolates were race 2 (virulent for the race 1 resistant gene). Subsequently, the percentage of race 2 isolates dropped with the introduction of a race 2 resistant cultivar. Similar reports have come from Missouri (43), India (2), Africa (45), and Australia (19).

Mutation is the most likely mechanism producing the differences in virulence in X. malvacearum; however, genetic recombination might produce similar changes (5). Recombination mechanisms have been described

as transformation (21), conjugation (40), and transduction (58). Most of the work on recombination has been done with non-plant pathogens, but the findings will probably hold true for all bacterial species. Present information on the extent of the role played by recombination in genetic variability of X. malvacearum is lacking; however, Klein and Klein (28, 29) transformed Agrobacterium tumefaciens (E.F. Sm. & Town.)Conn. for pathogenicity, and Corey and Starr (13,14) transformed X. phaseoli, a close relative of X. malvacearum, for different colony types and for streptomycin resistance.

X. malvacearum also has been found to be variable in characters other than virulence. Brinkerhoff (5) found variants in nutrient agar colonies ranging from different type of growth (watery vs. viscid), pigmentation (mottled vs. normal), type of margins (irregular vs. smooth), and size and shape (large, appressed vs. normal, raised). He additionally found cultures resistant to the antibiotics streptomycin sulfate, penicillin G, and oxytetracycline.

Resistance to the antibiotics was readily obtained by screening large populations of X. malvacearum on an agar medium containing the antibiotic. Brinkerhoff (5) found two types of resistance common: One type originated as multiple-step resistance and was unstable on host passage or when the organism was cultured on media without the antibiotic. The other type was thought to be a single-step resistance that was stable on host passage or when the organism was grown in the absence of the antibiotic.

There are several reports (5,9,47,49) that virulent races buildup in resistant host tissue. Brinkerhoff (5) found that increased virulence in X. malvacearum for Knight's oligogenes followed a pattern similar to

that observed for single-step mutations for resistance to antibiotics. After inoculation of a resistant host, resistant type lesions appeared. Then, after 10 days to several weeks, a few susceptible type lesions would appear. Isolates from these lesions were usually fully virulent for the host from which they were taken. Parallel inoculation with the parent culture produced the same resistant type reaction as it did the first time. He found cultivars with single oligogene resistance subject to this phenomenon.

In some instances there appears to be a definite sequence in the buildup of virulence in the pathogen somewhat analogous to the buildup of the presumed multi-step resistance to antibiotics. In work done by Brinkerhoff and Hunter (8) and Brinkerhoff (unpublished data), avirulent cultures derived from a single cell produced mutants for virulence on the susceptible Acala 44 variety. Acala 44 strains with Knight's oligogenes were not attacked. But, once virulence for the susceptible host was obtained, mutants for virulence for hosts with the different oligogenes were isolated. Furthermore, after this level of virulence was reached by the pathogen, higher levels of virulence was identified from single colony cultures from hosts with single oligogenes in a "tolerant" genotype, indicating virulence in X. malvacearum is enhanced by mutations for virulence on host polygenes (6). Brinkerhoff recovered only the parent race and avirulent isolates from immune plants. Cultures virulent for more than one host resistance gene were prone to revert to less virulent forms on synthetic media and under field environmental conditions that disfavored the pathogen. These cultures tended to revert to race 1 (back mutation) and generally survive only in the fully susceptible Acala 44.

Host-parasite compatibility probably follows a system much like that proposed by Flor (20): For each gene conferring resistance in the host, there is a gene conditioning virulence in the parasite. The disease reaction results from the interaction of the host gene system with the parasite gene system as affected by the environment upon each system and upon the interaction between these systems.

Stability of resistance may be affected by environmental factors. Water-soaking of host tissue by wind driven rain or sprinkler irrigation and mechanical injury with rain and high humidity greatly enhance disease severity (5,55). These conditions probably result in greater dissemination, more efficient inoculation, and render the host physiologically more susceptible to invasion. These conditions have been shown to promote the occurrence of new races of the pathogen (5).

