DISSEMINATION OF THE VERTICILLIUM WILT FUNGUS BY COTTON LEAVES

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By

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PREFACE

The author wishes to express his appreciation to Mr. Lloyd A. Brinkerhoff, Associate Pathologist, U. S. Department of Agriculture and Oklahoma Agricultural and Mechanical College, for suggesting the problem, and for his many helpful suggestions; to Dr. W. W. Hansen, Head of the Department of Botany and Plant Pathology, Oklahoma Agricultural and Mechanical College, for advice and for permitting the generous use of departmental equipment and facilities.

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TABLE OF CONTENTS

INTRODUCTION

Verticillium wilt of cotton has become one of the most serious disease problems of the irrigated agricultural regions of the west and the southwestern part of the cotton belt. Within this region of alkaline soils there has apparently been a very rapid spread of the disease in the past fifteen years.

Evidence that the pathogen can be transmitted to new locations has been obtained in several places. One instance that may be cited occurred at the United States Field Station at Sacaton, Arizona during the summer of 1948.[1] Verticillium wilt had not been observed during 36 previous years of cotton breeding and testing. Wilt first occurred in two isolated areas of a four acre block of land which had been used for testing cotton varieties. Cotton seed for at least the ten previous years had come from wilt-infested fields of both California and New Mexico.

Preliminary isolation and greenhouse studies by L. A. Brinkerhoff have established the presence of the pathogen in dry infested leaf tissue and the ability of this tissue to transmit the pathogen.[2] Leaves may be widely spread by wind, irrigation and rain water, and probably on agricultural implements, and might well constitute an important factor in the rapid build-up of the disease in the western cotton growing area.

The purpose of the following investigation was to substantiate Brinkerhoff's experiments with dry leaves, obtain data on the effect of aging and dessication of infested leaves, and histologically study infested leaf material.

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[1] L. A. Brinkerhoff. Unpublished data.

[2] *Ibid.*

LITERATURE REVIEW

History and Distribution.—The Verticillium wilt fungus, *Verticillium albo-atrum* Reinke and Berth., and its related forms are wide-spread vascular parasites. Although the pathogen was first described and named in 1879 (18) its pathogenicity on the cotton plant (*Gossypium* spp.) was unknown within the United States until Carpenter (3) in 1914 discovered two diseased cotton plants at Arlington, Virginia which yielded pure cultures of the fungus.

Verticillium wilt of cotton apparently was not again considered as a disease of cotton until 1927 when Shapalov and Rudolph (21) reported it in a field near Wasco, California. They postulated the introduction of the fungus by means of seed potatoes which had been planted and plowed under just prior to the planting of the cotton. From this first occurrence of the wilt pathogen a rapid spread was noted. By 1930 Verticillium infestations were observed in four counties of the San Joaquin valley (9). In a survey by Harrison and Brinkerhoff (8) in 1946, the disease was found to be widely distributed throughout the cotton growing area of central California. Estimates placed the amount of infection at 20 per cent and economic losses caused by the pathogen from 5 to 20 per cent.

In New Mexico, Verticillium wilt is now considered the most important cotton disease in the state (5). As in other western states, a very rapid increase in the amount of infection has been noted.

Verticillium wilt of cotton in Oklahoma was first reported in 1932 (10). McLaughlin (15) isolated *V. albo-atrum* from diseased plants from Geary and Mangum, Oklahoma. Other reports (12) have located the disease in Johnson and Tillman counties.

In 1929 Taubenhaus *et al.* (26), reported a cotton wilt in three counties of west Texas which was designated "waxahachie" wilt, and which later proved to be caused by *V. albo-atrum.* Other states which have reported Verticillium wilt infestations of cotton include Tennessee (22), Arkansas (28), Mississippi (17), Louisiana (1), Alabama[2], and Arizona[3]. A report by Lehman and Garriss (14), in 1948 identifying Verticillium wilt infected plants in North Carolina completes the known occurrence of the disease across the entire cotton belt.

Where the soils are alkaline a similarity between nearly all of the reports of Verticillium wilt is noted. Following the original discovery of the disease, which isusually an isolated spot, a rapid increase occurs. Seventy to ninety per cent of a new area may become involved with losses of 20 to30 per cent being common.

Not only the cotton producing areas of this country but every major cotton growing region of the world has reported losses from this disease. Sarrejanni (20) reported the infection of cotton in Greece in 1933. Experiment stations in Africa and Australia (7, 23, 27) report its occurrence in those countries. In the cotton regions of Russia (24) Verticillium wilt damage is very acute on susceptible varieties. It is of common occurrence in Peru (2), where much work is being attempted to control the disease.

