TECHNIQUES OF INOCULATING COTTON WITH THE BACTERIAL BLIGHT, *FUSARIUM* WILT AND *VERTICILLIUM* WILT ORGANISMS

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To my parents, brother and sister, this thesis is dedicated.

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CHAPTER I

INTRODUCTION

Bacterial blight (caused by Xanthomonas malvacearum (E. F. Sm.) Dowson), Fusarium wilt (caused by Fusarium oxysporum f. vasinfectum (Atk) S. and H.), and Verticillium wilt (caused by Verticillium albo-atrum Reinke & Berth.) are important diseases of cotton. Planting resistant varieties is considered to be an effective means of controlling bacterial blight and Fusarium wilt. Verticillium wilt can probably be controlled by resistant varieties provided the resistance in existing lines can be transferred to commercial varieties. Screening for resistance in cotton, however, requires the use of effective inoculation techniques so that resistance can be properly evaluated. Since host interactions to plant pathogenic organisms are known to change with temperature (5, 11, 47), selection and standardization of temperatures are also prerequisites to an efficient screening program.

In the past, occurrence of Fusarium wilt and Verticillium wilt was thought to be dependent upon soil type and other factors. It is now known that both can occur in the same field on sandy soils (Brinkerhoff, unpublished). In addition, blight is usually present in Oklahoma fields. Therefore, resistance to all three diseases is needed.

Workers have used many different methods of inoculating cotton with the three organisms, but usually only one disease has been evaluated on the same plants.

The present investigation was planned to inoculate single plants with all three organisms in order to evaluate resistance to all three diseases. Previous work of Brinkerhoff (unpublished) has shown that bacterial blight resistance in cotton can be evaluated by inoculating the cotyledons, provided the temperature regime favors pronounced disease expression. For the present study, it was hoped that methods could be devised in which individual leaves could be inoculated to evaluate resistance to Fusarium wilt and Verticillium wilt also.

CHAPTER II

REVIEW OF LITERATURE

Occurrence and Losses

Bacterial blight and Fusarium wilt were two of the earliest recognized diseases of cotton in the United States. Verticillium wilt was more recently found (12).

Brown (12) reported that bacterial blight of cotton occurred throughout the "Cotton Belt" in the United States. This disease was more severe on Sea Island and American-Egyptian, Gossypium barbadense L., cotton than on upland cotton, Gossypium hirsutum L. About 1900, Fusarium wilt was recognized as causing great damage to the cotton crop in the United States.

More recently, reports of the Cotton Disease Council's Committee on Losses estimated losses in production of cotton for three years (1952-1954) in the United States to be 0.9% from bacterial blight, 1.3% from Fusarium wilt, and 1% from Verticillium wilt (12). In 1967, the Council's committee reported that the reduction in yield of cotton was 0.6% from Fusarium wilt, 4.4% from Verticillium wilt, and 0.9% from bacterial blight (31).

Bacterial Blight

The first extensive artificial inoculations were made by Knight and Clouston (26) in the Sudan. They prepared inoculum by soaking

infected leaves in water and applied the bacterial suspensions with hand operated sprayers.

In 1948, Weindling (41) described many methods of inoculating cotton with X. malvacearum under field conditions. These methods were: spraying the bacterial suspension on the leaves by means of a knapsack sprayer; sprinkling the plants from a small can which contained the organism; atomizing onto the seedling by the use of an atomizer; rubbing bolls with a toothbrush or with a piece of cheesecloth wetted with a bacterial suspension; directing a coarse spray of a bacterial suspension to the boll surface by the use of a knapsack sprayer; applying the inoculum over the lower side of the leaves; and using a hypodermic syringe to direct a stream of inoculum to the leaves. He also suggested that standardization of inoculation procedures should minimize the effects of variable natural conditions and provide uniformly severe disease conditions throughout.

Brinkerhoff and Fink (9) inoculated field plantings of cotton by water soaking leaves with spray from single nozzle guns from a power sprayer. Cotyledons and leaves of seedlings in the greenhouse were inoculated with a rubber tube-tipped syringe through the stomata or through a wound made with a dissecting needle (8). Brinkerhoff and Fink (9) also used cheese cloth bags containing sand which were dipped into an aqueous suspension of inoculum and rubbed on the lower side of cotyledons.

Bird (Personal Communication) completely mechanized inoculation of plants in the field by directing bacterial suspensions from jet nozzles from runners of a boom-type sprayer operated by the power takeoff of a tractor. He also inoculated individual leaves by hand by scratching

the lower surface with a toothpick that had been dipped in inoculum.

In the Sudan, Gunn (21) inoculated cotyledons by introducing several drops of a concentrated aqueous suspension of X. malvacearum into the cup formed by the first emerging leaves. Innes (24) studied resistance mechanisms in bacterial infected plants that had been inoculated by means of foliar sprays and vein inoculations under conditions of high temperature and humidity. He also inoculated leaves of cotton in the main vein with X. malvacearum in his program of breeding for resistance to bacterial blight (23).

Last and Dransfield (29) inoculated the youngest fully expanded leaves of cotton with bacterial suspensions by the use of a rubbing technique (fingers used with carborundum as an abrasive).

Logan (30) worked on bacterial boll rot of cotton (X. malvacearum), comparing two inoculation techniques for the assessment of host resistance. He used a fine brush to apply the bacterial suspensions to the surface of the young bolls immediately after corolla drop, and he also injected the wall tissue of bolls, three to four weeks old, with a bacterial suspension by the use of a fine needle. Both techniques gave good disease symptoms, but the former technique showed symptoms earlier.

Wernham (42) inoculated the leaf with X. malvacearum by smearing the leaf with a bacterial suspension. He introduced the organism into the stem by the use of a sterile scalpel. He also used an atomizer or camel's hair brush to apply the inoculum to the leaves. In each instance Wernham incubated the inoculated plants at 100% relative humidity.

The effect of temperature on bacterial blight of cotton was

reported by Stoughton (39). He concluded that the disease is severe when the temperatures are high throughout the period of its development and when humidity is high during a period of about two days after inoculation, but Weindling (41) concluded that Stoughton's work is good only for the conditions Stoughton used, but not under some other conditions or with other plant materials. This seems to agree with Brinkerhoff and Presley (11), who reported that the relatively low night (19 C) and high day (36.5 C) temperatures were the cause of breaking down of resistance conferred by single genes in cotton varieties. The 19 C night coupled with a 25.5 C day temperature enhanced disease symptoms in susceptible varieties. But the 25.5 C nights, when coupled with each of three day temperatures (25.5 C, 26.5 C, and 36.5 C), enhanced disease resistance.

Bacterial blight disease symptoms are discussed by Brown (12). He notes that when the seedlings are infected, circular, or irregular water soaked spots appear first on the lower side of the cotyledons. On the leaves, angular, discolored, or deadened spots are bounded by veinlets.

Last (28) reported that the disease symptoms include internal and external stem discoloration. Tissues adjacent to affected veins become water soaked, and leaf sectors dependent upon these veins become pale and eventually dry out and die.

Fusarium Wilt

Fusarium wilt of cotton caused by F. oxysporum f. vasinfectum has been studied by many workers. According to Young (47), Elliott inoculated cotton seeds with the fungus. Elliott reported that the artificially inoculated seed carried the fungus in a viable form on the

seed lint for at least five months and the disease was introduced into soil by means of artificially infected seeds.

Oshanina and Gubanov (35) inoculated cotton with the organism by using a soil inoculation technique. An oat medium infested with the Fusarium organism was applied to the soil. Similar work was done by Krasiehkov and Menlikiev (27), using Fusarium-infested barley grains to inoculate the soil before the plants were grown.

Perry (36) used a root-inoculation technique to determine the reaction of cotton plants to Fusarium wilt.

A pot-inoculation method was used by Wickens (43). The plant to be tested was grown in a small pot that was placed within a larger pot. The roots of the plant grew through the hole at the bottom of the small pot into the soil in the large pot. The small pot was lifted up, which injured the roots, and the fungus suspension was placed into the soil in the large pot. The small pot was then returned to the large pot.

Bugbee and Sappenfield (14) injected the hypocotyl of the cotton plants with conidial suspensions of Fusarium by using a 23-gauge hypodermic needle and 5 ml syringe. The needle was injected into the stem until the beveled point was just visible. A drop of suspended conidia was sucked into the stem immediately or shortly after the needle was removed. They also used a modified pot method. Bottomless flint glass tubes were placed vertically into larger pots of soil. A seed was planted in each tube. After two weeks, the tubes were removed from the pots. The injured roots were dipped into a conidial suspension for 30-45 seconds.

Jenkins and Taylor (25) reported that when Fusarium was introduced into soil together with sting nematodes, Belonolaimus gracilis, or root

knot nematodes Meloidogyne spp., the disease symptoms were more severe than when Fusarium alone was present. The same kind of work was reported by Minton and Minton (32). In Egypt Abo-El-Dahab (2) also obtained the same results inoculating cotton with F. oxysporum f. vasinfectum and the nematode Meloidogyne hapla.

Temperature plays an important role in Fusarium wilt development. Young (47) reported that a root temperature of 30-32 C for greenhouse-grown plants was the optimum temperature range for the development of cotton wilt. He also pointed out that for practical purposes 25 C was the minimum and 26-28 C was rather favorable. According to Young (47), the optimum temperature for growth of cotton was reported by Balls to be 32 C, and the optimum temperature for growth of the pathogen on both a liquid medium and on an agar medium was reported by McRae to be between 25 C and 30 C.

In the Leninabad region of the U. S. S. R., Stepantsev (38) reported that with the thin lint cotton varieties, Fusarium wilt appeared only when the temperature was less than 36.8 C and the optimum temperature was 29 C.

Atkinson (3), who proposed the name F. vasinfectum, reported that the symptoms of Fusarium wilt in cotton were yellowing, drying, death of portions of the leaves, defoliation, and dwarfing. The plants either died, or partial recovery sometimes occurred. Darkening of the vascular system and the development of mycelium in the xylem of diseased plants were also reported.

Brown (12) noted that the first external evidence of the disease was yellowing at the edges of the lower leaves and in the area midway between the main veins of the leaf. The yellowing spread, and the

leaves became brown or shriveled. The leaves finally fell to the ground and the stem sometimes died back. In other cases, the plant did not shed its leaves but became stunted or dwarfed. Cross-sections of infected stems showed a brownish ring inside the cambium at early stages, and the ring became darker in advanced stages.

Verticillium Wilt

Brinkerhoff (7) used a hypodermic needle to inoculate cotton plants in the field. He used a No. 24 needle to inject approximately 0.25 ml of inoculum into the hypocotyl of young seedlings just below the soil level.

Evans (19) devised three methods of inoculating cotton with V. albo-atrum:

1. He inoculated the plants by pouring a fungus suspension over roots that had been injured with a sterile scalpel.

2. He inoculated plants by inserting a sterile dissecting needle through the stem above the soil level and then squeezing inoculum into the puncture from a sponge as the needle was removed.

3. He injected the hypocotyl about 2 cm below the soil level by means of a hypodermic needle. He concluded that all three methods were efficient in transmitting the disease. But the needle puncture-sponge method was done more rapidly than the other two.

Wiles (44) grew cotton seedlings in sand and inoculated them by carefully lifting the plants and dipping the roots in a suspension of V. albo-atrum prepared in a blender. The seedlings were carefully transplanted after inoculation.

Erwin et al. (18) inoculated the cotyledonary node with a suspension

of spores of V. albo-atrum by puncturing with a No. 20 hypodermic needle. They also compared their stem-puncture method with Wiles' root-dipping method. The disease symptoms from the stem-puncture method were more severe than from the root dipping method.

Bugbee and Presley (13) used a 23-gauge syringe to inject the stem of the cotton plant. The Verticillium inoculum was injected into the lower stem at an angle of approximately 45 degrees to the stem until the bevel of the point was just visible. The drop of inoculum that formed in the axis of stem and needle disappeared into the plant, giving visual evidence of inoculation. They concluded that resistant plants (Seabrook Sea Island) were only slightly stunted and chlorotic, while susceptible varieties showed symptoms as early as four days after inoculation.

Abdel-Raheem and Bird (1) inoculated cotton with V. albo-atrum through cut roots and by injection into stems. The roots were cut with a spatula inserted into the soil to make a slit and 10 ml of inoculum was poured into each slit, which was sealed by pressing the soil back together and watering. The stems were inoculated by inserting a hypodermic needle into the center of the stem above the cotyledonary node and then covering the wound with masking tape. They concluded that from the stem inoculations, the plants showed symptoms earlier and had more severe external symptoms, internal discoloration, and vertical extension of the wilt pathogen than from the root inoculations.

Wilhelm (45) grew cotton seedlings in small containers and inoculated by carefully removing the root balls from the containers and then spraying the exposed roots with a conidial suspension of V. albo-atrum. Afterwards the plants were carefully returned to the containers for incubation.

In the U. S. S. R., Mirpulatova and Nagornaya (33) introduced a fungal suspension into the central vein of a leaf. They reported that the fungus spread upwards through the leaf and gradually spread through the entire blade but did not move downward into the petiole.

Berry and Thomas (5) reported that the growth of V. albo-atrum in culture was limited at 10 C to 30 C.

In (S-Y) (22) in China, reported that the development of symptoms was dependent upon temperature; the disease was favored at 25 C, developed less at 28 C, and was absent at 30 C.

Stepantsev (38) made a detailed study of Verticillium wilt of cotton in the Leninabad region of the U. S. S. R. He found that temperature played a very important role in the development of wilt; 25.2 C was optimum for wilt development and 36.8 C was maximum.

Edgington and Walker (17) reported that the optimum temperature for growth of V. albo-atrum was 22 C. It grew very slowly at 28 C and not at all at 32 C. The disease symptoms were more severe on tomato plants at a soil temperature of 28 C than at a lower temperature when air temperatures were 16 C, 20 C, and 24 C. When soil and air were both at 28 C, no symptoms occurred.

In Peru, De Segura (16) reported that cotton leaves infected with V. albo-atrum had purple veins; adjacent tissues later on turned necrotic and sometimes purple also. Brown (12) has described the symptoms of Verticillium wilt at the different developmental stages of cotton. At the seedling stage, the cotyledons become yellowish and are often quite desiccated; vascular discoloration appears at the base of the hypocotyl, and the seedlings usually die. In young plants that have three to five leaves, stunting occurs when infection takes place. The leaves are dark

green and show some crinkling between the veins. In larger plants, the chlorotic areas on the leaf margins and between the principal veins appear mottled. As the symptoms develop further the chlorotic area usually becomes larger, paler and necrotic. Defoliation often follows. In the larger plants the symptoms usually occur first in the lower leaves and extend upward to the middle and upper leaves. If the disease is severe, all the leaves and bolls shed and some plants die. Vascular discoloration of the stem at the ground line appears to be more evenly distributed through the stele and to be lighter brown than the discoloration produced by Fusarium wilt.

Petiole inoculation has been used as a method of infecting alfalfa with bacterial wilt. Graham (20) inoculated alfalfa with Corynebacterium insidiosum by injecting the petiole with the use of a fine hollow glass needle. He inserted the needle, which had been dipped into young bacterial colonies, into the petiole at the junction of the two lateral leaflets. Susceptible plants began to show symptoms in two weeks, while the resistant ones were still green.

Toothpicks have been used to inoculate corn plants. Young (46) tested the reaction of lines in corn to fungi causing ear rots and stalk rots. He grew the organisms (Diplodia zeae, Gibberella zeae, and Helminthosporium sp.) on toothpicks which had been placed in potato dextrose broth in test tubes. Fungus-invaded toothpicks were inserted into corn stalk tissues but the rind tissue had first to be punctured by a sharp metal instrument. He concluded that the advantages of this method were as follows: a uniform amount of inoculum was introduced into the plants; different sections of the stalk or ear could be inoculated with the same or different organisms at the same time; the

point of inoculation was detected readily; the spread of the pathogen from the peg was easily traced; and large populations could be inoculated rapidly.