High air temperatures (35-36 C) with and without high relative humidity increase disease severity in susceptible G. barbadense lines (50,51,52). Brinkerhoff and Presley (10) found disease reaction was greater in susceptible and resistant upland cotton under conditions of low night temperatures (20 C and below) and moderate to high day temperatures. Resistance conferred by single oligogenes either in a susceptible or tolerant background was ineffective under low night and high day temperature regimes (i.e. 20 C or below and 36 C or above, respectively). Temperature had no noticeable effect on immunity in immune types and blight reactions were less severe with high night and high day temperatures.

Arnold and Brown (1) estimated 44-83% of the variation in host-pathogen interaction in Uganda was due to differences in average temperature. They also reported differences in disease expression associated

with variation in solar radiation and shading prior to or after inoculation.

There are many reports (10,39,41,55,57) of variance in disease reaction with the age and kind of host tissue. In general, young succulent tissue and bolls are most susceptible. Bolls seem to remain susceptible longer than stems or leaves. Leaf, stem, and boll reactions frequently do not appear to be correlated, especially for different host genes and different races of the pathogen (27).

Stability of resistance is probably closely related to the ability of the pathogen to multiply while inside the host. Perry (44) has shown population levels increase much slower in resistant cotton as opposed to susceptible. Brinkerhoff (unpublished data) has had similar results and found the overall buildup of the pathogen was greatly reduced in immune tissue.

Variation in inoculum concentration too may vary the disease reaction (5,27,39,55). Induction of the macroscopic hypersensitive response (HR) has been shown to be directly related to the relative number of pathogen cells introduced into the host tissue (30). Brinkerhoff et al. (7) reported X. malvacearum inoculum levels of 3 X 10^5 cells per milliliter were unable to cause macroscopic symptoms of hypersinsitive cell death in immune cotton, but microscopic examination showed the HR was occurring at the cellular level though. They also found the bacterial population increased 30-300 fold in the 4-8 days following the interval of plant cell death and the cells may remain viable for as long as 48 days. Inoculum levels of 10^8 cells/ml produced a confluent HR in immune cotton (2 genes plus modifiers?) in 18-48 hours, but was somewhat slower in resistant cotton (1 gene). Work reported by

Turner and Novacky (53) with tobacco showed similar results.

Other sources of variation in disease reaction are: method of inoculation (26), plant spacing (10), available soil moisture (57), and host nutrition (57).

CHAPTER III

HOST SPECIFICITY IN X. MALVACEARUM

Materials and Methods

Plants used in these experiments were started from seed planted in the Cotton Disease Nursery at the Plant Pathology Farm, Stillwater, Oklahoma on May 14, 1974. (See Table 1 for list of host lines.) The source of the seed was from self-pollinated plants grown in the nursery the previous season. All seed was acid delinted and treated with a 1:1 mixture of Arasan and Captan fungicide just prior to planting. Planting was done in rows approximately 25 feet long with 3 feet spacing between rows. The entire population was surrounded with other cotton, grain sorghum, and sweet corn as an environmental buffer zone. After emergence, rows were thinned to one plant per foot.

On July 3, when individual plants had four to eight true leaves, the whole test population was inoculated with X. malvacearum race 1. Inoculum was derived from a single colony isolated from naturally infected cotton leaf tissue collected at the Oklahoma Agricultural Experiment Station, Tipton, Oklahoma during the 1973 season. The leaf tissue was air dried and stored at 10 C. Isolation of the culture was made by a manner similar to the technique described below. Identification of the culture was made by L.A. Brinkerhoff, Plant Pathologist, Oklahoma State University and Langston University, using standard differentials (24).

TABLE I

UPLAND COTTON ACALA 44 TYPE HOST LINES WITH CORRESPONDING BLIGHT RESISTANCE GENES

Host Lines	Blight Resistance Genes
1. Acala 44	None
2. Acala 121	b_7
3. Acala 161	B_N
4. Acala 13	B_2
5. Acala B_2	B_2
6. Acala B_3	B_3
7. Acala B_4	B_4
8. Acala B_5	B_5
9. Immune 216	2 or 3 oligogenes + unknown modifiers?
10. $F_6$2B	unknown
11. $F_6$4B	unknown

Lines 2-8 were obtained by crossing a parent containing the resistant gene with Acala 44, followed by four or more backcrosses to Acala 44. Immune 216 was derived from 101-102B, which in turn was derived from G. barbadense with multiple backcrosses to the G. hirsutum cultivar "Empire." Lines 10 and 11 are resistant inbred Acala 44 types selected from the cross Immune 216 X Acala 44.