Methods of Dissemination.—Several means of dissemination of the pathogen have been proposed. Among the more common of these are infested plant

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[2]Presley, John T. Unpublished paper presented to a joint meeting of the Southern and Potomac Sections of the American Phytopathological Society.

[3]Brinkerhoff, L. A., and B. H. Waddle. Mimeographed report presented to the Southwest 4-state Cotton Growers Association, Sacaton, Arizona. October, 1948.

debris (19), asexually propagated crops, and crops in which seed infestation is known to occur.

Internal infestation of cotton seed was reported by Taubenhaus (25), while Brown[4] claimed to have isolated the pathogen from the lint of cottonseed. The common occurrence of these two types of seed infestation has largely been discounted by Rudolph and Harrison (19). In extensive laboratory isolation tests with infected cotton plants they were unable to show penetration of the fungus into the seed, although its occurrence in the bracts, receptacles, and placentae was established. They also reported that the fungus died fairly rapidly from the tops of cotton plants after frost.

In some instances seed transmission of the fungus has been postulated to account for a new occurrence of the disease. Sarrejanni in letters to Miles (16) proposed such a theory to explain an outbreak in Greece of Verticillium wilt which first appeared in a field of cotton grown from seed imported from North Carolina. However, the possibility of the pathogen already being present in the soil but unable to attack native varieties was also pointed out. The disease in Greece has been confined to introduced varieties.

Further support of the theory that the pathogen occurs indigenously in some soils has been obtained by Presley.[5] In 1937 an outbreak of Verticillium wilt occurred in a crop of cotton planted to the land for the first time in Hidden Valley, Arizona. This valley is well isolated from other cultivated areas and is irrigated by pump water. For three years, using precau-

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[4]Rudolph, B. A., and J. G. Harrison. Cited from newspaper accounts. (Ref. 19 Biblio.)

[5]Presley, John T. Unpublished paper presented to the Southern States Cotton Growers Association. Memphis, Tennessee. 1942.

tions to eliminate any chance of seed-borne infection, Presley studied further occurrence of the disease in the valley, and concluded that infection resulted from the indigenous occurrence of the pathogen.

Hansford (7), from his observations on the spontaneous occurrence of the disease on first-cropped land, concluded also that V. albo-atrum must be indigenous to certain soils in Uganda. In 1938 he reported 90 per cent infection of cotton crops when planted for the first time.

Seed dissemination, both internal and external, has been proven in tomatoes and eggplants (11). Transmission of the fungus by infested potato tubers also occurs. When crops such as these are used in crop rotations, introduction of the fungus may occur.

Pathological Histology.— Few histological studies have been made on Verticillium wilt infested plants. Reinke and Berthold (18) demonstrated the fungus hyphae within the vascular and parenchyma tissues of the potato plant. The presence of microsclerotial forms of the fungus were shown as they occurred in the vascular elements. Carpenter (3) demonstrated V. albo-atrum within the tissues of okra plants. Both of these works showed hyphae of swollen and abnormal forms. Neither of them showed the characteristic verticillate branching of the conidiophores within the tissues of the host.

The pathological histology of Verticillium wilt of cotton has not been reported in the literature.

METHODS AND MATERIALS

Greenhouse experiments.—It has been reported that optimum soil temperature for the development of Verticillium wilt is 73 to 75 degrees Fahrenheit, and that above 86 degrees the disease is inhibited (13). In order to determine if temperatures were favorable for the experiments reported herein, a continuous record of soil temperatures was obtained by a thermograph. Table 1 lists the weekly maximum and minimum means for the duration of the experiments. On several occasions the temperature exceeded 86 degrees, but never remained above that for more than four hours. As can be seen in Table 1, temperatures were close to the optimum for the most part.

Table 1. Greenhouse soil temperatures for winter 1948-49. Weekly means recorded in degrees Fahrenheit.

Week ending		Maximum	Minimum	Week ending		Maximum	Minimum
November	29	75	70	March	2	79	78
December	7	72	64		9	70	67
	14	77	74		16	69	66
	21	69	65		23	77	75
	28	67	62		30	74	70
January	5	63	62	April	4	70	67
	12	71	69		13	71	69
	19	77	74		20	64	58
	26	77	75		27	70	63
February	2	77	75	May	4	76	70
	9	72	66		11	77	71
	16	73	71		18	78	75
	23	74	70				

In order to determine whether leaves can disseminate V. albo-atrum, and to investigate conditions which affect the fungus in the leaf, a series of greenhouse experiments was initiated in November of 1948. These experiments are briefly outlined as follows: (1) addition of infested leaf material to sterilized soil prior to planting; (2) injection of microscopic particles of leaves into healthy plants; and (3) the addition of minute particles of leaf material to the fuzz of cotton seed before planting.