CHAPTER III

MATERIALS AND METHODS

BACTERIAL BLIGHT STUDIES

Xanthomonas malvacearum (E.F. Sm.) Dowson, Race 1 (6) was used in the study. The inoculum was prepared by transferring a loopful of the stock culture to each of 10 ml of Potato-Carrot-Dextrose Agar (PCDA) slants contained in a 20 ml bottle. The PCDA medium was prepared as follows: Potato-Dextrose-Agar, 39 g; carrot juice, 15 ml; yeast extract, 0.5 g; $CaCo_3$, 0.2 g; $MgSO_4$, 0.3 g; and distilled water to make 1 liter (PDA from Difco Laboratories, Detroit, Michigan). Two 3-day-old cultures that had been incubated at 18 C were thoroughly mixed with 100 ml of sterile distilled water and used as inoculum. The approximate number of bacteria per ml was determined by flooding the surface of nutrient agar plates with bacterial suspensions as outlined by Brinkerhoff (8).

Four plants of each of the cotton varieties, Acala 414, Stoneville 62, Westburn, and Seabrook Sea Island (Table II) were grown in each 30 cm pot containing steamed soil. Acala 414 was immune to bacterial blight and the remaining three varieties were susceptible. The tests were made in two temperature-controlled rooms at temperature regimes of 32 C day and 25 C night and 30 C day and 19 C night. Fourteen-hour days with approximately 500 ft-c illumination were used. Six pots of each variety were provided for each temperature regime.

When the plants were 12 days old, inoculations were made by a method described by Brinkerhoff (8). A 5 ml glass syringe was used in which the needle was replaced by a short rubber tube (5 mm diam.) held in place by a rubber band. The rubber tube-tipped syringe containing the inoculum was pressed against the lower side of a cotyledon until a water-soaked area appeared. Bacterial concentrations of about 8×10^6 and 1.6×10^8 cells per ml were used. One cotyledon of each plant was injected in the same manner with sterile distilled water to serve as a control. After inoculation, the plants were observed daily. As soon as the obvious symptoms of the disease were visible, usually within 5-20 days depending on bacterial concentrations, both the injected and non-injected cotyledon were removed and disease readings recorded.

FUSARIUM WILT STUDIES

General Methods

To reduce possible genetic variability of the inoculum, single conidial cultures were made and tested for pathogenicity. All subsequent studies were made with one of the pathogenic isolates. To obtain single conidial cultures of F. oxysporum f. vasinfectum (Atk.) S. and H., a loopful of a stock culture was transferred to fresh PDA in 9 cm petri dishes. After seven days, dilution plates were made on water agar. Germinating conidia were transferred singly to fresh PDA 24-48 hours later by the use of a very tiny loop with flattened and sharpened edges.

After obtaining single conidial cultures of the organism, preliminary tests were conducted to determine the temperature relationships and pathogenicity of the isolates. In the temperature study, 2.5 mm diameter plugs were cut from seven-day-old colonies on PDA and placed at

the centers of petri dishes containing PDA. Three petri dishes were then placed at each of eight temperatures: 5 C, 10 C, 18 C, 22 C, 25 C, 28 C, 30 C, and 32 C. After seven days, the diameter of each colony was measured.

Preliminary trials indicated that conidia from a three- to seven-day-old fungus culture started by flooding plates with conidial suspensions gave better germination than conidia from three- to five-week-old cultures.

Therefore, in the pathogenicity tests the inoculum used was usually only four to five days old. Sterile tap water was poured on the fungus culture in a petri dish. A sterile glass rod was used to rub the surface of the culture to loosen the conidia. The petri dish was agitated for one to two minutes and the conidial suspension was then filtered through two layers of sterile cheese cloth. To estimate the concentration of conidia used, both the dilution plate technique and haemocytometer count method were used. Ten-day-old plants, grown in vermiculite, of the cotton varieties Stoneville 62 (susceptible) and Westburn (resistant) were used as test plants. Inoculation was made by cutting the root tips of the seedlings and then dipping the roots for one minute into the conidial suspensions which contained approximately 3×10^4 conidia per ml. Plants whose roots were treated in the same manner, but dipped in water, were provided as controls. Ten inoculated plants of each variety were placed in a temperature-controlled room under a regime of 32 C, 14-hr day (with approximately 500 ft-c illumination) and 25 C night. Two weeks after inoculation, the number of infected plants was recorded. All the plants tested in the temperature-controlled room were grown in steamed soil. Plants in the growth chamber and temperature

controlled tanks were grown in modified Hoagland's solution (Table I) as used by Guinn (Personal Communication).

Details of Specific Techniques of Inoculation

A. Studies in a Temperature-Controlled Room in Soil

Experiments were designed to compare various techniques of inoculating cotton with F. oxysporum f. vasinfectum. The cotton varieties were Acala 414, Seabrook Sea Island, Stoneville 62, and Westburn, as previously described. Since inoculation with X. malvacearum and F. oxysporum f. vasinfectum did not indicate interactions in preliminary trials, the same plants used in the bacterial blight study were re-used after they had produced four to five true leaves. In all experiments, the upper leaves were inoculated.

In the first experiment, a seven-day-old culture was used as inoculum using the following techniques:

1. Injection of the fungus suspension into the nectary of the midvein at the lower side of the leaf with a 5 ml glass syringe (needle size No. 26).

2. The same as in Technique 1, except that injection was in the petiole, about 2 cm from the junction of the leaf blade and petiole.

3. A dissecting needle was injected through the petiole and a 2.5 x 4 cm sponge saturated with the fungal suspension was squeezed against the petiole for one minute to force the inoculum into the hole created by the needle.

4. A fungus-saturated toothpick was inserted into each petiole and left in this position until the termination of the experiment. The toothpicks used for inoculation were prepared in the following manner:

TABLE I

MODIFIED HOAGLAND'S SOLUTION USED FOR GROWING COTTON PLANTS

Chemical	Concentration (mg/liter)
$Ca(NO_3)_2 \cdot 4H_2O$	472.00
$MgSO_4 \cdot 7 H_2O$	246.00
KNO_3	202.00
KH_2PO_4	68.00
H_3BO_3	2.40
$MnSO_4 \cdot H_2O$	1.20
$ZnSO_4 \cdot 7 H_2O$	0.36
$CuSO_4 \cdot 5 H_2O$	0.03
$CoCl_2 \cdot 6 H_2O$	0.06
$Na_2MoO_4 \cdot 2 H_2O$	0.20
FeEDTA[1]	3.5 ppm[1]
HCl	0.01 ml

[1]10 g $FeCl_3$ and 10.5 g EDTA were dissolved in 1 liter of water. Ten ml of this solution were added to each 10 liters of nutrient.

Flat toothpicks were cut into halves and the pointed upper part was steam-sterilized at 121 C (15 psi) for 30 minutes. About 25 to 30 sterilized toothpicks were placed on the surface of a petri dish containing PDA. Approximately 0.5 ml of a conidial suspension was pipetted into each dish and the dish was shaken carefully to spread the suspension uniformly. After four to seven days, the toothpicks were covered with fungus mycelium.

Plants were inoculated by each technique. The concentration of the conidia used in Techniques 1 to 3 was estimated to be 8×10^6 per ml. Data were taken nine days after inoculation. Plants inoculated with sterile water were provided as controls for Techniques 1 to 3. In Technique 4, plants injected with sterile toothpicks were used as controls.

In the second experiment, a four-day-old fungus culture was used as inoculum, using the following techniques:

1. The fungus suspension was injected into the leaf blade-petiole junction by means of a 50 µl syringe. To facilitate the entry of the inoculum, two to three punctures were made in each petiole. The amount of inoculum used per inoculation was 20 µl but only about 10 µl entered the petiole.

2. Inoculation of the petiole was done by means of a fungus-saturated toothpick (same as Technique 4 in Experiment 1).

3. Inoculation was made by means of a 5 ml rubber tube-tipped syringe containing the fungus suspension. The syringe was pressed against a wounded area in the leaf until a water-soaked area appeared. The wound was made by scratching the lower side of the leaf with a sterile dissecting needle.

Plants were inoculated by each technique and control plants were provided for each technique. The symptoms of the disease were visible in six to seven days, but data were not taken until 10 days after inoculation. Isolations were made to determine the rate of fungus spread from the point of inoculation. Four infected leaves of each variety inoculated by each technique were randomly selected. Twelve pieces of approximately 3 x 7 mm were cut from each leaf at the points illustrated in Figure 1. Tissue pieces were surface sterilized in 0.6% sodium hypochlorite for two to five minutes, after which they were placed in petri dishes containing PDA in the order shown in Figure 2.

The third experiment was designed to determine whether inoculation with one wilt fungus would affect the response of the plant to inoculation with a second wilt fungus. The hypothesis to be tested was that phytoalexin or some other inhibitors might have been translocated from the site of the parasite-host interaction to the new leaves.

Four plants of one variety were grown in one pot at a continuous temperature of 30 C. Two of these plants were inoculated with a suspension of F. oxysporum f. vasinfectum (3×10^6 conidia/ml) with a microsyringe at the junction of leaf blade and petiole. The other two plants were not inoculated. Four pots were provided for each variety. Seven days after inoculation, the number of plants that became infected was recorded, after which all of the inoculated leaves were removed. The temperature of the room was lowered to 27 C day and night and the plants were inoculated with V. albo-atrum in the same manner. The conidial suspension of the inoculum was estimated to be approximately 2.5×10^7/ml. The number of infected plants was recorded seven days after inoculation. Five leaves of each variety were randomly selected and

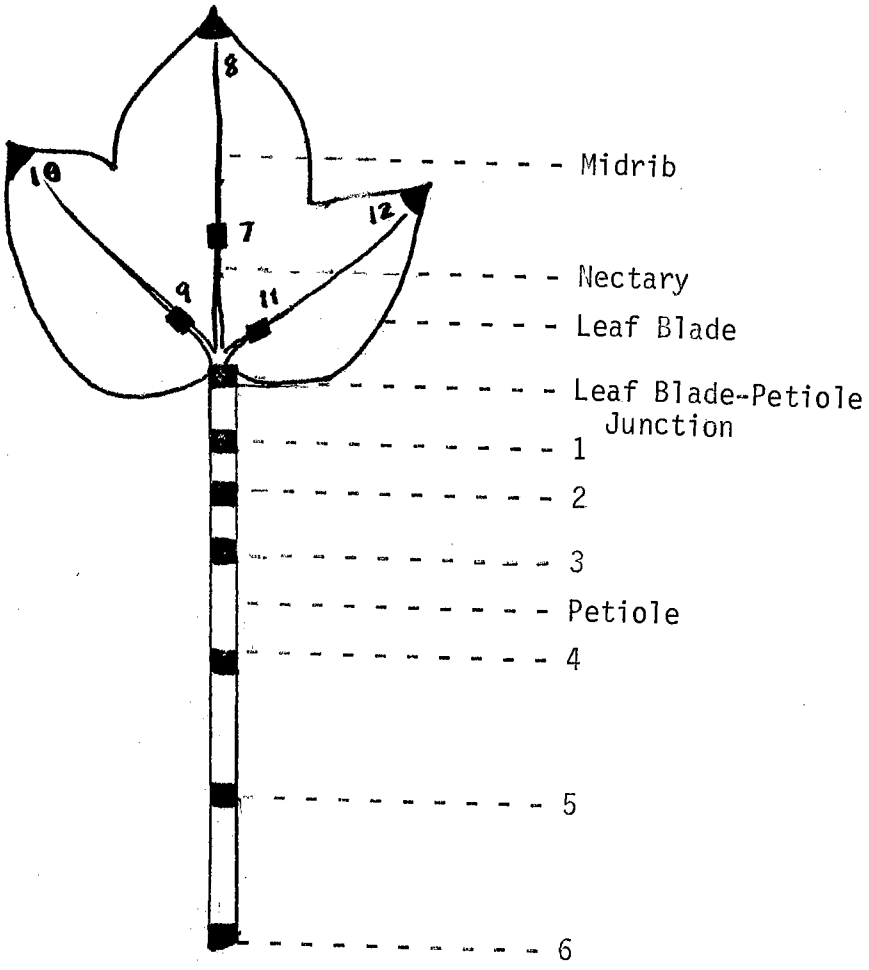


Figure 1. Diagram Showing the Points at Which Pieces of Tissues Were Obtained for Isolation

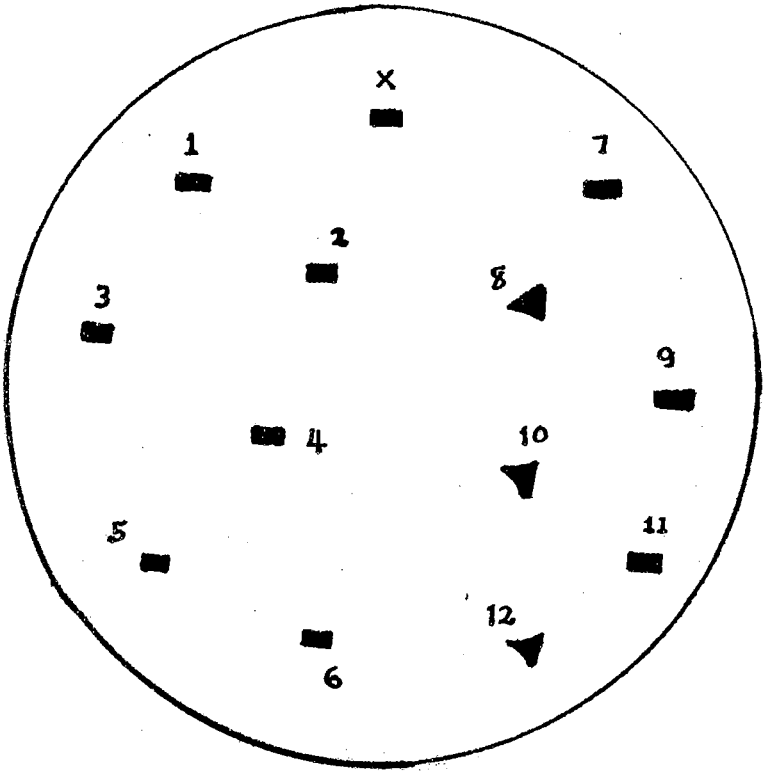


Figure 2. Diagram Showing the Arrangement of Pieces of Tissues in the Isolation Plate

isolations were made as previously described.

B. Studies in a Growth Chamber in Nutrient Solutions

Again, the experiments were designed to compare various techniques of inoculating cotton with F. oxysporum f. vasinfectum. Because of space limitation only three varieties were used. These were Seabrook Sea Island, Stoneville 62, and Westburn. The plants were grown in plastic buckets containing modified-Hoagland's solution which was aerated from porous plastic tubes with air supplied by an electric pump. Each bucket contained four plants of one variety. In all experiments the upper leaves were inoculated. The growth chamber was set for 14 hr of light with an intensity of 2500 ft-c at the top of containers. The inoculum contained approximately 3×10^6 conidia/ml.

In the first experiment, the temperatures were 30 C days and 20 C nights. A seven-day-old culture of F. oxysporum f. vasinfectum was used as inoculum, using the following techniques:

1. Injection of the fungal suspension into the nectary of the mid-vein at the lower side of the leaf with the use of a 5 ml glass syringe.

2. The same as Method 1, except that the point of inoculation was at the petiole, about 2 cm from the junction of leaf blade and petiole.

Plants were inoculated by each technique, and control plants were provided for each technique.

The symptoms of the disease became visible in six to eight days, but data were not recorded until 10 days after inoculation.

Isolations were made to determine the rate of fungus spread from the point of inoculation. Six leaves of each variety inoculated by each technique were randomly selected. Isolations were also made from control leaves.

In the second experiment, a three-day-old culture of F. oxysporum f. vasinfectum was used as inoculum, using the following techniques:

1. Approximately 20 µl of fungus suspension was injected into the leaf blade-petiole junction with a microsyringe.

2. Inoculation was made the same way as Technique 1, except the point of inoculation was in the midvein.

3. The needle-sponge technique was used to inoculate the petiole about 2 cm from the leaf blade.

The temperature in the growth chamber for this test was 30 C, continuously.

The symptoms of the disease became evident in six days and the data were taken seven days after inoculation. Five leaves of each variety inoculated by each technique were randomly selected for isolations as previously described.

C. Studies in Nutrient Solutions in the Greenhouse in Temperature-Controlled Tanks

An experiment was designed to determine the effect of temperature and the effect of covering the inoculation wound with tape. This test was made in two temperature-controlled tanks. Petioles were injected with a microsyringe 2 cm from the leaf blade-petiole junction.