Isolation Technique

Two 6 mm diameter leaf discs were punched from previously inoculated host tissue, placed in a tea strainer, and washed 60 seconds under a stream of tap water to remove as much surface contamination as possible. The discs were then placed in 3-4 ml of sterile water and macerated with a mortar and pestle. One ml of the supernatant was then serially diluted to 10^{-9} original concentration with one ml sterile pipettes in 9 ml sterile water in perfume bottles with plastic caps. One-half ml each of the dilutions from 10^{-3} through 10^{-9} were spread over the surface of Difco Bacto Nutrient Agar in petri dishes with aluminum covers containing absorbant paper discs. The plates were then put in plastic bags and stored at room temperature (approx. 24 C). The paper discs prevented condensation and subsequent smearing of the surface, while storage of the plates in plastic bags prevented excessive drying and was a safeguard against invasion by "culture mites." A duplicate series of plates was made in each isolation to render a sufficient surface area for single colonies to grow without coalescing and to allow for contamination if it occurred.

Preparation of Inoculum

Five days after the petri dishes had been inoculated, single colonies were picked from the agar surface with a sterile wire hook and transferred to perfume bottles containing 9 ml Difco Bacto Nutrient Broth. The cultures were then incubated overnight in a Eberbach mechanical shaker at room temperature and taken to the field for inoculation the next morning. The inoculum used in the original field inoculation

was diluted to ca. 10^6 cells/ml. Inoculum used subsequent to this was full strength, average number of cells/ml ca. 10^8-10^9 by plate count method.

Inoculation Technique

The first inoculation of the total test population was made with a spray gun operated at about 300 p.s.i. driven by a tractor power-take-off. Plants were blown to the ground by the spray stream and the leaves partially infiltrated to give a water-soaked appearance. Care was taken to produce as little mechanical damage to the plant tissue as possible. The adjustment of the spray stream was more critical for successful water-soaking than was the pressure; a coarse spray water-soaked more readily when the stomata were open.

Succeeding inoculations were made by hand employing Monoject disposable plastic syringes without needles. The syringe tip was placed firmly against the underside of the leaf and injected until water-soaking could be seen at the contact point. Each leaf used in the experiments was inoculated 25 times, and, in order to keep track of each individual inoculation, they were made in a specific order (Fig. 1). Individual plants and leaves were selected with an effort made to use young vigorous leaves and to avoid plants showing excessive mechanical damage or signs of Verticillium wilt or bacterial blight. Due to the great number of inoculations, syringes had to be used over and over again. To avoid mixing cultures and contamination, the syringes were washed with a 1:10 Chlorox-water solution followed by a 3:10 ethanol-water solution and then rinsed in tap water and air dried; all these steps were carried out in the field.