For these experiments a collection of infested cotton leaves was made by L. A. Brinkerhoff and L. M. Blank. This infested material, which varied in age and degree of decomposition, was obtained from various sections of five southwestern states where Verticillium wilt is most prevalent. A description of this material and the source is given below:

1. Snider, Texas; collected on October 4, 1948. All leaves showed severe mottling and scorching which characterize the advanced stages of Verticillium wilt of cotton. All leaves were taken directly from infected plants.

2. Shafter, California; collected July 30, 1947. Leaves taken from infected plants as in Number 1 just prior to the time they would have been shed.

3. Shafter, California; collected August 1, 1947. These leaves were picked from the ground about infected plants and were partially decomposed.

4. Roswell, New Mexico; collected directly from the plants September 28, 1948.

5. Thatcher, Arizona; picked directly from the plant August 13, 1948.

6. Thatcher, Arizona; picked as Number 5, but due to being placed in storage while still moist the leaves were stained and had developed a fungus growth on the surface.

7. Tipton, Oklahoma; collected directly from the plant October 14, 1948.

In addition to these leaves, infested cotton stems collected at Tipton, Oklahoma on November 10, 1948 were used. All collections were stored at room temperatures in paper sacks under dry conditions.

The first experiment was designed to determine whether dry infested cotton leaves could transmit Verticillium wilt to uninfected plants. For this planting fifty 1/2 gallon glazed pots were cleaned and disinfested with a mercuric chloride solution. A heavy clay-loam soil mixed with well rotted manure, both of which had been steam sterilized for 10 hours, was added to the pots. Hydrated lime was added to the sterilized soil-manure mixture to give an initial pH of 8.2.

Six leaf sources (numbers 1, 2, 3, 4, 6, and 7 as listed above), two stem sources, one inoculated control, and one uninoculated control, constituted the planting. The 10 treatments were randomized and each replicated five times. The leaf blades were separated from the petioles, crushed, and spread in an even layer at a depth of two inches below the surface of the soil. Equal amounts of leaf material were weighed for each replication of a single source. Depending on the amount of leaf material available, the amount used varied. Table 2 lists the amount of leaf material used. The first stem source was cut into two inch sections and arranged radially in the pots at a depth of two inches. The second stem source was cut into fine shavings and spread at the same depth. For the inoculated control, an agar slant of a two weeks growth of the fungus was added to the soil at the same depth as the plant material. Immediately after the addition of these materials to the pots, four seeds per pot of a susceptible cotton variety, Acala Roundboll, were planted. These seed were grown in an area that was known to be free of the Verticillium wilt fungus. Germination was good and the number of seedlings was reduced to one per pot.

Table 2. Amount and source of Verticillium wilt infested cotton plant material added to pots in experiment 1.

	Leaf Sources						Stem Sources	
	1	2	3	4	6	7	8	9
Amount in grams	8	4	4	2	2	1	10	5

The second greenhouse infection experiment was set up in order to determine the effect of longevity and dessication upon pathogen within the leaf. Since there are various ways in which dry leaves can be shattered and broken into small fragments in the field, it was felt that a knowledge of the effect of severe grinding of the infested tissue was desirable.

Forty 6-inch clay pots were cleaned, disinfested and filled with soil in the same manner as in experiment 1. Disease-free seeds were planted on November 20, 1948. The plants made normal growth and seedlings were reduced to one per pot. Six leaf sources were used, numbers 1, 2, 3, 4, 5, and 6 as listed above. Under aseptic conditions a quantity of leaf tissue from each source was thoroughly pulverized with a mortar and pestle. Each source was stored in a separate test tube at room temperatures until the date of injection.

Just prior to the injection of the ground tissue into the plants, the dry material was sifted through four thicknesses of cheesecloth. A suspension in sterile water was prepared using 0.5 gram of leaf tissue to 10 ml. water. Injection into the plant was made with a Berton, Dickinson Veterinary syringe equipped with a 20 gauge Yale-Type needle. The needle was inserted into the stem at about a 45-degree angle at the soil line. To reduce the possibility of contamination, the sterile water controls were injected first. Between each series of injections the syringe and needle were thoroughly washed in water and sterilized with 95 per cent ethyl alcohol. A series of check plants was injected with a water suspension of a pure culture of *V. albo-atrum*, prepared by mixing one agar slant with 200 ml. of water, in a Waring Blender, and straining through eight thicknesses of cheesecloth. Approximately one milliliter of both the culture inoculum and leaf material was injected into each of the plants. Figure 1 is a photomicrograph of the suspension of leaf material.