The expanded upper opposite leaves were inoculated. One inoculated leaf of each pair was covered by white gummed tape (Time Tape, Professional Tape Co.) at the point of inoculation.

The inoculum contained approximately 3×10^6 conidia/ml. Plants inoculated with sterile water were provided as controls.

Disease symptoms developed in leaves of plants in the tank at 20 C about 15 days after inoculation and readings were taken after 16 days.

The plants in the tank at 25 C showed symptoms just seven days after inoculation, but readings were also taken 16 days after inoculation. In the tank at 25 C, some plants showed symptoms on non-inoculated leaves. This was probably because of translocation of conidia (36) from petioles to stems and other parts of the plant. Hence, all the plants in the 25 C tank were not used for the subsequent tests with V. albo-atrum.

Five leaves of each variety with both taped and non-taped wounds from both tanks were selected for isolations.

VERTICILLIUM WILT STUDIES

General Methods

The previously discussed technique for obtaining a single conidial culture was also used for V. albo-atrum.

The plants were inoculated with V. albo-atrum sometimes before and sometimes after inoculation with F. oxysporum f. vasinfectum. In the temperature-controlled room, the same plants were inoculated with all three pathogens. The opposite leaf of the inoculated leaf of each plant was provided as a control by injecting with sterile water or left non-inoculated.

After obtaining a single conidial culture of V. albo-atrum, a preliminary test was made to determine the effect of temperature on growth of the organism.

Seven-day-old colonies on PDA were used to seed plates in the same manner as in the Fusarium studies, except that two petri dishes were placed in each of five incubators at 14 C, 20 C, 25 C, 28 C, and 32 C. After seven days, the diameter of each colony was measured.

Three- to six-day-old cultures were used as inoculum. The fungus

was grown on Czapek's agar. The conidial suspensions were prepared in the same manner as for the Fusarium inoculum.

Details of Specific Techniques of Inoculation

A. Studies in the Temperature-Controlled Room in Soil

Experiments were designed to compare techniques of inoculating cotton with V. albo-atrum and the effect of temperature on disease severity. The plants were re-used after studies with Fusarium wilt. The temperature was maintained at 32 C days (14 hr of light) and 25 C nights.

In the first experiment, a six-day-old culture was used as inoculum, and the following techniques were tried:

1. Injection of the fungus suspension into the leaf blade-petiole junction with a microsyringe. The suspension contained approximately 2.4×10^7 conidia /ml.

2. The point of a fungus-infested toothpick was inserted into each petiole and left in this position until the end of the experiment. Control plants were also provided in this experiment.

The disease symptoms were recorded 20 days after inoculation.

The second experiment was performed to determine whether inoculation with F. oxysporum f. vasinfectum affects the reaction of the plant to V. albo-atrum. The procedures for this were discussed with the Fusarium wilt inoculation techniques.

B. Studies in a Growth Chamber in Nutrient Solutions

The first experiment was designed to compare techniques of inoculating cotton with V. albo-atrum. The plants used had been inoculated with F. oxysporum f. vasinfectum and the leaves were removed. The regime for

this test was 14 hr of light and 30 C days and 20 C nights.

The upper opposite leaves were inoculated as discussed before. The inoculum contained approximately 1 x 10^7 conidia. The inoculation techniques used were as follow:

1. Injection of the fungus suspension into the nectary with the use of a 5 ml glass syringe. It was observed that a sugary exudate covered some of the nectaries. In this case, the injection was done in the midvein near the nectary.

2. Inoculation was made in the same manner as Technique 1, but injection was in the petiole, 2 cm from the leaf blade-petiole junction.

The disease symptoms were first observed in six days, and the data were recorded seven days after inoculation. Five leaves of each variety inoculated by each technique were randomly selected for isolation, as previously described for Fusarium.

The second experiment was designed to determine the effect of temperature on disease development and the effectiveness of the microsyringe leaf blade-petiole junction technique.

V. albo-atrum inoculum, containing 2.5 x 10^7 conidia/ml, was injected into the leaf blade-petiole junction with a microsyringe. The amount of inoculum used for each injection was 20 µl (0.02 cc).

Two plants of each variety in one bucket were inoculated, while the other two plants in the same bucket served as controls. The temperature in the growth chamber was set at 27 C both day and night.

Disease symptoms were visible six days after inoculation. The data were recorded after seven days. Six leaves from each variety in both control and inoculated plants were randomly selected for isolation.

C. Greenhouse Studies in Nutrient Solutions in Temperature-Controlled Tanks

This experiment was designed to compare techniques of inoculation. Inoculum of *V. albo-atrum*, containing 2.4×10^7 conidia/ml, was injected into plants by the following techniques:

1. Injection of a conidial suspension into the leaf blade-petiole junction by a microsyringe.

2. Inoculation was done in the same manner, but the point of inoculation was in the petiole 2 cm from the leaf blade.

3. Injection of a conidial suspension into the petiole by the needle-sponge technique.

The amount of inoculum used for Techniques 1 and 2 was approximately 20 µl (0.02 cc) for each injection.

The experiment was conducted in two temperature-controlled tanks which were maintained at 27 C for the roots. Plants in one experiment had been inoculated previously with *F. oxysporum* f. *vasinfectum*.

The symptoms first appeared eight days after inoculation and the data were recorded after 12 days.

Five leaves of each variety for each inoculation technique were selected for isolation.

CHAPTER IV

RESULTS

Reaction of Cotton Varieties to Three Common Diseases

Acala 414 was resistant to bacterial blight and susceptible to Fusarium wilt and Verticillium wilt. Seabrook Sea Island showed resistance to Fusarium wilt and Verticillium wilt and susceptibility to bacterial blight. Stoneville 62 appeared susceptible to all three diseases. Westburn was susceptible to bacterial blight and intermediate to both Fusarium and Verticillium. The sources of cotton varieties and their reactions to the diseases are shown in Table II.

TABLE II

SOURCE OF COTTON VARIETIES AND THEIR REACTIONS TO THREE DISEASES

Variety	Source	Reaction to Disease[1]		
		Xm.	Fofv.	Va.
Acala 414	Okla. Breeding Line	R	S	S
Stoneville 62	Commercial Variety	S	S	S
Westburn	Okla. Released Variety	S	I	I
Seabrook Sea Island	U. S. Coastal Variety	S	R	R

[1]R = Resistant, S = Susceptible, and I = Intermediate

Growth of Verticillium and Fusarium on PDA

The isolate of F. oxysporum f. vasinfectum grew well over the temperature range of 22 to 30 C, less at 18 and 32 C, and only slightly at 10 C. The optimum temperature was 30 C (Table III and Figure 3).

The V. albo-atrum isolate grew more slowly than the Fusarium isolate. The fungus grew over the range of 14 to 28 C and had an optimum temperature of 25 C (Table IV).

TABLE III

EFFECT OF TEMPERATURE ON GROWTH OF F. OXYSPORUM F. VASINFECTUM ON POTATO DEXTROSE AGAR

Temperature (C)	Growth in Diameter[1] (cm)
5	0.0
10	1.2
18	6.8
22	8.3
25	8.5
28	8.5
30	9.0
32	4.0

[1]Recorded seven days after transfer.

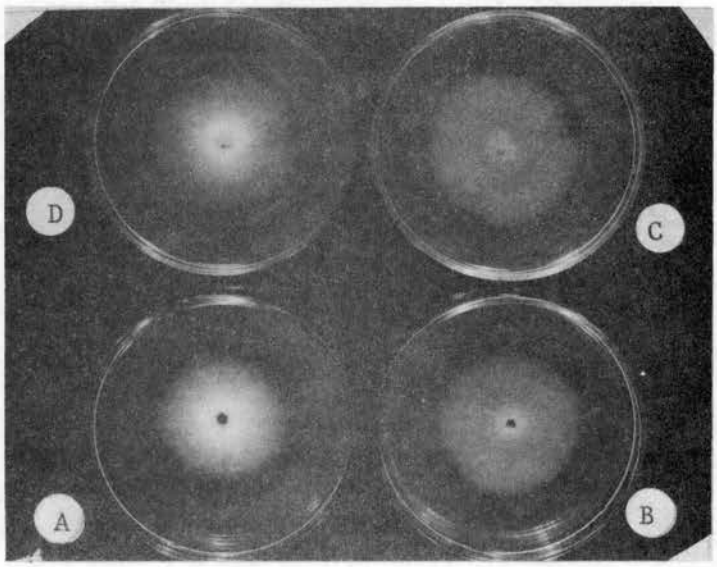


Figure 3. Growth of F. oxysporum f. vasinfectum on PDA. A, 22 C; B, 25 C; C, 28 C; D, 30 C.

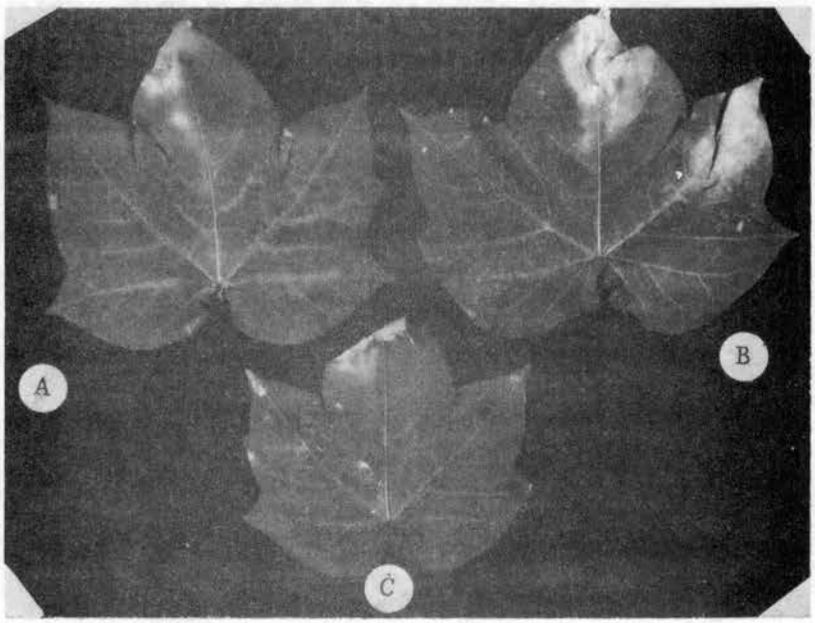


Figure 4. Symptom Patterns Obtained in Leaves Inoculated with F. oxysporum f. vasinfectum. A, Microsyringe at Midvein; B, Microsyringe at Petiole; C, Needle-Sponge at Petiole.

TABLE IV

EFFECT OF TEMPERATURE ON GROWTH OF V. ALBO-ATRUM ON POTATO DEXTROSE AGAR

Temperature (C)	Growth in Diameter (cm)[1]
14	1.8
20	2.7
25	2.9
28	2.4
32	0

[1]Recorded seven days after transfer.

Disease Symptoms

A. The bacterial blight resistant variety Acala 414 showed no disease development but was dry and necrotic at the point of inoculation, while the susceptible varieties (Stoneville 62, Westburn and Seabrook Sea Island) had water-soaked lesions. The bacterial blight symptoms from this type of inoculation have been described by Brinkerhoff (8).

B. In the Fusarium and Verticillium wilts, Stoneville 62 was susceptible in every test, except the one in which a rubber tube-tipped syringe was used for inoculation. The symptoms in this variety were as follows: The infected leaf became yellow, often at the tip or the edge of the leaf blade. The part of the leaf showing symptoms was yellow and soft when touched. When the disease was severe, those parts became dry and necrotic. Westburn was intermediate in the number of infected plants, compared with Stoneville 62 and Seabrook Sea Island.

In addition to the number of infected leaves, the size of the chlorotic area, and the intensity of discoloration on infected leaves were also characteristic of each variety.

Westburn showed the same type symptoms as Stoneville 62 but the number of infected leaves was less than Stoneville 62. Seabrook Sea Island showed mild symptoms - veins and veinlets became purple on the part that showed symptoms, and that part of the leaf was pale yellow (Figure 6).

Disease symptoms were recorded as follows: 0 = no symptom, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% infected area per leaf; m = mild discoloration (Table V). The results of the tests in succeeding sections are presented as the number of infected leaves but are not specific as to the degree of disease symptoms.

TABLE V

DISEASE SYMPTOMS EXPRESSED IN THREE COTTON VARIETIES FROM LEAF INOCULATIONS WITH V. ALBO-ATRUM IN A GROWTH CHAMBER (30 C DAYS, 20 C NIGHTS)

Variety	Number of Leaves in Each Disease Category[1]									
	Microsyringe Inoculation Site									
	Nectary					Petiole				
	0	1	2	3	4	0	1	2	3	4
Stoneville 62	8	21	0	0	0	4	11	3	5	4
Westburn	8	17	2	0	0	7	14	2	3	1
Seabrook Sea Island	24	3(M)	0	0	0	18	8(M)	0	1(M)	0

[1]0 = No symptoms, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of the leaf area showed chlorosis; M = Mild discoloration.

The symptoms of Fusarium wilt disease patterns are shown in Figures 4 to 6.

Pathogenicity Test of Fusarium and Verticillium

For seedlings that were inoculated by dipping the roots in a conidial suspension of Fusarium, Stoneville 62 showed disease symptoms one week after inoculation, and the plants were dead in three weeks. Westburn appeared green and healthy at the time of observation. The check plants of both varieties were free of disease symptoms. The temperatures for this test were 32 C days and 25 C nights. Both varieties were severely diseased in the Verticillium test, but symptoms did not appear until 12 days after inoculation.

Bacterial Blight Studies

The inoculation technique enabled one to effectively differentiate between resistant and susceptible varieties. Stoneville 62, Westburn, and Seabrook Sea Island plants were 100% infected under the two temperature regimes. The susceptible plants showed symptoms earlier at 32 C days and 19 C nights than at 32 C days and 25 C nights. When two cell concentrations (8×10^6 and 1.6×10^8) were used at the same temperature regime, the susceptible plants showed symptoms earlier with the use of the more concentrated inoculum (Table VI).

Results of Fusarium Wilt Studies in a Temperature-Controlled Room in Soil

From 66 to 100% of the plants of the susceptible varieties (Acala 414 and Stoneville 62) inoculated with a syringe at the nectary in

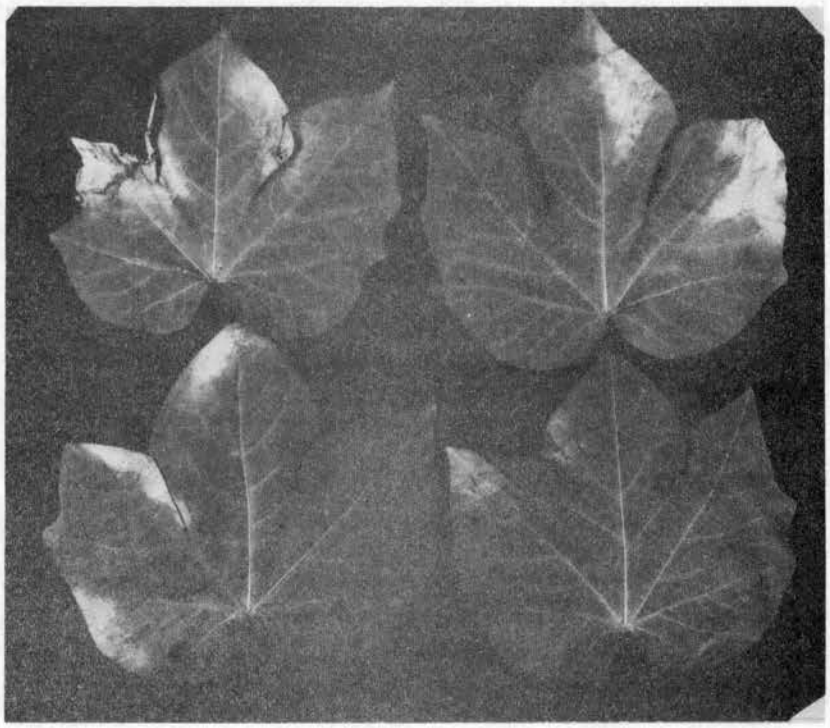


Figure 5. Various Symptom Patterns Obtained in Leaves Inoculated with F. oxysporum f. vasinfectum by Using Microsyringe Injected at Leaf Blade-Petiole Junction.