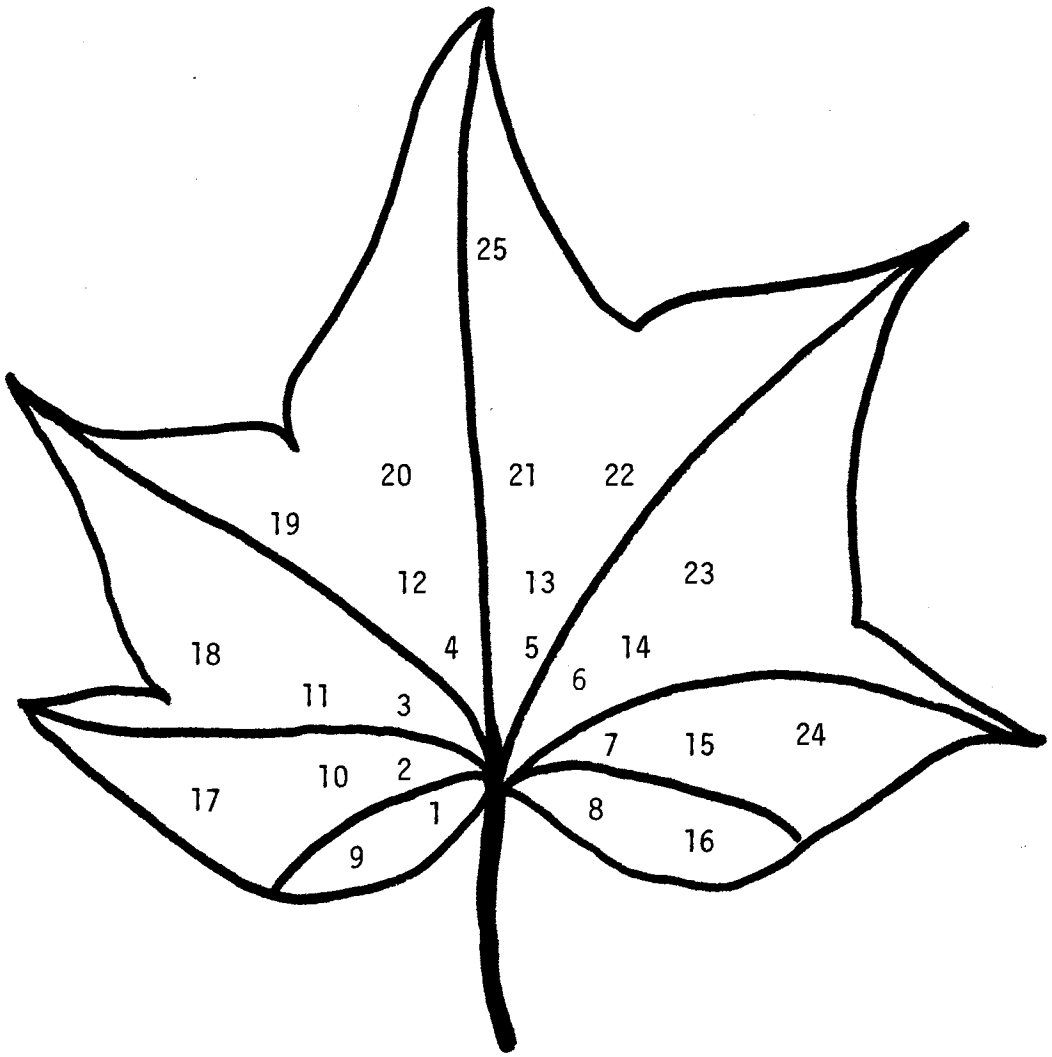

Figure 1. Order of Inoculations on Underside of Cotton Leaf.

Classification of Disease Reactions

Disease reactions were classified as compatible or incompatible. The compatible reaction was marked by water-soaking which often spread beyond the inoculated area and sometimes accompanied partial necrosis of inoculated tissue. Incompatible reactions were differentiated mainly by the absence of water-soaked tissue at the point of inoculation. Reactions showing chlorosis and/or dry brown necrotic tissue with no water-soaking were graded as incompatible. No visible reaction was also graded as incompatible. Lesion size was not a criterion for this system of classification since concentrated inoculum usually does not produce individual lesions (5). Additional explanation of materials and methods are given with specific experiments.

Host Specificity in X. malvacearum After 6 Days Incubation in Host Tissue

On July 9, 6 days after the general field inoculation with race 1, host tissue was collected from varieties Acala 44, Acala 13, Acala B_2, and Acala 121. One hundred single colony isolates were obtained from each variety for a total of 400 isolates. They were increased in nutrient broth and on July 15-16, each of the cultures was inoculated into each of the host lines: Acala 44, Acala 13, Acala B_2, and Acala 121. The inoculations were replicated twice making a total of 3,200 single inoculations. Six days after host inoculations were completed, disease readings were made.

Host Specificity in X. malvacearum After 20 Days Incubation in Host Tissue

On July 23, 20 days after initial inoculation with race 1, 100

single colony isolates were obtained from each of the four test lines as before. At this time, "mutant lesions"[1] were developing on some plants in the population, but these lesions were deliberately avoided in punching out leaf discs since the cultures derived from them would likely be 100% virulent for the host from which they were obtained. The object of the experiment was to check the virulence of cultures obtained from areas where no active disease symptoms had yet appeared at the this point in time. Inoculum was prepared as before, inoculations made July 29- Aug 2, and disease readings taken Aug 7.