The pithy interior of the plant stem had become woody and toughened so that injection was very difficult. Also the needle used for injection continually became clogged, so that there was a question as to whether leaf particles were actually introduced into all of the plants. A different technique was attempted some ten weeks later in the hope that more consistent results could be obtained. The ground leaf material, which had been stored in plugged test tubes at room temperatures was used. A V-shaped cut was made at the base of the stem of plants showing no symptoms from the previous injection; this allowed a flap to be pried away from the stem. With a fine-pointed pair of forceps a small pinch of the ground leaf inoculum was placed in the exposed woody cylinder. The flap was replaced and bound tightly with several layers of friction tape.

The third greenhouse experiment was undertaken to determine if infested leaf material carried on the fuzz of cotton seed is able to transmit Verticillium wilt. Five leaf sources (numbers 1, 3, 4, 5, and 6 as listed above) and one uninoculated control were used. Again the leaf sources were randomized and each replicated five times. Thirty clean, disinfested pots were filled with soil as in experiment 1 except that ground limestone (one per cent by weight) was used in place of hydrated lime. Very small pieces of the leaf tissue were attached to the lint of the cotton seed just prior to planting. In replications 1, 2, and 3, small pieces from the petiole-blade junction were attached, and in replications 4, and 5, pieces of the blade proper were used. By moistening the seed, the particles adhered to the fuzz. This planting was made on November 14, 1948.

Early in January, 1949, this experiment was repeated. The new pots of soil were sterilized by autoclaving at 15 pounds pressure for two hours. Instead of using the old leaf sources, infected leaves from experiment 1 were used as a source of inoculum. The method of attachment of the leaf particles was similar to the first planting. The soil, a fertile sandy type, was not packed tightly in the pots. Lime was not added since the original soil reaction was alkaline. Continual irrigation caused the soil level to drop in the pots. On two occasions freshly autoclaved soil was added to the pots to maintain the soil at the top of the pots.

Isolation studies.—Prior to using the leaf sources listed previously for the greenhouse infection tests, laboratory isolation studies were made in an attempt to determine whether the fungus was viable, and in what part of the plant it occurred most frequently.

Pure cultures were obtained more easily and consistently from fresh root and stems rather than from leaves or dry plant material. Isolations from young stems were made by peeling the bark from a one-half inch length of tissue with sterile forceps and scalpel, dipping into a 1:10 solution of sodium hypochlorite (Chlorox) for five to ten seconds and planting on potato-dextrose agar slants. With large stem sections, a small block of the discolored xylem was cut away with a sterile scalpel, surface sterilized and planted on the agar.

Isolation of the fungus from leaf material was more difficult. Two general areas of the leaf were used for the isolations. One, the junction of the leaf blade with the petiole, where there is a maximum of vascular tissue; and two, yellowed and necrotic areas of the leaf blade proper.

First isolations were attempted on the dry leaf sources listed previously which were from one to thirteen months old. Surface sterilization of the tissue was accomplished by immersing in 95 per cent ethyl alcohol followed by dipping in a 1:10 solution of Chlorox. The alcohol immersion seemed to facilitate a thorough wetting of the margins of the tissue, and gave a better surface disinfestation. Time schedules for length of immersion in the two liquids varied with the general size and condition of the tissue, but usually 5 to 10 seconds in the alcohol followed by 30 to 45 seconds in the Chlorox solution proved satisfactory.

For isolation from the ground tissue used in the second greenhouse experiment, a sterilized natural medium as described by Hansen and Snyder (6) was used. Treatment of the straw for 24 hours with propylene oxide at the rate of 1 ml. per liter capacity of the container sterilized the tissue. Approximately 15 ml. water agar and 0.2 gm. of ground straw were used for each petri dish. This method eliminated the necessity for surface sterilization, which would have been impractical for the ground tissue. Although this type of isolation is designed to reduce contamination, many different fungi were evident, making it difficult to obtain V. albo-atrum in pure culture.

In all cases of infection in the greenhouse experiments, positive identification of the causal organism was made by isolation of the fungus from the diseased plants. These isolations were all from stem sections as described above.

<u>Pathological Histology</u>.--Leaf material from one infected plant of experiment 1 which had shown severe wilt symptoms was selected for sectioning. The leaf, which had been killed by the disease, was stored in a paper sack and dried approximately two weeks prior to sectioning.

The tissue was fixed in FFA solution for 48 hours. It was then passed through the ethyl alcohol series and into chloroform for paraffin infiltration. The material was subjected to a vacuum for a short time to remove air from the tissues before adding the paraffin. A temperature of 55 degrees C. was maintained for imbedding in the paraffin blocks. A rotary microtome was used for the sectioning.