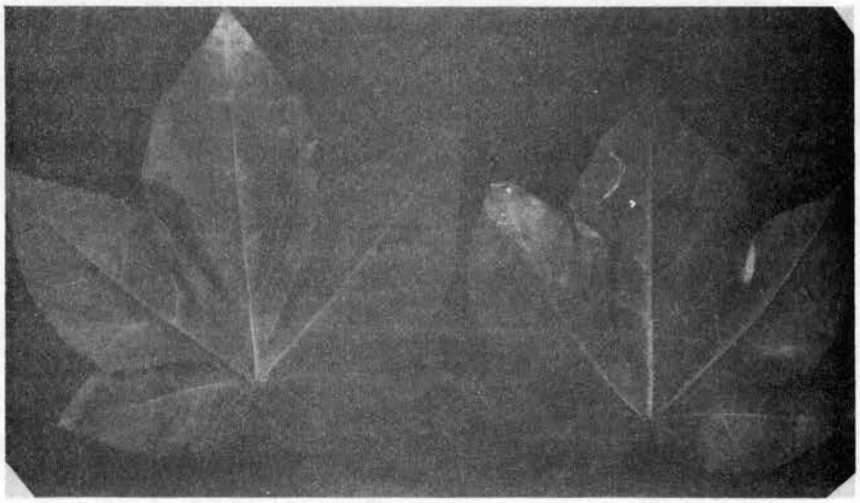


Figure 6. Symptoms of V. albo-atrum on Leaves of Seabrook Sea Island. Left, Microsyringe at Leaf Blade-Petiole Junction; Right, Microsyringe at Petiole.

TABLE VI

EFFECT OF DAY AND NIGHT TEMPERATURES AND BACTERIAL CELL CONCENTRATIONS ON PATHOGENICITY OF *X. MALVACEARUM* TO COTTON VARIETIES

Variety	Mean Number of Infected Leaves[1]		
	32 - 25 C		32 - 19 C
	8×10^6	1.6×10^8	1×10^7
Stoneville 62	6(18-22)[2]	6(6-8)	6(4-5)
Westburn	6(18-22)	6(6-8)	6(4-5)
Seabrook Sea Island	6(18-22)	6(6-8)	6(4-5)
Acala 414	0	0	0

[1]Four replicates of six leaves.

[2]Number of days until symptoms first appeared.

Experiment 1 showed disease symptoms. When the inoculations were made at the petiole, the susceptible varieties still had 66 to 100% of the plants showing disease symptoms. From the needle-sponge inoculation, disease symptoms showed in only 50% of the plants of the susceptible varieties. Toothpick inoculations gave 83-100% diseased plants. All three techniques permitted one to differentiate between susceptible and resistant varieties, but using the syringe injection at the nectary and at the petiole and inoculating by means of toothpicks gave better results than the needle-sponge technique (Table VII).

In Experiment 2, the three varieties showed no symptoms when a rubber tube-tipped syringe was used to inoculate the leaf blade. With the use of a microsyringe, Stoneville 62 showed 100% disease symptoms, while 80% of the plants in this variety showed wilt with the use of the toothpick technique. Both techniques permitted one to differentiate the three varieties from each other by the number of infected leaves. But the microsyringe injection at leaf blade-petiole junction gave better results than when toothpicks were inserted into the petiole (Table VIII).

In the isolation plates, no fungus growth occurred from tissues taken from uninoculated leaves and from leaves inoculated with a rubber tube-tipped syringe. Fungal growth from tissues from infected leaves of of the three varieties that were inoculated with microsyringe at the leaf blade-petiole junction showed that the fungus spread down the petiole farther and more often than when a toothpick inoculation was made. Following microsyringe and toothpick inoculations, the fungus also moved into the veins of the leaf (Figures 7 and 8).

In Experiment 3, plants of four varieties showed typical responses to the two fungi, but both susceptible varieties had only about 90-95%

TABLE VII

MEAN NUMBER OF INFECTED LEAVES INOCULATED WITH F. OXYSPORUM F. VASINFECTUM USING FOUR INOCULATION TECHNIQUES. PLANTS WERE GROWN IN SOIL IN A TEMPERATURE-CONTROLLED ROOM (32 C DAYS, 25 C NIGHTS)[1]

Variety	Technique of Inoculation					
	Syringe Nectary	Syringe Petiole	Syringe Nectary	Needle-sponge Petiole	Syringe Nectary	Toothpick Petiole
Acala 414	6	4	5	3	4	6
Stoneville 62	6	6	6	3	4	5
Westburn	2	2	2	1	2	1
Seabrook Sea Island	1	0	2	0	0	1

[1]Four replicates of six leaves.

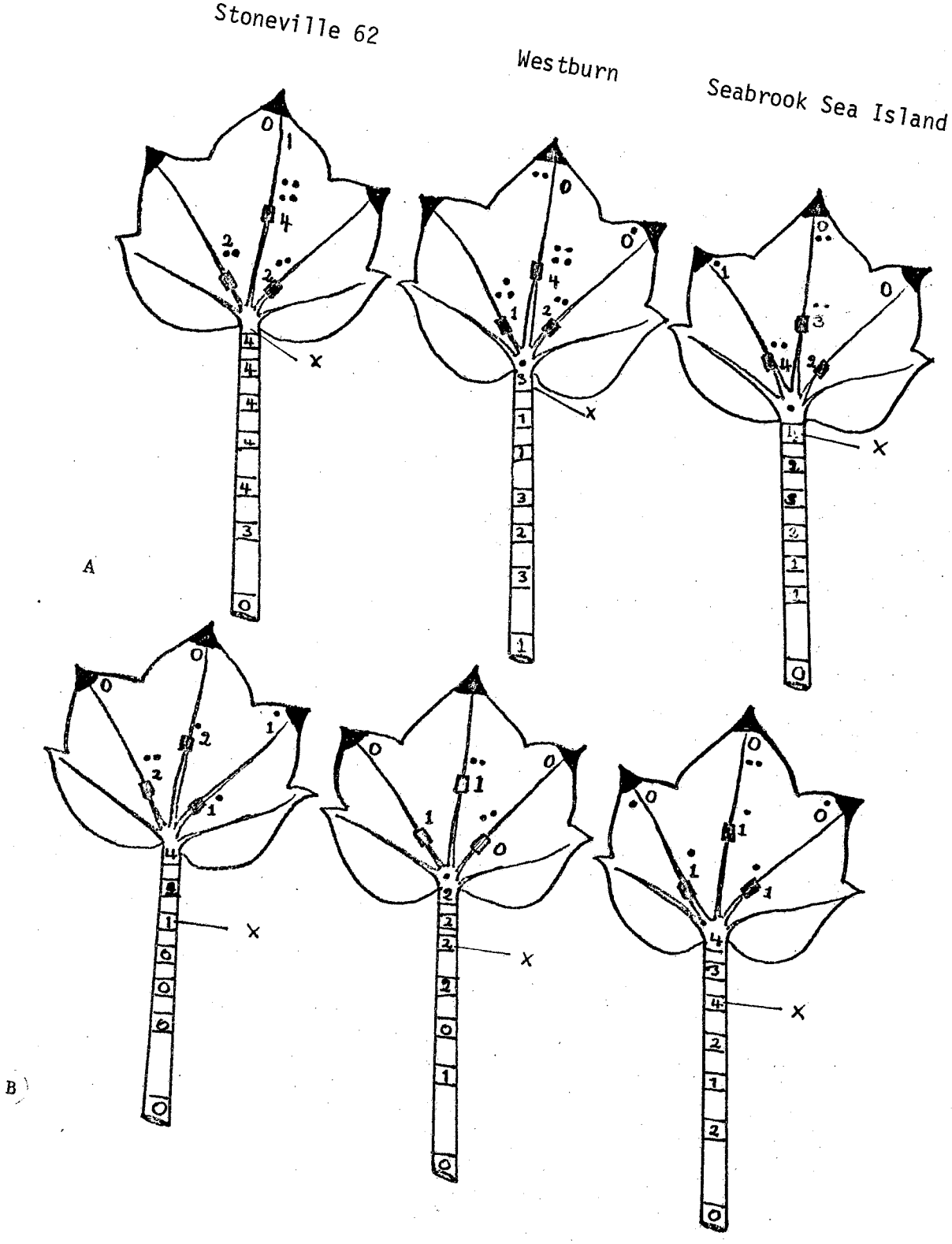
TABLE VIII

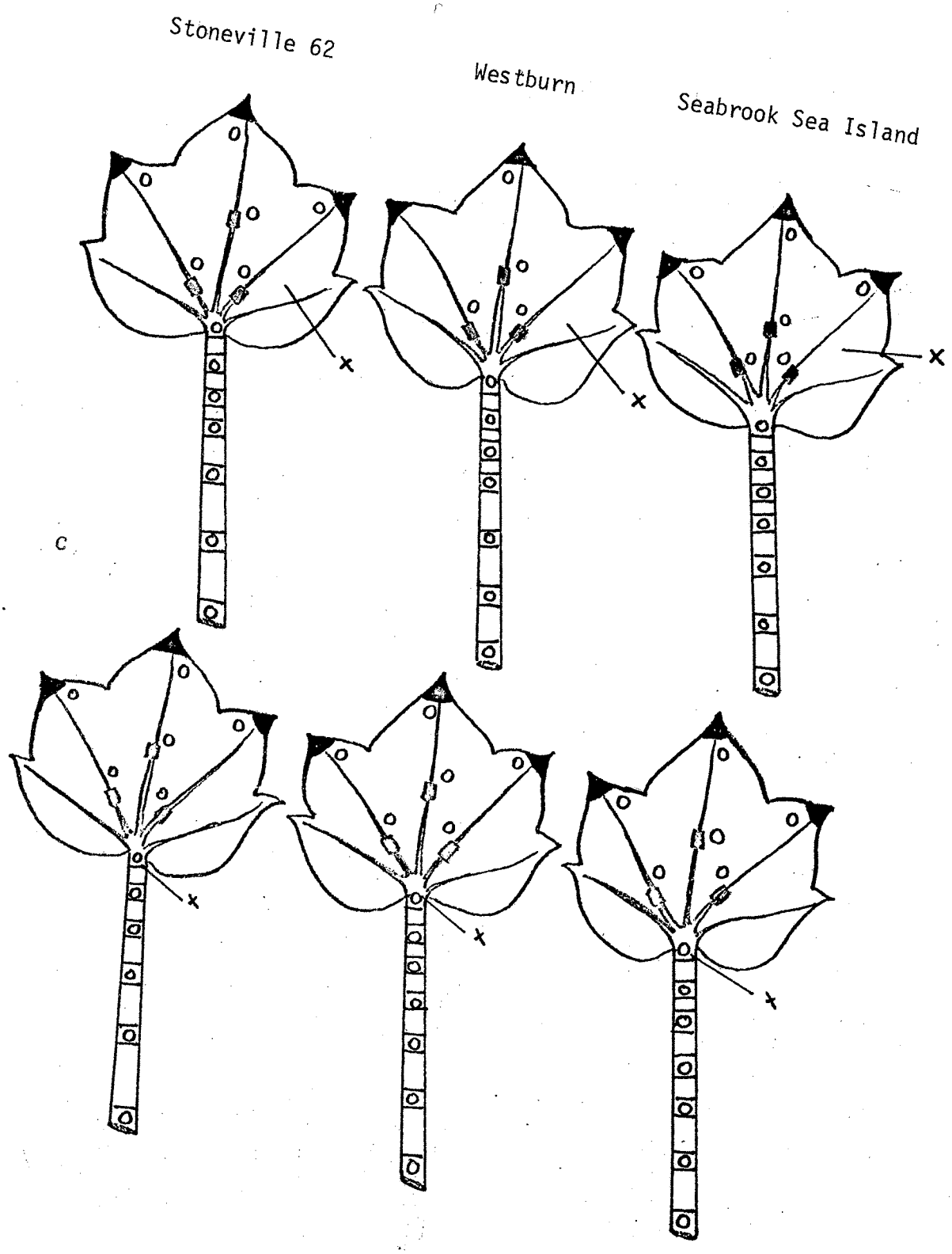
MEAN NUMBER OF INFECTED LEAVES INOCULATED WITH F. OXYSPORUM F. VASINFECTUM USING THREE INOCULATION TECHNIQUES. PLANTS WERE GROWN IN A TEMPERATURE-CONTROLLED ROOM (32 C DAYS, 25 C NIGHTS)[1]

Variety	Technique of Inoculation		
	Microsyringe at Leaf Blade-Petiole Junction	Toothpick at Petiole	Syringe at Leaf Blade
Stoneville 62	9.0	7.2	0
Westburn	7.7	2.0	0
Seabrook Sea Island	5.7	0.7	0

[1]From total of nine leaves, four replicates.

Figure 7. Number of Leaves from Which F. oxysporum f. vasinfectum Was Isolated 10 Days After Inoculation at the Locations Indicated. Isolations Were Made from Four Leaves/Variety/Technique; x Indicates Inoculation Point, Spots Indicate the Number of Leaves That Showed Symptoms in That Area. A, Microsyringe at Leaf Blade - Petiole Junction; B, Toothpick at Petiole; C, Rubber Tube-Tipped Syringe at Leaf Blade; D, Control.


Stoneville 62
Westburn
Seabrook Sea Island
A
B
X


Stoneville 62
Westburn
Seabrook Sea Island
C
D

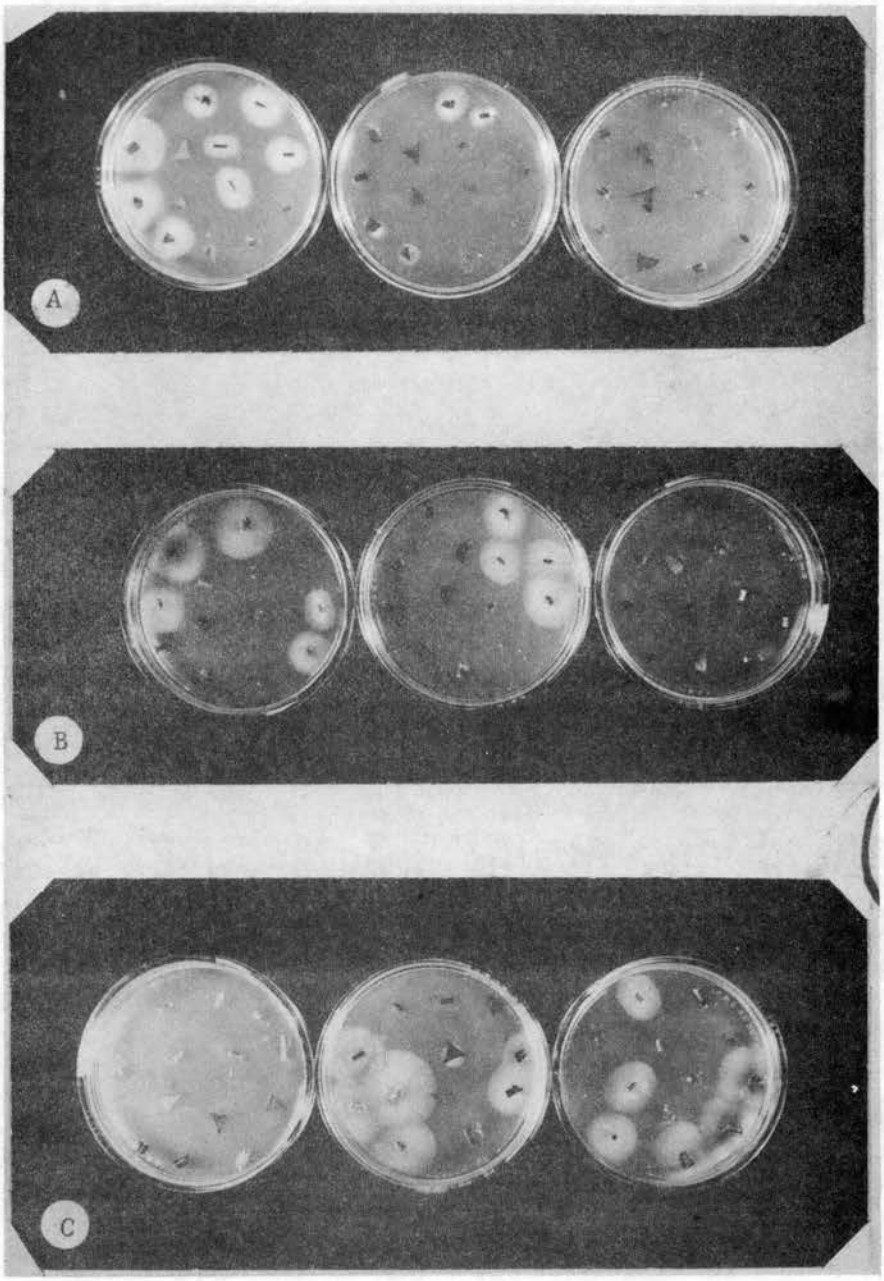


Figure 8. Growth of F. oxysporum f. vasinfectum from the Tissues on PDA from Three Techniques of Inoculation. Left, Microsyringe at Petiole-Leaf Junction; Middle, Toothpick at Petiole; Right, Rubber Tube-Tipped Syringe at the Lower Side of Leaf Blade; A, Stoneville 62; B, Westburn; C, Seabrook Sea Island.

infected leaves. The plants that had been inoculated with Fusarium and then inoculated with Verticillium had the same number of infected leaves as the plants that were inoculated with Verticillium alone. Acala 414 and Stoneville 62 were susceptible to both fungal diseases, Westburn was intermediate, and Seabrook Sea Island was resistant (Table IX). Isolation showed that the fungus grew into both leaf blades and petioles. Differences between resistant and susceptible plants could not be detected by the data from the isolations (Figure 9).