Host Specificity of X. malvacearum Isolated from Different Sources of Resistance

On Aug 21, leaf and boll tissue showing mutant lesions was collected. On Aug 22, isolations were made. Instead of selecting single colonies for use in preparation of inoculum, smears or mass transfers were made by drawing a culture transfer needle across the surface of the agar through many colonies. It was thought that this would indicate the average virulence of the mutant bacteria causing the mutant lesion. This had proven to be true for race 1 and race 2 cultures isolated from dry leaf tissue by Brinkerhoff (unpublished data). Host inoculations were made Aug 27-28. Disease readings were made at 7 and 10 days later.

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[1]The term "mutant lesion" is used to describe a lesion presumed to be caused by bacterial mutants. The lesion has a water-soaked appearance indicating full susceptibility of host tissue and usually occurs singly amony many other dried brown resistant type lesions. The mutant bacteria must undergo many cell divisions in order to attain sufficient numbers to cause macroscopic disease symptoms.

Results and Discussion

Host Specificity in X. malvacearum After 6 and 20 Days Incubation in Host Tissue

All 400 isolates obtained 6 days after inoculation with race 1 incited typical race 1 reactions on the host lines tested (Table II). Only Acala 44, the susceptible strain, was attacked indicating no apparent change in host specificity in 6 days time.

Host specificity of isolates obtained 20 days after initial inoculation with race 1 remained typical of race 1 (Table III). All 400 isolates were virulent on Acala 44 but incompatible with Acala 13, Acala 121, and Acala B_2.

The results of the first two tests indicate that under the experimental conditions that prevailed, X. malvacearum remained host specific at the race level for as long as 20 days. The unchanged host specificity at 6 days is complementary to results obtained by other workers. However, Brinkerhoff in unpublished but similar experiments has found shifts in host specificity at 14, 28, and 48 days subsequent to original inoculation. Schnathorst (47) found no change at 14 days but reported 77% of the isolates recovered 8 weeks after inoculation with race 1 were race 2. Although mutant lesions were developing in the test population, indicating a change in virulence in the pathogen, no new virulence was found in isolates obtained from outside the mutant lesions.

No simple explanation is known that can account for the contrast in results. Perhaps chance in sampling is responsible, but if the sample size were much larger, it would be difficult for one person to do the work in the span of time that allows for best experimental uniformity.

TABLE II

DISEASE REACTIONS OF 400 SINGLE COLONY ISOLATES OBTAINED 6 DAYS AFTER INOCULATION OF X. MALVACEARUM INTO HOST TISSUES[1]

Host Source of Isolates	Blight Resistance Genes	Host Lines			
		Acala 44	Acala 13	Acala 121	Acala B_2
Acala 44	None	100 (+)[2]	100 (-)[2]	100 (-)	100 (-)
Acala 13	B_2	100 (+)	100 (-)	100 (-)	100 (-)
Acala 121	b_7	100 (+)	100 (-)	100 (-)	100 (-)
Acala B_2	B_2	100 (+)	100 (-)	100 (-)	100 (-)

[1]Experiment replicated twice, both replications yielded same results.

[2]Compatible reaction indicated by (+); incompatible reaction indicated by (-).

TABLE III

DISEASE REACTIONS OF 400 SINGLE COLONY ISOLATES OBTAINED 20 DAYS AFTER INOCULATION OF X. MALVACEARUM INTO HOST TISSUES[1]

Host Source of Isolates	Blight Resistance Genes	Host Lines			
		Acala 44	Acala 13	Acala 121	Acala B_2
Acala 44	None	100 (+)[2]	100 (-)[2]	100 (-)	100 (-)
Acala 13	B_2	100 (+)	100 (-)	100 (-)	100 (-)
Acala 121	b_7	100 (+)	100 (-)	100 (-)	100 (-)
Acala B_2	B_2	100 (+)	100 (-)	100 (-)	100 (-)

[1]Experiment replicated twice, both replications yielded same results.