After fixing the ribbons, the paraffin was dissolved from the sections in xylol and the slides passed back through the alcohol series to water. Safranin and fast green dissolved in 50 per cent alcohol were found to be satisfactory stains. Safranin dissolved in 50 per cent alcohol, and light green dissolved in clove oil gave better results however. The slides remained in safranin 12 hours and were then passed into xylol after which light green was applied. The light green destained the safranin, and was in turn destained by pure clove oil. It was then put into xylol and mounted in nevillite.

Leaves from healthy plants as well as leaves from infected plants grown in sterile soil and inoculated by a pure culture injection were sectioned by the free-hand method. The sections were stained with acid fuchsin.

RESULTS

Greenhouse experiments.—Table 3 shows the results of experiment 1, giving the amount of infection induced by infested leaf trash. The leaf sources from Snider, Texas; Roswell, New Mexico; and Tipton, Oklahoma, resulted in the highest rate of infection, with all replicates becoming infected.

This experiment was primarily a test of infested leaf material, but the two stem sources, numbers 8 and 9, were included as a comparative check. Although each stem source was of identical material, noticeable differences were found between the treatments. Number 8, where whole sections were planted, produced the disease in 4 of 5 plants, while number 9, in which the source was cut into fine shavings, produced the disease in only 2 of 5 plants.

The series inoculated with a pure culture were slow in being attacked and showed only slight symptoms of the disease. The cultures which were used for inoculation were transferred from a culture isolated from an infected cotton plant at Sacaton, Arizona. This fungus had been continuously cultured on agar for 4 months, and, although sclerotial cultures of this isolate have been obtained, the cultures used here produced no sclerotia.

Leaf sources 1, 4, and 7, which were one month, two months, and one month old respectively, gave the most consistent results even though the amount of leaf material in the pots varied from 8 grams to 1 gram. Numbers 2 and 3, which were 13 months old, and number 6, which was three months old, gave the lowest amount of infection. This may indicate that age of the leaf material rather the n the amount is of more importance in producing infection.

In all cases of infection, except the pure culture inoculated series, the symptoms were severe. Complete shedding of the leaves and subsequent infection of new leaves was common. General stunting of the affected plants occurred. Figure 2 shows a typical infected plant as compared with an uninoculated control. In all replications the uninoculated controls remained healthy.

Table 3. Number of plants of five single plant replicates infected by *V. albo-atrum* at monthly intervals in experiment 1.

Source	January 10	February 10	March 10	April 10	Total
1	1	0	3	0	4
2	0	0	3	0	3
3	0	0	0	3	3
4	2	2	0	1	5
6	0	0	1	2	3
7	0	5	0	0	5
8[a]	2	1	1	0	4
9[a]	1	0	1	0	2
10[b]	1	0	2	0	3
11[c]	0	0	0	0	0
Per cent of plants infected	14	16	22	12	64

[a] Stem sources.
[b] Culture inoculated control.
[c] Uninoculated control.

The results of experiment 2 determining the viability of the fungus in the crushed leaf material are shown in Table 4. The experiment was terminated on May 27, 1949. All of the plants were cut at the base of the stem and inspected for typical discoloration. Within the area of injection on most plants a very black discoloration was common, extending up and down from the injection point a distance of 2 to 5 centimeters. These plants were not considered to be infected with Verticillium wilt.

Following the second inoculation, a tendency for the wounds to dry fairly rapidly, despite the binding of tape, was noticed. Later in the experiment, relatively large cankers, galls, and scars were in evidence. It was thought that in some cases an impermeable corky layer had been deposited by the plant which inhibited development of the fungus.

In all cases the uninoculated plants remained healthy. The control series injected with the pure culture of V. albo-atrum were all very severely infected.

Table 4. Number of plants infected by inoculations with crushed leaf particles.

	Leaf Source					Sterile water	Healthy plant material	Pure culture
	1	2	3	4	5			
Number infected	2[a]	1[a]	0	2[b]	2[b]	0	0	5[b]

[a] Plants infected subsequent to second inoculation.
[b] Plants infected prior to second inoculation.

The first planting of the third experiment dealing with transmission of leaf material by the seed was continued until the plants reached maturity. At this time none of the plants had shown any symptoms typical of Verticillium wilt. Two plants had shown yellowing and scorching early in the experiment but later recovered. When the experiment was two months old severe red spider attacks occurred on the cotton in the greenhouse, resulting in marring and discoloration of the leaves. Under these conditions, mild leaf symptoms of Verticillium wilt would have been impossible to observe. The plants made good growth and appeared normal when discarded, except for the red spider damage. At the time of discarding, the stems were cut to check for vascular discoloration. None was found so it was concluded that no infection had occurred during this experiment.