Results of Fusarium Wilt Studies in a Growth Chamber in Nutrient Solution

Experiment 1: The use of the syringe inoculation technique at the nectary and at the petiole, under a 30 C day - 20 C night regime, effectively differentiated the three varieties in their response to the disease. However, the susceptible variety (Stoneville 62) showed only 80% infection from the syringe inoculation at the nectary and only 63% from the syringe inoculation at the petiole. Seabrook Sea Island showed no symptoms (Table X).

Isolations showed that the fungus spread more into the leaf blade areas than into the petiole following inoculation by a syringe at the nectary. The infected leaves from the syringe inoculation at the petiole showed that the fungus spread both up to the leaf blade and down to the petiole. The frequency and location of isolates did not indicate the relative susceptibility or resistance of the cotton varieties (Figure 10).

In Experiment 2, at 30 C continuous temperature, the microsyringe inoculation at the leaf blade-petiole junction permitted differentiation

TABLE IX

MEAN NUMBER OF INFECTED LEAVES FROM A MICROSYRINGE INJECTION AT THE PETIOLE WITH F. OXYSPORUM F. VASINFECTUM FOLLOWED BY A SIMILAR INOCULATION ONE WEEK LATER WITH V. ALBO-ATRUM. PLANTS WERE GROWN IN SOIL IN A TEMPERATURE-CONTROLLED ROOM (30 C CONTINUOUSLY FOR FUSARIUM AND 27 C CONTINUOUSLY FOR VERTICILLIUM)[1]

Variety	Mean Number of Infected Leaves[1]			
	Inoculated With:			
	Fusarium Only	Verticillium (Following Fusarium)	Verticillium Only	Control
Acala 414	3.8	3.7	3.8	0
Stoneville 62	3.9	3.7	3.6	0
Westburn	1.3	2.1	2.0	0
Seabrook Sea Island	0.4	0.3	0.5	0

[1]Six replicates of four leaves.

Figure 9. Number of Leaves From Which F. oxysporum f. vasinfectum and V. albo-atrum Were Isolated Seven Days After Inoculation at the Locations Indicated. Isolations Were Made From Five Leaves/Variety; x Indicates Inoculation Point, Spots Indicate Number of Leaves That Showed Symptoms in That Area. A, Fusarium Inoculated Leaves; B, Verticillium Inoculated Leaves.

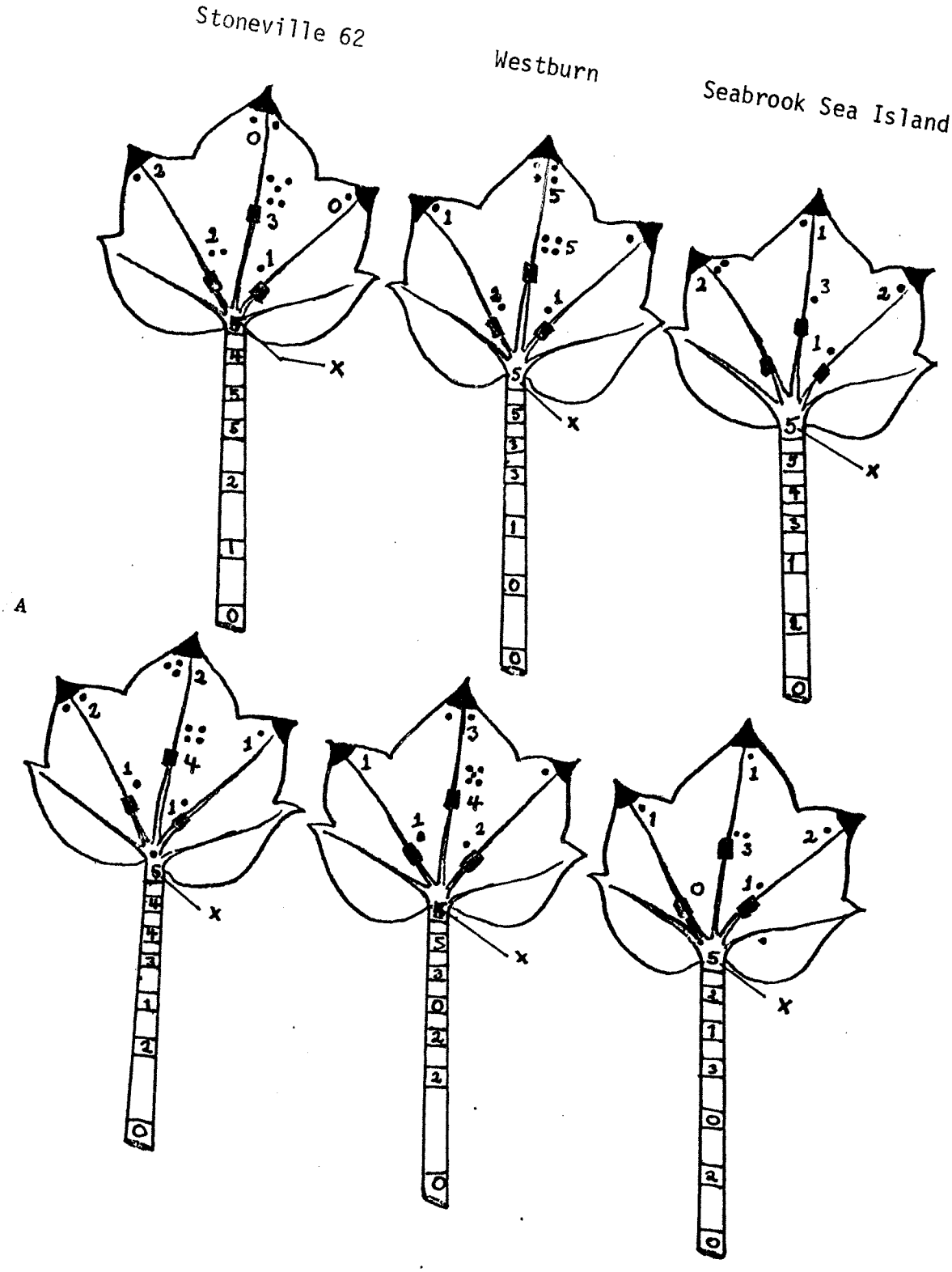

Stoneville 62
Westburn
Seabrook Sea Island
A
B
X

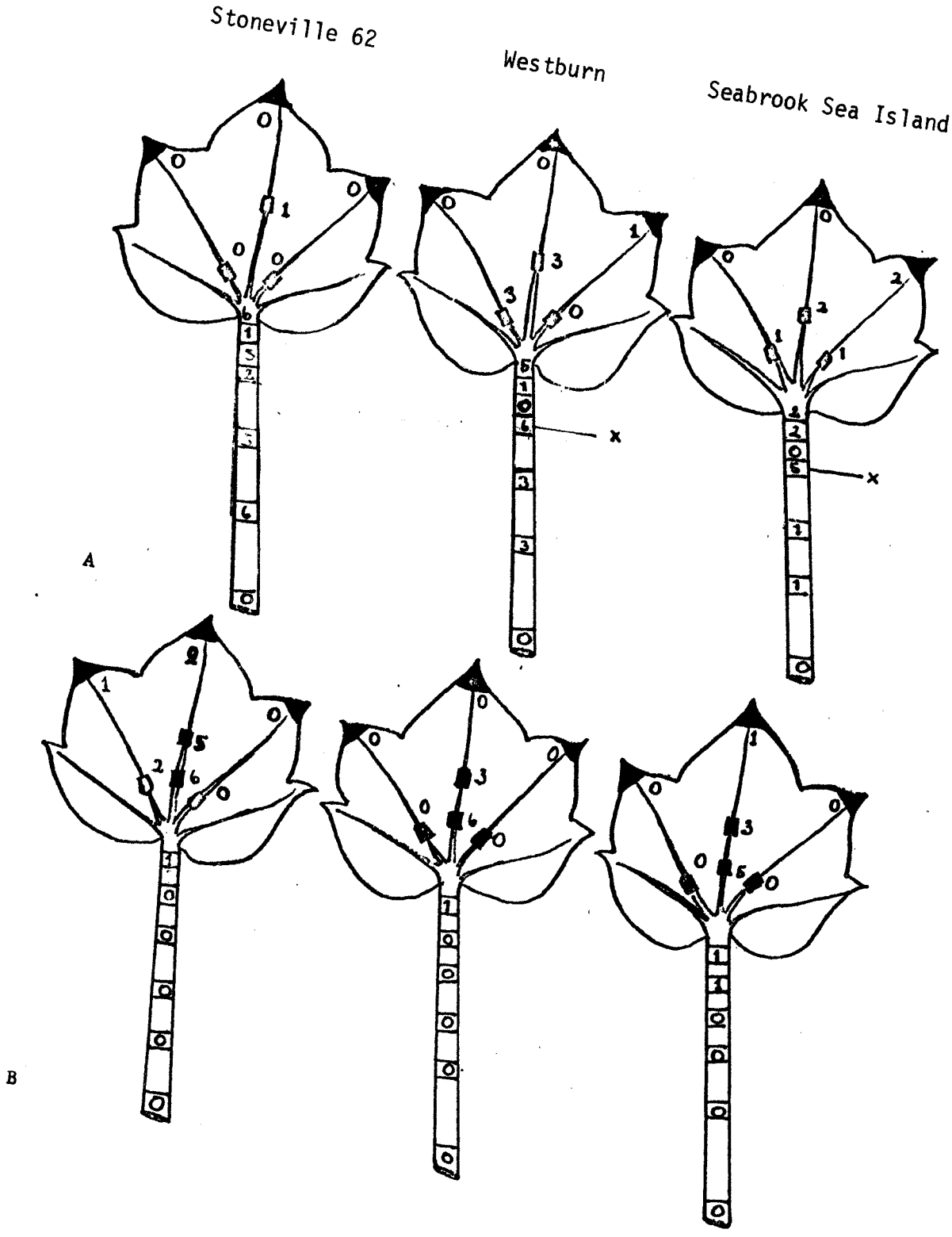
TABLE X

MEAN NUMBER OF INFECTED LEAVES INOCULATED WITH F. OXYSPORUM F. VASINFECTUM USING SYRINGE INJECTION. PLANTS WERE GROWN IN NUTRIENT SOLUTION IN A GROWTH CHAMBER (30 C DAYS, 20 C NIGHTS)[1]

	Site of Inoculation	
Variety	Nectary	Petiole
Stoneville 62	7.3	5.7
Westburn	3.0	1.3
Seabrook Sea Island	0	0

[1]Three replicates of nine leaves.

Figure 10. Number of Leaves From Which F. oxysporum f. vasinfectum Was Isolated 10 Days After Inoculation at the Locations Indicated. Isolations Were Made From Five Leaves/Variety/Technique; x Indicates Inoculation Point. A, Syringe at Petiole; B, Syringe at Nectary.


Stoneville 62
Westburn
Seabrook Sea Island
A
B

among the resistant, intermediate, and susceptible varieties, but the microsyringe inoculation at the midvein and the needle-sponge inoculation at the petiole did not (Table XI).

Isolations from infected leaves showed that the fungus spread downward into the petiole and upward to the leaf blade with every technique. Fungus growth was observed at the base of infected leaves in the microsyringe injected at leaf blade-petiole junction technique (Figure 11).

Results of Fusarium Wilt Studies in the Greenhouse in Temperature-Controlled Tanks

The effect of two root temperatures (20 and 25 C) on the number of visibly infected leaves following inoculation of the petiole with a microsyringe was slight. However, at 20 C, Westburn did have more infected leaves than Seabrook Sea Island but there was no difference at 25 C. Stoneville 62 had more infected leaves than the other two varieties at both temperatures and more infected leaves at 25 C than 20 C. The effect of covering the inoculation wound was slight and inconsistent (Table XII).

Isolations from infected leaves of plants in 20 C and 25 C tanks showed fungal growth both down the petiole and up into the leaf blade in either covered or uncovered inoculation wounds. The number of infected leaves showing fungal growth at the end of the petiole was greater in the 25 C tank than 20 C tank (Figure 12).

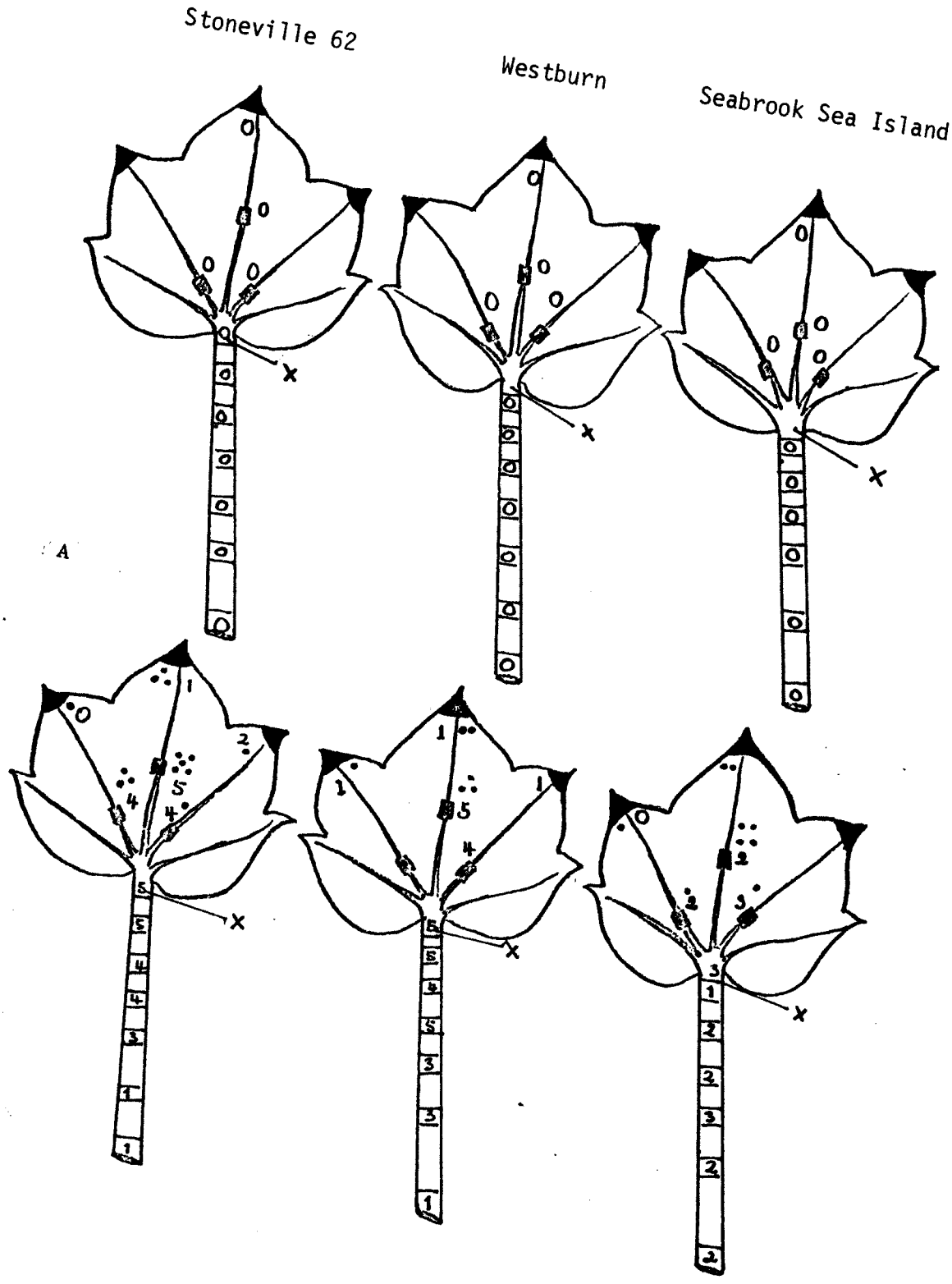
TABLE XI

MEAN NUMBER OF INFECTED LEAVES INOCULATED WITH F. OXYSPORUM F. VASINFECTUM USING THREE INOCULATION TECHNIQUES. PLANTS WERE GROWN IN NUTRIENT SOLUTION IN GROWTH CHAMBER AT 30 C CONTINUOUS[1]

Variety	Techniques of Inoculation		
	Microsyringe at Petiole Junction	Microsyringe at Midvein	Needle-sponge at Petiole
Stoneville 62	6.0	2.0	1.0
Westburn	4.0	1.3	0.3
Seabrook Sea Island	1.3	1.3	0

[1]Three replicates of six leaves each.