[2]Compatible reaction indicated by (+); incompatible reaction indicated by (-).

Another possibility is of course environmental effects. As shown in the review of literature, the environment may affect the disease reaction considerably. Disease developmental conditions during the course of the first two experiments were not optimum, but were definitely favorable.

Host Specificity of X. malvacearum Cultures Isolated From Different Sources of Resistance

Graphic results of the host specificity test comparing isolates from mutant lesions from cotton lines with different genes for resistance are shown in Table IV. Most of the inoculum was virulent as indicated by compatible reactions on Acala 44, but in three cases the inoculum was apparently avirulent. Many of the isolates obtained from mutant lesions were not virulent on the host from which they came. There were some disagreements between disease readings made in Replicate 1 and Replicate 2 even though the inoculum came from the same syringe and the leaves were on the same plant. In a few instances, the decision as to compatibility or incompatibility almost became arbitrary due to the lack of definitive symptoms. Possibly the technique used to prepare the inoculum caused the unexpected variability in readings. In making the mass transfers when preparing inoculum, cells from many colonies were undoubtedly inoculated into the broth culture. Maybe avirulent cells were favored in the growth medium or perhaps some variants acted in a "cross protective" capacity when put into the host medium.

Environmental factors may have played a role in causing the variation of results too. With a frontal passage the day inoculations began, cool temperatures prevailed for several days. The cool temperatures, in addition to the direct effect it may have had on the development of

TABLE IV

HOST SPECIFICITY OF X. MALVACEARUM CULTURES ISOLATED FROM DIFFERENT SOURCES OF RESISTANCE[1]

Host Source[4] of Culture	Host Lines																
	Ac44	Im216	AcB_4	Ac121	Ac121[2]	AcB_5	$AcB_2(1.2)^3$	$AcB_2(2.3)^3$	$F_6 2B$	$F_6 2B^2$	$AcB_3(1.2)$	$AcB_3(2.3)$	Ac161(1.2)	Ac161(2.3)	Ac13(1.2)	Ac13(2.3)	$F_6 4B$
Acala 44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acala 161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acala 161	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acala 161	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B_2 Cross	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
B_2 Cross	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-
B_2 Cross	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acala B_2	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-
Acala B_3	+	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-
$F_6 2B$	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acala B_4	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-

TABLE IV (CONTINUED)

B_3 Cross	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B_3 Cross	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Acala 121	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-
Acala 13	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acala B_5	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Acala B_5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]Experiment replicated twice.

[2]Two separate lots of these host lines were tested.

[3]Host lines with (1.2)(2.3) indicated were segregating for different levels of blight resistance. (1.2) and (2.3) represent their disease grades according to Brinkerhoff's system (5).

[4]Host sources with the word "cross" are hosts whose genotype contains one of Knight's "B" genes as indicated.

bacterial blight, also made evident symptoms of Verticillium wilt in some of the blight-inoculated plants. Verticillium wilt tends to obscure bacterial blight symptoms.

CHAPTER IV

ACQUISITION OF CULTURES RESISTANT TO ANTIBIOTICS

Materials and Methods

Some experiments with X. malvacearum involve a long period of time, have special parameters, or require a high degree of accuracy. With the threat of unwanted X. malvacearum contaminants ever present, especially in facilities or areas where the organism is being experimented with, a method is sometimes needed to help assure that the original culture or its clonal descendants are maintained throughout an experiment.

Brinkerhoff (5) used streptomycin resistance as a genetic marker in his earlier experiments. To carry this method one step further and to give greater certainty in future experimentation, the author designed experiments with the objective in mind of obtaining cultures of X. malvacearum "marked" by simultaneous resistance to two antibiotics.

The plan was to develop the cultures achieving resistance to one antibiotic at a time. First, leaf tissue from Acala 44 was collected and isolation made from a lesion showing a fully susceptible disease reaction. From the dilution plate series, a single large colony was selected and increased as done in the previous experiments.