In the second planting of this experiment numerous small adventitious roots developed and ramified throughout the new layers of soil which were added to the pots. Nine weeks after planting the seed and four weeks after adding the fresh soil to the pots, one plant showed disease symptoms. Yellowing, followed by scorching, occurred first in the angles of the leaf formed by the leaf lobes. These symptoms were not entirely typical of Verticillium wilt which had occurred in the other experiments of this investigation. Typical symptoms were yellowing around the entire margin of the leaf, resulting in scorching. Isolation from the stem of this plant yielded pure cultures of typical V. albo-atrum.

None of the other plants of this experiment produced any symptoms of Verticillium wilt. At the present time this experiment is being repeated with 100 plants by the author and L. A. Brinkerhoff.

In order to determine the amount of plant material naturally occurring on the fuzz of cotton seed, an examination was made of a commercial source of reginned cotton seed. Five hundred seed were selected at random from a bag of the seed and examined under a low power (x25) microscope. Comparatively large fragments clinging to the fuzz were counted and classified as leaf, bract, or "unidentified." The unidentified group consisted probably of both leaf and bract material which could not be definitely idenified. From the 500 seed inspected, 31 particles were identified as leaf tissue, 27 as bract material and 25 were placed in the unidentified group. Total particles, excluding numerous micro-particles, equalled 83, or about 16 per cent of the seed carried plant material on the fuzz.

Isolation studies.--The results obtained from the isolations are summarized in Table 5. In the isolations from both stem and leaf tissues the growth of the fungus usually occurred as a fluffy white ball of hyphae growing from one or both ends of the tissue. Occasionally growth appeared from the sides of the tissue. At other times no mycelium was evident. Instead, a smooth, glistening, appressed growth, soon turning to typical black microsclerotia, covered the agar surface.

Table 5. Number of isolations of *Verticillium albo-atrum* from infested dry leaf and stem tissues collected from five different southwestern states.

Source of tissue		Part of plant	Medium	*V. albo-atrum*	Contaminants[a]	Total Attempts
Leaf source	#1	Leaf	PDA	1	4	5
	#2	"	"	0	5	5
	#3	"	"	0	5	5
	#4	"	"	2	0	5
	#5	"	"	2	3	5
	#6	"	"	3	2	5
	#1	Ground leaf material	Straw agar	3	1	5
	#2	"	"	0[b]	4	4
	#3	"	"	0[b]	4	4
	#4	"	"	0[b]	4	4
	#5	"	"	0[b]	4	4
Snider, Texas		Leaf	PDA	4	11	15
Roswell, N.M.		Stem	"	3	2	5
Tipton, Okla.		Stem	"	4	1	5
Totals				23	44	79

[a] Contaminants include *Alternaria* sp., *Neurospora* sp., *Penicillium* sp. and some *Eubacteriales*.

[b] Although many contaminants were present, sclerotia of *V. albo-atrum* were identified microscopically in most of these plates; pure cultures of the fungus were not obtained, however.

In all cultures listed in Table 5 and in numerous other cultures of *V. albo-atrum*, sclerotial and mycelial secotrs were common. In the majority of instances a mycelial transfer produced a mycelial culture; and conversely, when sclerotia were transferred the resulting culture would be predominantly or totally sclerotial.

Most of the cultures were incubated at 20 to 22 degrees C. which Barducci (2) states to be the most favorable temperature for growth of the fungus in culture. Cultures which were incubated at room temperatures, i.e. 25° to 27° C., showed no striking differences in growth patterns, but contaminants, when present, grew more rapidly.

Re-isolation of the fungus from the diseased plants of the greenhouse experiments yielded pure cultures of V. albo-atrum characterized by a predominant sclerotial growth with very little fluffy mycelium. Little difficulty was experienced with contaminants in these isolations when reasonable care was taken to maintain aseptic conditions.

Pathological Histology.—Hyphae were fairly abundant within the vascular system of all the diseased plants sectioned. Cross-sections of the petioles of the plants showed hyphae in many of the vessels. Within the petiole proper, no complete plugging of vessels was found, although many vessels were blocked with dark-stained deposits. All vascular tissues of the longitudinal sections stained a deep red, the parenchyma and epidermal layers stained green, while the fungus stained red. The hyphae were coiled around the inside of the vessels. Wall penetration between vessels was observed in a few sections but no hyphae were found in cells other than the larger vessels of the xylem tissue.

Best results were obtained on one slide containing eight serial sections of the area of the blade-petiole junction. These sections had been cut longitudinally through the protoxylem elements of one of the leaf veins and then directly across the vascular area of two other veins. Both longitudinal and transverse vascular areas were clearly exposed. On the longitudinal sections the hyphae were easily visible. Many of the sections, which were between 15 and 20 microns thick, were cut so as to remove the upper one-third of the vessels, clearly revealing their contents. Spiral and scalariform thickenings of the vessel walls were evident in all of the vessels. The fungus hyphae were stained red. In many places within the hyphae, very dark-stained minute objects which resembled nuclei were evident.