Figure 11. Number of Leaves From Which F. oxysporum f. vasinfectum Was Isolated Seven Days After Inoculation at the Locations Indicated (Isolations Were Made From Five Leaves/Variety/Technique; x Indicates Inoculation Point, Spots Indicate Number of Leaves that Showed Symptoms in That Area. A, Controls; B, Microsyringe at Petiole Junction; C, Needle-Sponge at Petiole; D, Microsyringe at Midvein.


Stoneville 62
Westburn
Seabrook Sea Island
A
B

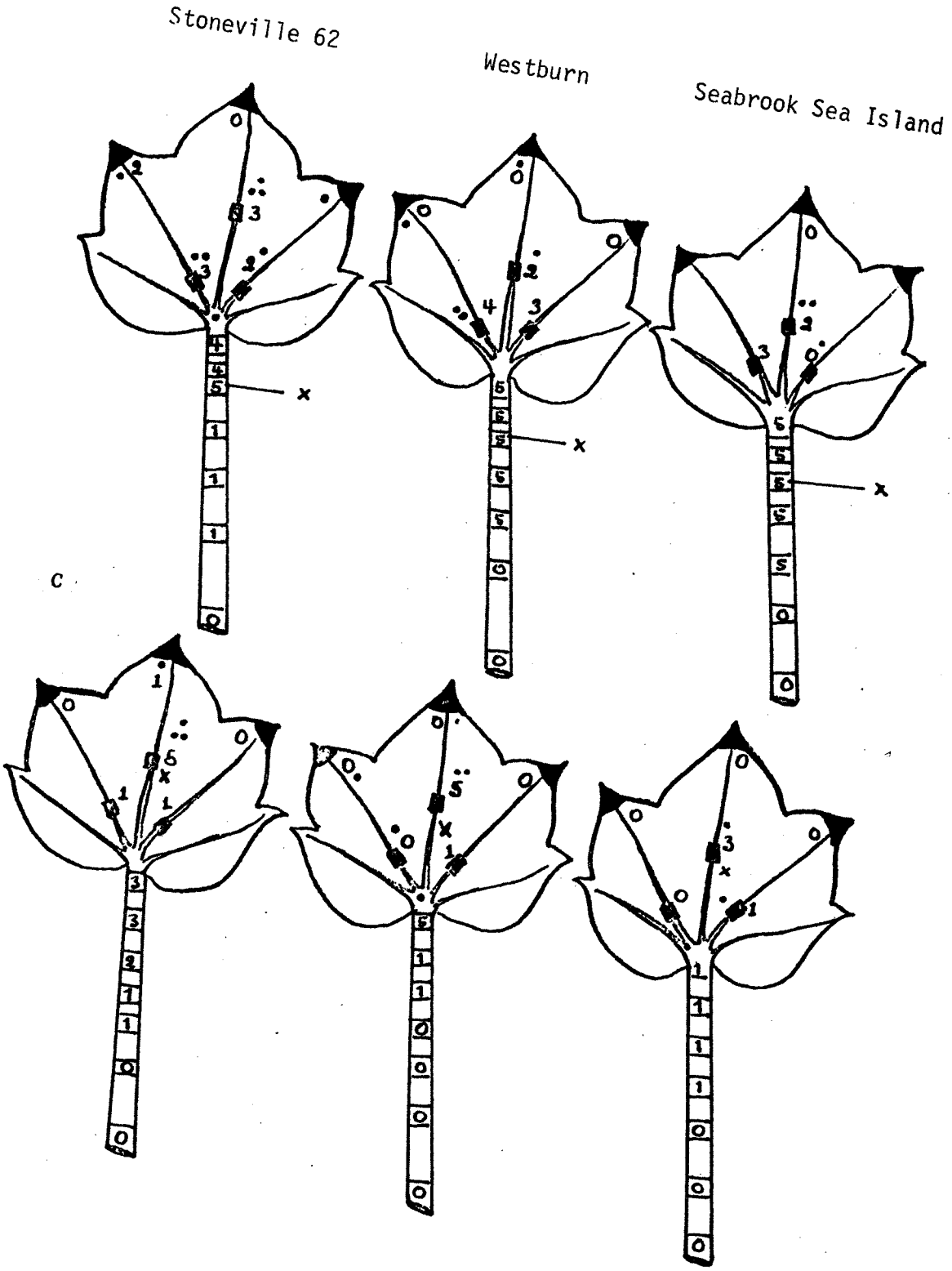

Stoneville 62
Westburn
Seabrook Sea Island
C
D

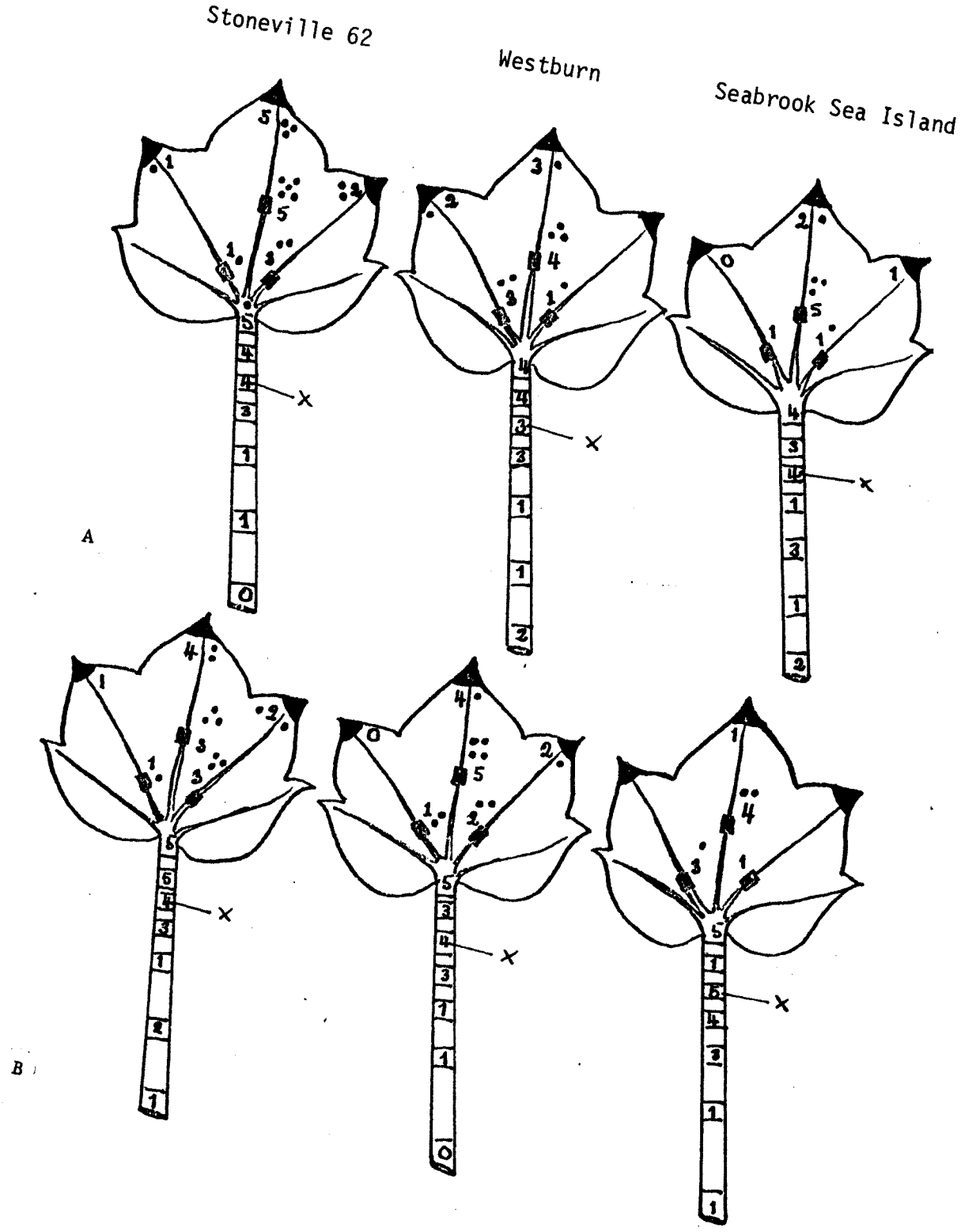
TABLE XII

EFFECT OF TEMPERATURE AND COVERING THE POINT OF INJECTION ON THE NUMBER OF PLANTS INFECTED BY F. OXYSPORUM F. VASINFECTUM IN A GREENHOUSE NUTRIENT SOLUTION. INOCULATION WAS WITH MICROSYRINGE[1]

	Covered		Uncovered	
Variety	20 C	25 C	20 C	25 C
Stoneville 62	3.4	4.0	3.6	3.8
Westburn	2.2	1.8	2.0	1.6
Seabrook Sea Island	2.2	1.8	1.4	1.6

[1]Three replicates of four leaves.

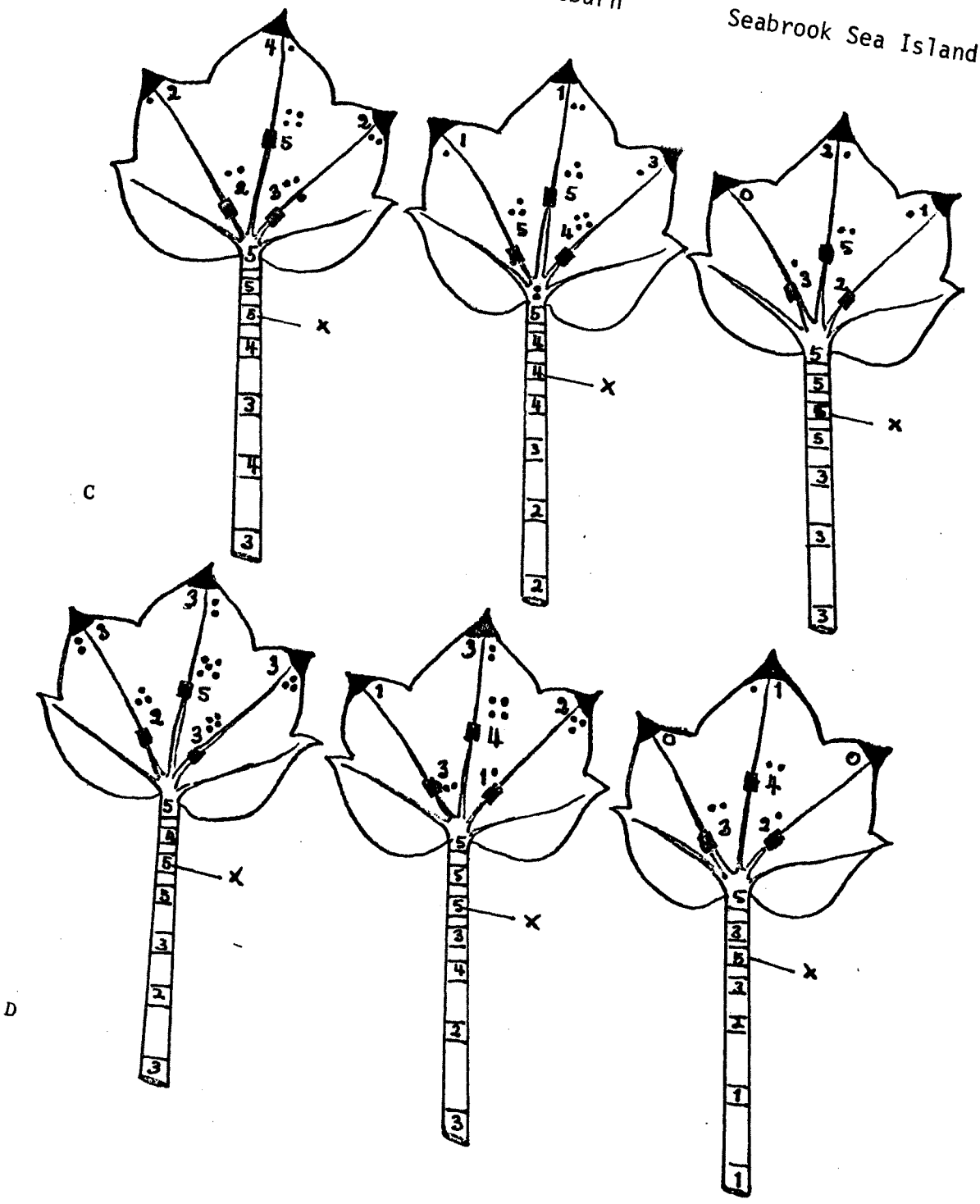
Figure 12. Number of Leaves From Which F. oxysporum f. vasinfectum Was Isolated 16 Days After Inoculation at the Locations Indicated. Isolations Were Made From Five Leaves/Variety; x Indicates Inoculation Point, Spots Indicate Number of Leaves That Showed Symptoms in That Area. A, 20 C, Taped; B, 20 C, Untaped; C, 25 C, Taped; D, 25 C, Untaped.


Stoneville 62
Westburn
Seabrook Sea Island
A
B

Stoneville 62

Westburn

Seabrook Sea Island



C

D

Results of Verticillium Wilt Studies in a Temperature-Controlled Room in Soil

At the temperature regime of 32 C days - 25 C nights, two techniques of inoculations (microsyringe at leaf blade-petiole junction and toothpick at petiole) gave effective differentiation of susceptible, intermediate, and resistant varieties. However, not 100% of the inoculated leaves of the susceptible Stoneville 62 became infected (Table XIII).

Results of Verticillium Wilt Studies in a Growth Chamber in Nutrient Solution

In the first experiment at 30 C days and 20 C nights, Stoneville 62 appeared susceptible but Seabrook Sea Island was resistant. The syringe inoculations at the nectary and petiole permitted one to distinguish between the resistant, intermediate, and susceptible varieties (Table XIV).

Isolations from infected leaves showed fungus growth through the leaf blade and down the petiole but no fungus growth at the end of the petiole. There was no difference between varieties (Figure 13).

In Experiment 2, at a continuous temperature of approximately 25 C, Stoneville 62 showed 100% infection with the disease and both Westburn and Seabrook Sea Island showed fewer leaves with symptoms from the microsyringe inoculation at the leaf blade-petiole junction (Table XV).

Isolations from infected leaves showed fungus growth down to the end of the petiole in all three varieties and also to the tips of the leaf blades. The number of infected leaves that showed fungus growth in different areas did not differ for the three varieties (Figure 14).

TABLE XIII

MEAN NUMBER OF INFECTED LEAVES OF PLANTS GROWN IN SOIL AND INOCULATED WITH V. ALBO-ATRUM BY TWO TECHNIQUES IN A TEMPERATURE-CONTROLLED ROOM (32 C DAYS, 25 C NIGHTS)[1]

Variety	Technique of Inoculation	
	Microsyringe at Petiole Junction	Toothpick at Petiole
Stoneville 62	4.0	4.5
Westburn	2.7	2.7
Seabrook Sea Island	0.7	0.5

[1]Four replicates of five leaves each.