Petri dishes were prepared containing nutrient agar and 100 ppm of the antibiotic to be tested. (See Table V for a list of antibiotics screened.) The correct amount of the antibiotic was weighed, dissolved in 10 ml sterile water and added to the nutrient agar 5 minutes after it

TABLE V

INITIAL SCREENING FOR RESISTANCE TO ANTIBIOTICS

Antibiotic	No. of Colonies[1] at 12 Days	Colony Color	Colony Morphology
Neomycin (Lot 1)	1.3	White	Rough
Neomycin (Lot 2)	11.6	White	Rough
Acriflavin	200 plus	Yellow	Smooth
Penicillin G	20.3	Yellow	Rough
Chloramphenicol	38.8	Yellow	Smooth
Streptomycin (Lot 1)	8.0	White	Rough
Streptomycin (Lot 2)	0.0	-	-
Oxytetracycline	0.0	-	-
Control (no antibiotic)	Colonies overran plates in 4 days	Yellow	Smooth

[1]Average based on 8 petri plate replicates.

had been removed from the autoclave. After the agar had solidified in the petri dishes, 0.8 ml of a 24 hour broth shake culture (ca. 10^8-10^9 bacterial cells/ml) was delivered by pipette and spread over the surface of the medium. As a check, two plates of nutrient agar without antibiotic were inoculated also. Petri dishes were placed in plastic bags and incubated at room temperature for 12 days. At this point, data were recorded as to number of colonies per plate and colony morphology.

A pathogenicity trial was also made to see if the cultures had lost virulence while being maintained on the synthetic media. Inoculum was prepared by washing the colonies off the surface of the growth medium with 50 ml sterile water, mixing well, and inoculating the Acala 44 host by the syringe technique described earlier. Disease readings were made 6 days later and showed all inoculations resulted in compatible reactions. Bacteria were recovered from lesions produced by isolating with the previously described technique. One hundred ppm of antibiotic to which the bacteria had resistance was included in the growth medium. This gave some degree of assurance that contaminants would not be isolated.

In 5 days time, sufficient growth had occurred and cultures were ready to be screened for resistance to the second antibiotic. Petri dishes were prepared containing nutrient agar and 100 ppm each of two antibiotics. Mass transfers were made from plates containing bacteria with single-antibiotic resistance and spread over the surface. The plates were incubated 12 days, then a pathogenicity trial was made as before using Acala 44 as the host.

One week later disease readings were made; all reactions were compatible. Diseased leaf tissue was collected and isolations made by

dilution series on plates containing nutrient agar with the two antibiotics on which the cultures had been derived.

Finally, after single colonies had developed, host inoculations were again made (Acala 44); this time using single colonies as the source of inoculum. Using a sterile knife blade, the colony was lifted from the agar surface, put into a sterile syringe, thoroughly mixed with 3 ml sterile water, and injected as described in earlier experiments.

Results and Discussion

Final inoculations resulted in fully compatible disease reactions and host tissue was collected, air dried, and stored at 10 C. Cultures of X. malvacearum are now available for further use that have simultaneous resistance to two antibiotics in the following combinations: streptomycin-acriflavin; streptomycin-penicillin G; streptomycin-neomycin; streptomycin-chloramphenicol; and penicillin G-neomycin.

The attempt was made to incorporate streptomycin resistance as one of the genetic markers because of its proven stability in previous work with cultures of X. malvacearum (5).

Although no quantitative data were collected to support such a statement, acquisition of antibiotic resistance seemed to occur faster when streptomycin resistance was aquired first. The drugs had an effect on the colony morphology and color as shown in Table V, but had no apparent effect on virulence.

CHAPTER V

SUMMARY

1. Historically, resistant cotton varieties have readily been attacked by new virulent races of Xanthomonas malvacearum.

2. Cotton strains most susceptible to this phenomenon are those whose resistance to bacterial blight is conditioned by a single major gene.

3. The host specificity of X. malvacearum has been reported to change at the race level while inside resistant host tissue.

4. Host specificity was not found to change after either six or 20 days incubation in host tissue under the environmental conditions of these tests.

5. Environmental factors play a major role in disease expression, and probably also in the occurrance of mutants in resistant cotton.

6. X. malvacearum can be readily screened and selected for resistance to antibiotics.

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