Branching of the hyphae did not occur very frequently. The cells of the hyphae were swollen in many instances and at times had separated so that a single swollen cell was isolated from other fungus tissue. Thick-walled, chlamydospore-like forms were present in abundance. These swollen hyphal types somewhat resemble the microsclerotial hyphae of an _in vitro_ culture of _V. albo-atrum._ Figure 3 and figure 4 are photomicrographs of sclerotial forms from a culture and the form of the hyphae in the plant tissue.

In the cross sections adjacent to the longitudinal sections hyphae were also found in abundance. One vessel of the vascular bundle was so filled with hyphae as to be almost completely blocked, (Figure 5). Several vessels were filled with dark-stained deposits. Nearly all the vessels contained some hyphae. Between the vessels were wall configurations which were believed to be pits, although plant anatomy texts do not diagram this particular structure. Hyphae were found penetrating the walls at some of these points. Similar structures are shown in diagrams of Verticillium infected potato plants appearing in Reinke and Berthold's paper (18).

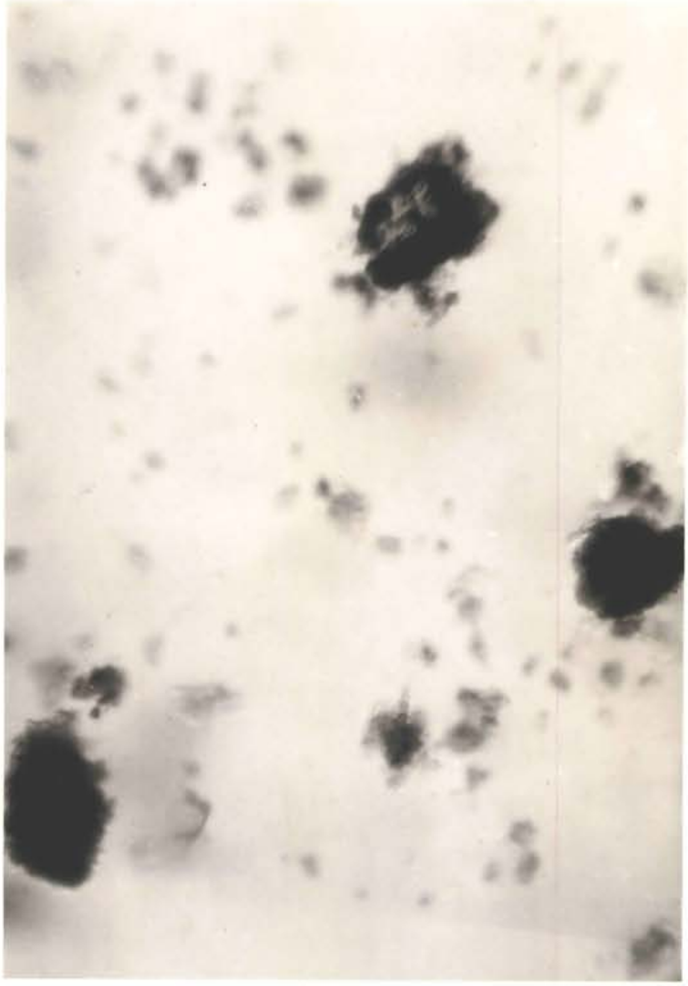

Fig. 1. Photomicrograph of crushed dry, cotton leaf material in water. x210.

Fig. 2. A cotton plant (right) showing typical Verticillium wilt symptoms. Left, a healthy control plant.

Fig. 3. Microsclerotia of V. albo-atrum grown on potato-dextrose agar. x2475.

Fig. 4. Longitudinal section of a part of the vascular area in a leaf vein showing hyphae of V. albo-atrum in the vessels. x2475.