TABLE XIV

MEAN NUMBER OF INFECTED LEAVES INOCULATED WITH V. ALBO-ATRUM USING THE SYRINGE-NEEDLE INJECTION. PLANTS WERE GROWN IN NUTRIENT SOLUTION IN A GROWTH CHAMBER (30 C DAYS, 20 C NIGHTS)[1]

Variety	Site of Inoculation	
	Nectary	Petiole
Stoneville 62	7.0	7.6
Westburn	6.3	6.6
Seabrook Sea Island	1.0	2.3

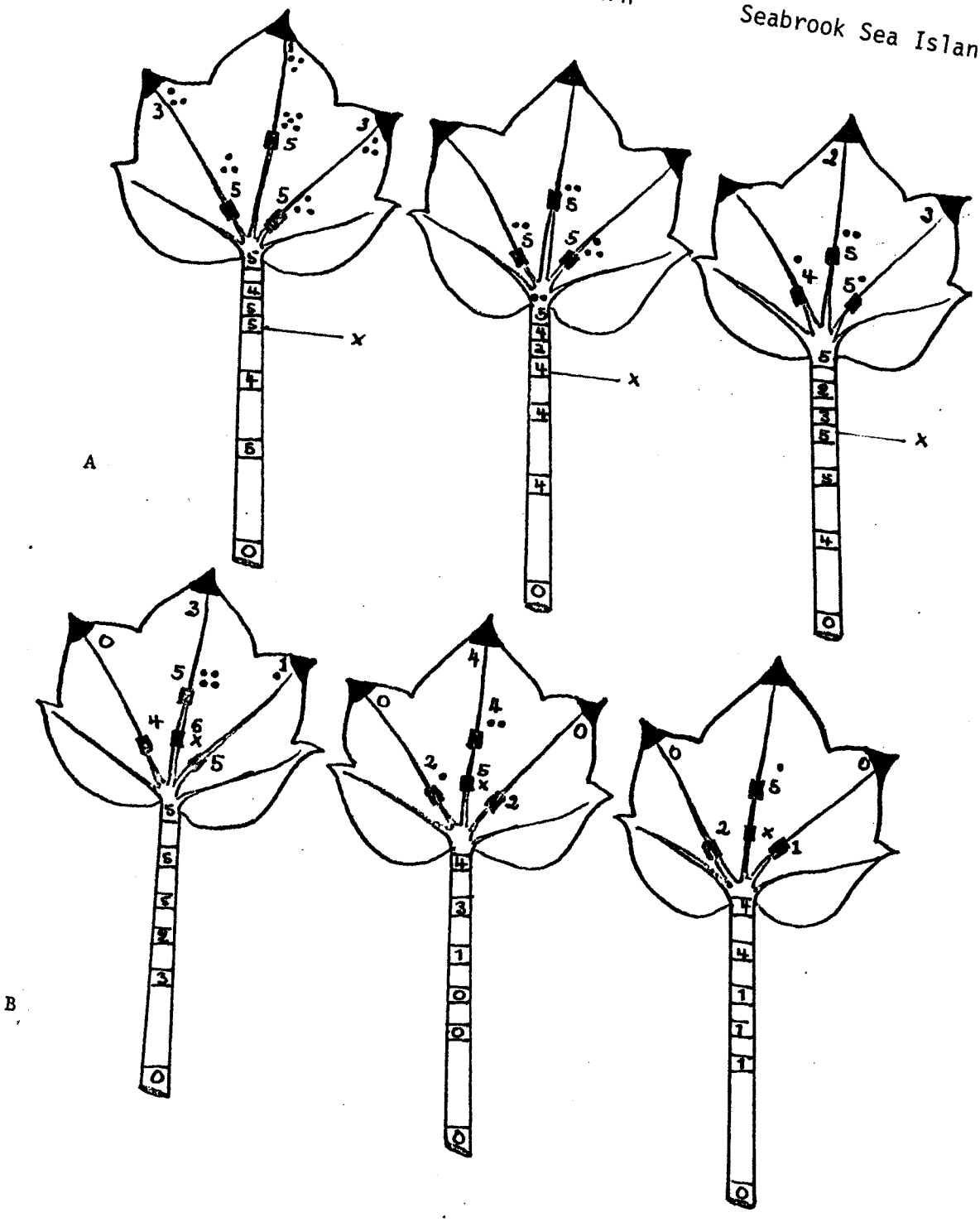
[1]Three replicates of nine leaves each.

Figure 13. Number of Leaves From Which V. albo-atrum Was Isolated Seven Days After Inoculation at the Locations Indicated. Isolations Were Made From Five Leaves/Variety/Technique; x Indicates Inoculation Point, Spots Indicate the Number of Leaves That Showed Symptoms in That Area. A, Syringe at Petiole Junction; B, Syringe at Nectary.

Stoneville 62

Westburn

Seabrook Sea Island



A

B

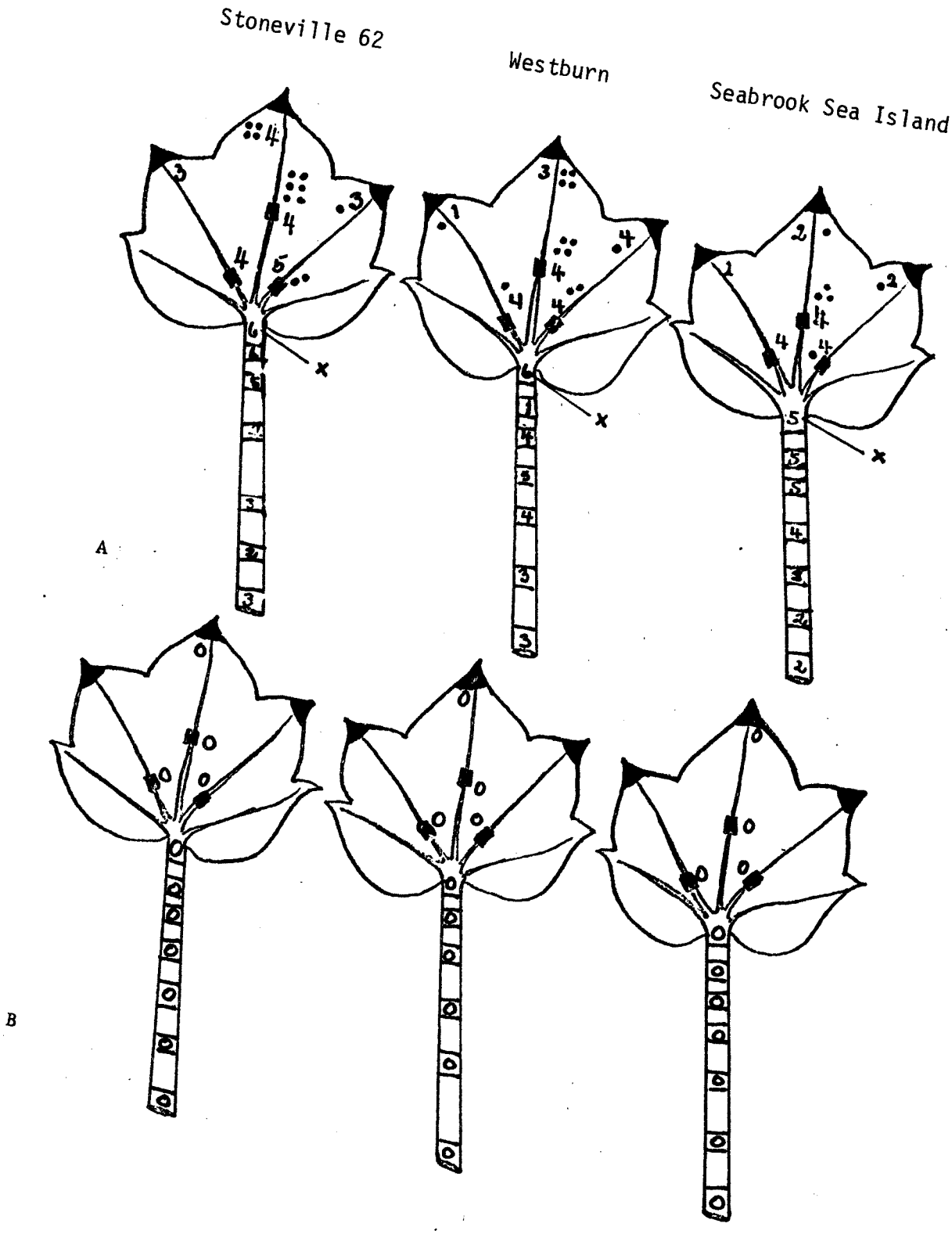
TABLE XV

EFFECT OF TEMPERATURE ON *VERTICILLIUM* WILT DEVELOPMENT AND THE EFFECTIVENESS OF INOCULATING WITH A MICROSYRINGE AT THE LEAF BLADE-PETIOLE JUNCTION. PLANTS GROWN IN NUTRIENT SOLUTION IN A GROWTH CHAMBER (AIR TEMPERATURE, 27 C)[1]

Variety	Mean Number of Infected Leaves	
	Control	Inoculated
Stoneville 62	0	6.0
Westburn	0	4.9
Seabrook Sea Island	0	4.5

[1]Three replicates of six leaves each.

Figure 14. Number of Leaves From Which V. albo-atrum Was Isolated Seven Days After Inoculation at the Locations Indicated. Isolations Were Made From Six Leaves/Variety; x Indicates Inoculation Point, Spots Indicate Number of Leaves That Showed Symptoms in That Area; Approximately 25 C Continuously.


Stoneville 62
Westburn
Seabrook Sea Island
A
B

Results of *Verticillium* Wilt Studies in the Greenhouse in Nutrient Solution

At 27 C continuous root temperature, microsyringe inoculation at the leaf blade-petiole junction permitted differentiation of varieties to the disease. Differences in the numbers of infected leaves between the three varieties were slight when inoculated with a microsyringe at the petiole or by the needle-sponge at the petiole junction (Table XVI).

Isolations showed fungus growth both downward in the petiole and upward into the leaf blade. Some infected leaves showed fungal growth to the petiole. The number of infected leaves that showed fungal growth did not permit one to differentiate resistance and susceptibility (Figure 15).

The growth of *Verticillium* mycelium from the pieces of infected leaves is shown in Figure 16.

TABLE XVI

MEAN NUMBER OF INFECTED LEAVES INOCULATED WITH V. ALBO-ATRUM USING THREE INOCULATION TECHNIQUES. PLANTS WERE GROWN IN NUTRIENT SOLUTION IN A GREENHOUSE (27 C CONTINUOUS ROOT TEMPERATURE)[1]

Variety	Technique of Inoculation		
	Microsyringe at Petiole Junction	Microsyringe at Petiole	Needle-Sponge at Leaf-Blade Petiole Junction
Stoneville 62	3.0	2.6	1.6
Westburn	2.2	2.0	0.6
Seabrook Sea Island	1.0	1.8	0.4

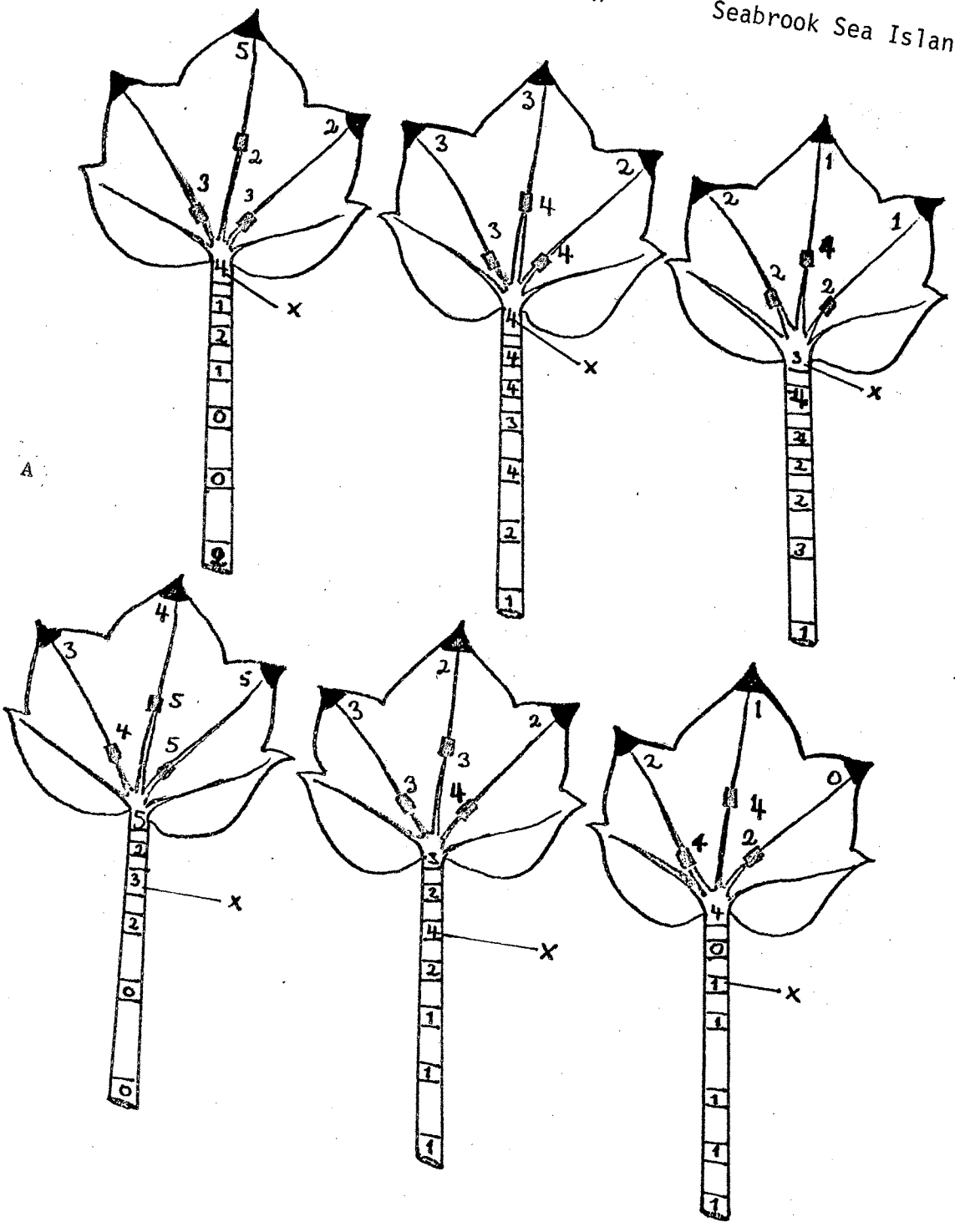
[1]Six replicates of three leaves each.

Figure 15. Number of Leaves From Which V. albo-atrum Was Isolated Eight Days After Inoculation at the Locations Indicated. Isolations Were Made From Five Leaves/Variety/Technique; x Indicates Point of Inoculation. A, Microsyringe at Petiole Junction; B, Needle-Sponge at Petiole; C, Microsyringe at Petiole; D, Control.

Stoneville 62

Westburn

Seabrook Sea Island



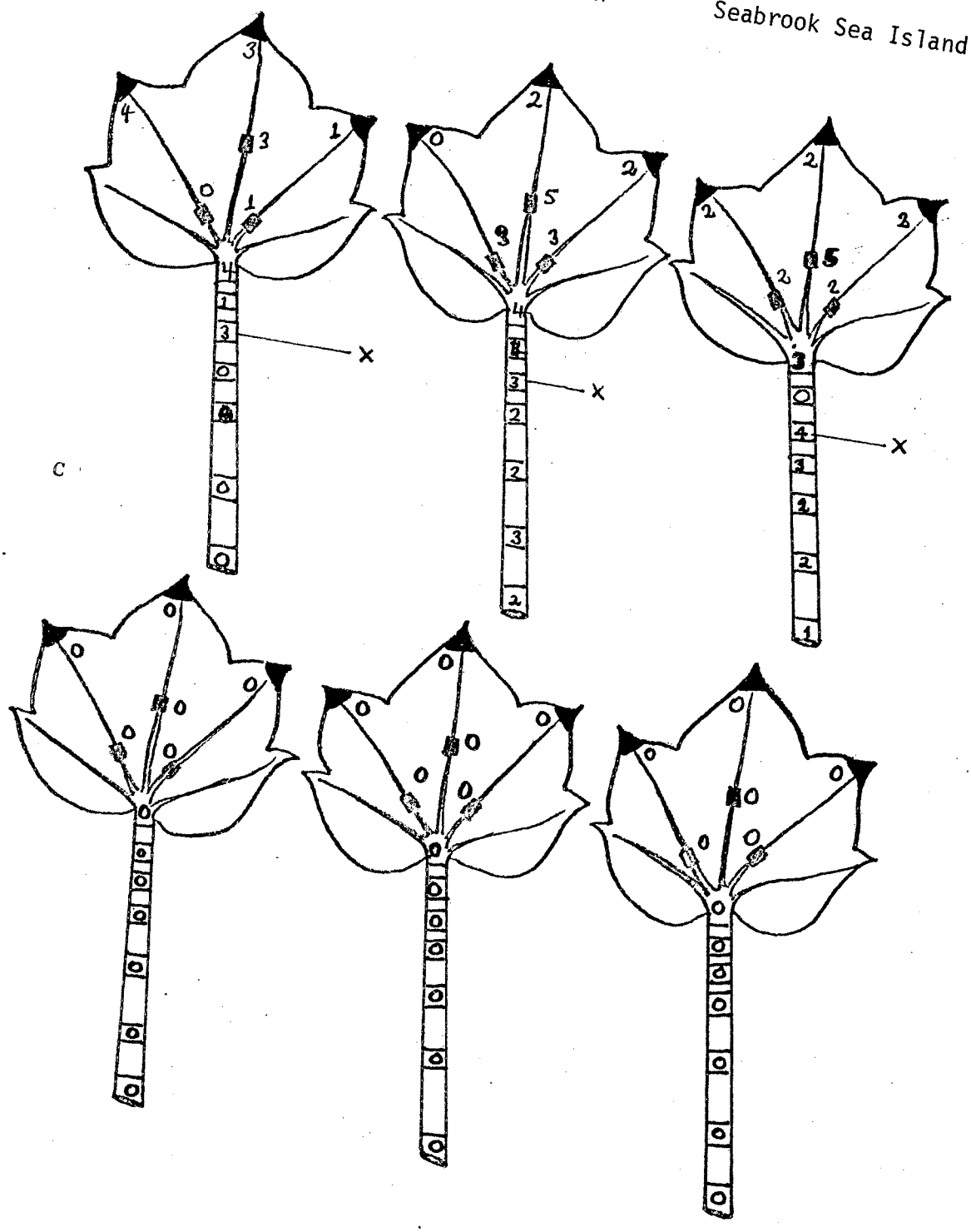
A

B

Stoneville 62

Westburn

Seabrook Sea Island



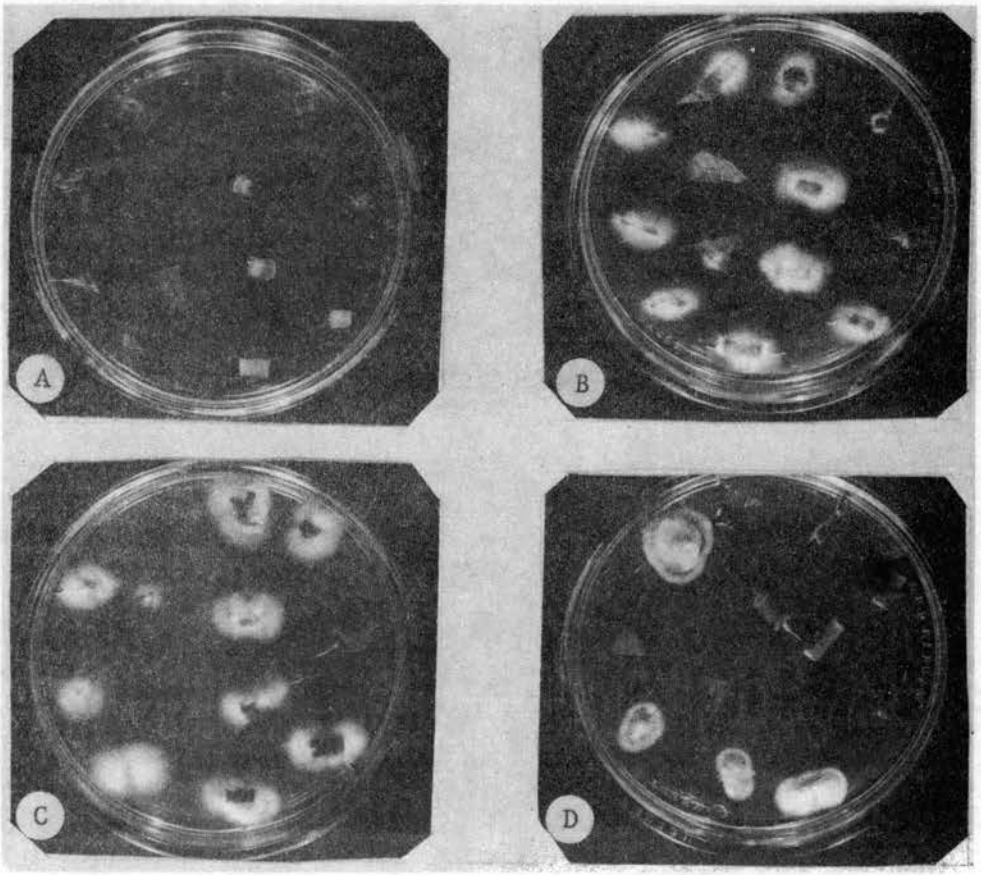


Figure 16. Growth of V. albo-atrum from Tissue Sections on PDA. A, Control; B, C, and D, Inoculated Leaves.

CHAPTER V

DISCUSSION

The technique of inoculating cotton with X. malvacearum by using a rubber tube-tipped syringe through the stomata or wounds in the leaf blade as used by Brinkerhoff (8) was effective in the present study. Removing the inoculated leaves as soon as the disease symptoms were observed prevented the spread of the organism from the inoculated leaf to the other parts of the plant.

Results of temperature tests on bacterial blight showed that 32 C days and 19 C nights were more favorable than 32 C days and 25 C nights for screening for resistance because disease symptoms showed earlier and were more pronounced. Inoculation of cotton plants with a high concentraion of bacteria (1.6×10^8 cells/ml) resulted in the earlier appearance of disease symptoms than inoculation with a lower concentration of bacteria (8×10^6 cells/ml).

A possible problem when inoculating with two wilt organisms consecutively was interference of the first organism with the second organism. It was believed that by inoculating leaves, the diseased leaves could be graded and then removed before the fungus could spread to other plant parts. Schnathorst (37) reported the bipolar movement of the Verticillium fungus in stems of cotton plants. Results from inoculation of the midvein have been reported by Mirpulatova and Nagornaya (33). They found that the Verticillium fungus spread upwards until the entire

blade became infected but the fungus did not move downward into the petiole. I found that within 7 - 16 days after inoculation of leaves or petioles with F. oxysporum f. vasinfectum or V. albo-atrum the fungi were present in the base of the petiole. The rate of movement depended on the location and technique of inoculation and on day and night temperatures. For instance, when plants were inoculated with F. oxysporum f. vasinfectum or V. albo-atrum at 30 C continuous and 27 C continuous temperatures, respectively, by using syringe injection at the nectary or at the upper part of the petiole, the fungus was isolated within seven days after inoculation from the base of the petiole. At 22 C root temperature, when plants were inoculated with F. oxysporum f. vasinfectum by means of a microsyringe injected at the upper part of the petiole, the fungus was found at the base of the petiole of some infected leaves 16 days after inoculation.

If leaf inoculations are to be successfully used to detect resistant plants, an obvious requirement is that all susceptible plants must show disease symptoms. It was found that environmental conditions and inoculation techniques which produced 100% disease expression on the susceptible variety also favored movement of the fungi toward the stem. Conversely, combinations of temperature regimes and techniques that restricted movement of the fungus did not produce visible disease symptoms in all inoculated plants of the susceptible variety. For instance, plants in the temperature-controlled room that were inoculated with F. oxysporum f. vasinfectum by means of microsyringe injection at the leaf blade-petiole junction under a 32 C - 25 C regime showed 100% disease symptoms in the susceptible variety Stoneville 62. Isolations showed that the fungus was present at the base of the petiole at the

time that the disease readings were taken. Further studies may show that readings could be taken earlier and still obtain 100% infection of leaves of the susceptible variety. On the other hand, plants which were inoculated with the same fungus at the same temperature regime by means of a fungus-saturated toothpick injected at the petiole did not have the fungus at the base of the petiole. However, the susceptible variety Stoneville 62 did not show 100% infection either.

Each of the techniques used to inoculate leaves with the wilt fungi has certain advantages. Use of a microsyringe was better than using a regular syringe for three reasons: (1) The amount of inoculum used each time could be measured easily; (2) There was no waste of inoculum during inoculation; and (3) The hole of the microsyringe needle did not become plugged with plant tissues. The needle-sponge technique did not give a high percentage of infected leaves in the susceptible variety. However, this technique could be used to distinguish between the three varieties by the number of infected leaves. Evans (19) successfully used the needle-sponge technique. This was probably because of the difference in the location of the injection site on the plant - the lower portion of the stem. I agree with Evans that the needle-sponge technique is rapid and a large population of plants can be inoculated in a short time. This technique would probably have been more effective if the needle had penetrated the vascular tissue in each instance. A fungus-saturated toothpick was found easy to use and also saved time in inoculating. Young (46) pointed out the advantages of using this technique to introduce the stalk rot organism into corn. The findings in the present study showed that this technique is suitable for the purpose of studying more than one disease in the same plant. Also,

the fungus did not spread very rapidly after the inoculation and the infected leaves could be removed before the fungus spread to the base of the petiole. Three disadvantages of this technique were found: (1) It was not satisfactory to inoculate small parts of the plant, such as veins; (2) It was not possible to increase or decrease the concentration of the inoculum; and (3) The concentration of inoculum was unknown. Probably, the method would be 100% effective if the point of the toothpick could be inserted into the vascular tissue each time. More investigations are needed to determine if this would be possible.

Each of the different locations for inoculations, the upper portion of the petiole, the leaf blade-petiole junction, and the midvein, has certain advantages or disadvantages. The further the point of inoculation was from the base of the petiole, the longer it took the fungus to spread to the stem. The midvein of the leaf was considered to be a good point of inoculation. However, I did not get satisfactory results from inoculating with a syringe or microsyringe at the midvein. The percentage of infected leaves of the susceptible variety was not high enough. Because the midvein is small, it is difficult to place the inoculum in the vascular tissue. It was found convenient to use the nectary as the point of inoculation and a high percentage of infected leaves in the susceptible variety was obtained. But at the nectary, it was sometimes difficult to make the injection because of the presence of sugary substances plugging the nectary. Inoculating the petiole about 2 cm from the leaf blade-petiole junction with a syringe, microsyringe or toothpick gave a high percentage of infected leaves. But inoculation at this location never gave 100% infected leaves of the susceptible variety. This was probably because the inoculum did not always get into

a vascular bundle. In a cross-section of the petiole, the vascular bundles are seen to be separated from each other (4). Thus, the point of the syringe or microsyringe might have entered the tissues between the vascular bundles. At the leaf blade-petiole junction, the vascular bundles are crowded and close together (4); therefore, the chance of injecting into the vascular bundle appeared to be better than elsewhere. The advantages of using a microsyringe to inoculate at the leaf blade-petiole junction are: (1) Inoculum was usually rapidly absorbed; (2) Inoculation was rapid; and (3) The highest percentage of infected leaves was obtained in the susceptible variety. Inoculation at the leaf blade-petiole junction has a disadvantage - the fungus spreads relatively rapidly from the point of inoculation to the base of the petiole.

The fact that differences between resistant, intermediate, and susceptible varieties could be determined by inoculations at the petiole or leaf vein is encouraging. It is feasible that under the proper conditions all susceptible plants within a mixed population could be successfully eliminated by the use of leaf inoculations. Further studies will have to be made, especially on the optimum concentration of inoculum. For these studies, it is suggested that the microsyringe technique of inoculation at the leaf blade-petiole junction be used.

CHAPTER VI

SUMMARY

Results of experiments designed to study the techniques of inoculating cotton with the bacterial blight, Fusarium wilt and Verticillium wilt organisms are as follows:

1. The resistance and susceptibility of four cotton varieties to the three organisms were evaluated by subsequent inoculations of single plants.

2. The use of a rubber tube-tipped syringe technique of inoculating plants at the cotyledon with suspensions of X. malvacearum permitted the differentiation between the resistance of Acala 414 and the susceptibility of Stoneville 62, Westburn, and Seabrook Sea Island to bacterial blight. Disease symptoms in susceptible plants appeared earlier under a 32 C day - 19 C night regime than under a 32 C day - 25 C night regime.

3. Syringe injection at the nectary and at the petiole, and toothpick inoculation at the petiole permitted a better differentiation between resistant and susceptible cotton varieties grown in soil under a 32 C day - 25 C night regime to F. oxysporum f. vasinfectum than the needle-sponge technique at the petiole. In a separate test, microsyringe injection at the leaf blade-petiole junction and toothpick inoculation at the petiole permitted one to differentiate between Stoneville 62, Westburn and Seabrook Sea Island. However, the microsyringe technique

appeared to be better than the toothpick technique, when judged on the number of infected leaves. However, the spread of the fungus to the base of the petiole was relatively fast. No plants inoculated at the leaf blade by means of a rubber tube-tipped syringe became infected.

4. Previous inoculation of plants with F. oxysporum f. vasinfectum did not affect the response of these plants to inoculation with V. albo-atrum, when judged on the number of infected leaves of plants inoculated with V. albo-atrum alone.

5. The disease reactions of Seabrook Sea Island, Stoneville 62 and Westburn grown in modified Hoagland's solution under a 30 C day - 20 C night regime to F. oxysporum f. vasinfectum were differentiated by the use of the syringe inoculation technique at the nectary and at the petiole. More leaves were infected when the inoculation was made at the nectary than at the petiole. The fungus spread more into the leaf blade than into the petiole following inoculation at the nectary; whereas, the fungus spread both up into the leaf blade and down into the petiole following inoculation in the upper part of the petiole. In a separate test at 30 C continuous temperature, the microsyringe inoculation at the leaf blade-petiole junction permitted differentiation among the resistant, intermediate and susceptible cotton varieties, but the microsyringe inoculation at the midvein and the needle-sponge inoculation at the petiole did not. Isolations from infected leaves showed that the fungus spread downward into the petiole and upward into the leaf blade with every technique.

6. The effect of two root temperatures (20 C and 25 C) in modified Hoagland's solution and covering the inoculation wound was slight and inconsistent for plants inoculated with the Fusarium wilt fungus.

7. The use of two techniques of inoculating cotton plants grown in soil under a 32 C day - 25 C night regime with V. albo-atrum (microsyringe inoculation at the leaf blade-petiole junction and toothpick inoculation at the petiole) distinguished the resistance of Seabrook Sea Island, the intermediate reaction of Westburn and the susceptibility of Stoneville 62. However, 100% infection of inoculated leaves of Stoneville 62 was not always obtained.

8. In a growth chamber in modified Hoagland's solution maintained under a 30 C day - 20 C night regime, the use of the syringe inoculation technique at the nectary and in the petiole of plants also differentiated the resistant, intermediate and susceptible reactions to V. albo-atrum. The fungus grew through the leaf blade and down the petiole, but not to the end of the petiole. When plants at 27 C continuous temperature were inoculated by a microsyringe at the leaf blade-petiole junction, Stoneville 62 showed 100% infection with Verticillium wilt, while Westburn and Seabrook Sea Island showed 82% and 75% infection, respectively. The fungus grew through the petiole and to the tips of the leaf blades in all three varieties. At 27 C continuous temperature, microsyringe inoculation at the leaf blade-petiole junction of plants grown in modified Hoagland's solution in the greenhouse permitted better differentiation of plant reactions to the disease than microsyringe inoculation at the petiole and needle sponge inoculation at the leaf blade-petiole junction. Resistance and susceptibility to the disease were not differentiated by isolations from infected leaves.

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