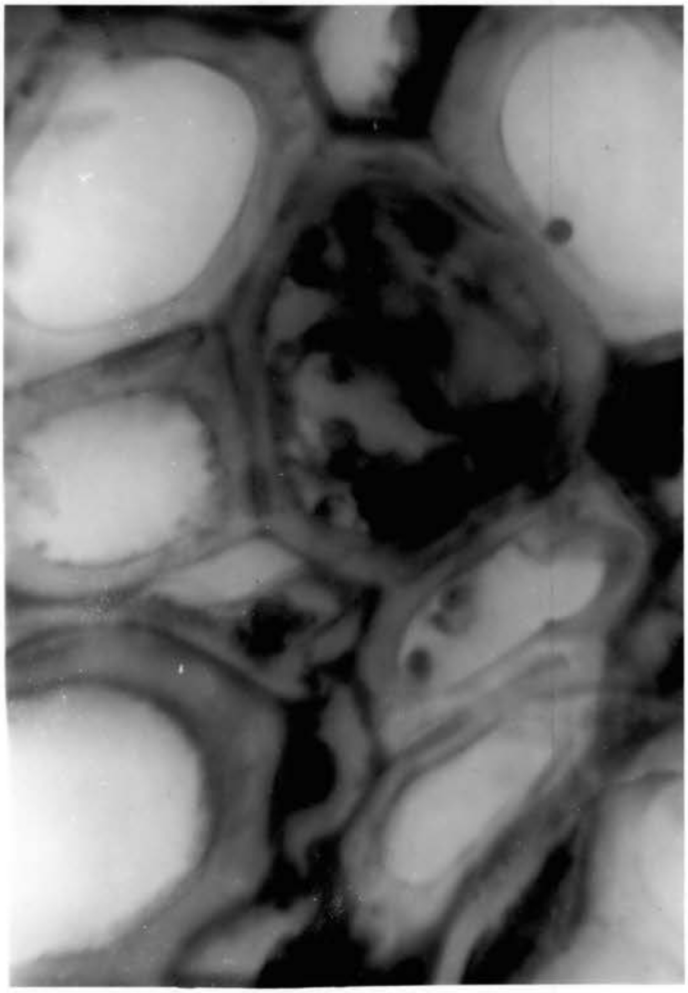

Fig. 5. Transverse section of a part of a leaf vein showing hyphae of V. albo-atrum in the vessels. x2475.

DISCUSSION

Any attempt to account for the dissemination or occurrence of the Verticillium wilt pathogen is difficult due to its wide host range, and the ability of the fungus to remain viable in the soil for great lengths of time.

Plant debris is considered as one of the most common means of dissemination. Although it is believed by some workers that the fungus dies fairly rapidly after frost in the above ground parts of the plant, it has been demonstrated in the present work that under dry storage conditions it can remain viable for over a year, at least in the leaf tissue. Crushing the leaf particles into small fragments does not appear to inactivate the fungus, although from the tests completed in this investigation the infective ability of such material seems to be decreased. Cultivating cotton after diseased plants have shed leaves would seem to be a method of rapidly increasing infection in a field.

Although internal and external infestation of cotton seed has been reported, doubt about the common occurrence of this has been established (19). In an examination of reginned cotton seed during this investigation, a large number of leaf and bract particles were found. By artificially infesting the fuzz of cotton seed with leaf particles the disease was produced. However, a true test of seed transmission would necessitate the planting of naturally infested seed in disease-free soil and later observing diseased plants.

Probably seed transmission of V. albo-atrum in the field is of uncommon occurrence, if it occurs. However, this investigation has demonstrated that the fungus may remain viable in small particles of infested leaf trash and that these particles are capable of transmitting the fungus when planted with seed in sterile soil. The introduction of the fungus to a new area by even one seed could possibly be responsible for serious outbreaks in following years.

Delinting cotton seed as a means of eliminating Verticillium infested plant debris might be worthy of investigation, especially where seed is being shipped from heavily infested areas to non-infested areas. Also the possibility of control by disinfectant dusts might be considered.

SUMMARY AND CONCLUSIONS

Experiments dealing with the occurrence of Verticillium albo-atrum R. and B. in infested cotton leaves have been completed. By laboratory isolation and histological studies, the presence, location and morphology of the fungus within the leaf have been determined. With dry leaves the fungus was most readily isolated from the vascular area at the junction of the petiole with the leaf blade, but it was also possible to isolate it from the midrib and lateral veins of the leaf. As far as could be determined the fungus was confined to the vessels of the vascular system in the petioles and leaves. In dry leaf tissue the fungus hyphae had many swollen and knotted areas, resembling the sclerotial forms of V. albo-atrum which occur in cultures on synthetic media.

In greenhouse experiments, infested cotton leaves mixed with the soil produced the disease in cotton in 64 per cent of the plants. Six leaf sources, varying in age from one month to 13 months were used, infections being obtained from all the sources. Crushing the leaves into extremely small particles reduced the infective ability of the fungus but did not entirely inactivate it.

One plant of 50 became infected with Verticillium wilt following attachment of infested leaf particles to the fuzz of the seed prior to planting. In examination of commercial reginned seed under a low power microscope, 16 per cent of the seed was found to carry plant particles which were entangled with the fuzz. These particles, most of which were identified as leaf or bract material, are known to be capable of harboring the fungus, and represent a possible means of transmitting the disease with the planting seed.

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