Temperature Effects upon Development and Pathogenicity of Defoliating and Nondefoliating Pathotypes of Verticillium dahliae in Leaves of Cotton Plants

Susan H. Temple, J. E. DeVay, and Linda L. Forrester

Department of Plant Pathology, University of California, Davis 95616.

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ABSTRACT

Development of symptoms of Verticillium wilt involving chlorosis and necrosis of leaves of cotton (*Gossypium hirsutum*) is preceded by invasion of leaf tissues by *Verticillium dahliae*, the plugging of xylem elements with a gel-like substance, and the wilting of limited leaf areas. At approximately 23 C, the pathogen was located mainly in xylem elements and xylem parenchma and was found generally throughout infected leaves of stem-inoculated plants. The pathogen was always isolated from leaf areas with symptoms of Verticillium wilt and usually from symptomless areas of the leaves. The defoliating pathotype (T9) of *V. dahliae* was isolated from all sections of petioles within 48 hr of stem inoculation, whereas the nondefoliating pathotype (SS4) was not isolated consistently from all sections of petioles until the 4th day; by the 10th day both pathotypes were recovered from all sections of infected leaves. Resistance of cotton to Verticillium wilt at 33 C (day temperature) was due mainly to the inhibition and death of *V. dahliae* in foliar tissues. In infected leaves the defoliating pathotype was better able to withstand high air temperature compared with the nondefoliating pathotype which could not be recovered from leaves after 16 days.

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Additional key words: defoliation, water stress, wilting, rate of infection, *Gossypium hirsutum*.

All varieties of selections of upland cotton (*Gossypium hirsutum* L.) tested for reaction to Verticillium wilt, have been susceptible to the defoliating or nondefoliating pathotypes of *Verticillium dahliae* Kleb. at mean temperatures of about 25 C (2, 3, 10, 20, 22). At 27 or 28 C, varieties such as 'Stardel' remain susceptible but 'Acala 4-42' may develop a temporary tolerance; but if the temperature is lowered, the Acala plants again become

highly susceptible (2). Tolerant and susceptible varieties of *G. hirsutum* are wilt resistant at about 32 C; diseased plants often developed new leaves and increased stem growth (2, 10, 11).

The symptomology of Verticillium wilt varies with the variety or species of cotton, the stage of plant development, the concentration of inoculum and pathotypes of *V. dahliae* present, and the environmental temperature (10, 20, 25). Factors such as soil potassium level, soil compaction, and soil moisture (19) also affect wilt development. Verticillium wilt in cotton is characterized by epinasty of the upper leaves followed by the appearance in leaves of irregular chlorotic patches which become necrotic. Infected plants are usually stunted, but stunting varies with disease severity and the age at which plants become infected. Discoloration of vascular tissues is also a characteristic symptom. Defoliation of infected plants may accompany the above symptoms, especially with a defoliating pathotype of *V. dahliae* (20).

Garber & Houston (8, 9) described the infection process in the roots and subsequent development of the pathogen in cotton plants both resistant and susceptible to wilt. Rapid movement of conidia through the vascular system with the invasion of leaf tissues (18, 22, 25), seems well established. Also reported (4) is rapid production of conidia in tracheal fluid and other liquids. Conidia are found in small leaf veinlets and give rise to additional conidial generations and germ hyphae (22); *V. dahliae* can be isolated from wherever chlorotic or necrotic lesions appear in leaves (8, 9, 22). The actual cause of leaf lesions is unknown although various workers have reported on the possible role of toxins (15, 21), blockage of vascular elements in leaves and petioles (8, 9, 22), and changes in concentrations of cytokinins (17). Susceptibility to Verticillium wilt is judged primarily by leaf symptoms since they are the most detrimental to the plant (8, 9, 20, 25). No appreciable damage results from infections that are limited to the stalks, as in resistant varieties of cotton *(G. barbadense* L.) (25).

The present work concerns the distribution and survival of *V. dahliae* in infected leaves as influenced by temperature. Also reported is the histology of infected petiole and leaf tissues in relation to symptom development.

MATERIALS AND METHODS.—*Growth and inoculation of plants.*—Cotton plants (*G. hirsutum*), 'Deltapine Smooth Leaf' and 'Acala SJ-1', were grown in a glasshouse (23 C ± 3 C), or in controlled-environment chambers [14-hr day; approx. 21,500 lux (2,000 ft-c);33-C day/26-C night or 26-C day/20-C night]. In the glasshouse and environment chambers, three plants were grown per 10.2-cm (4-inch) plastic pot containing U.C. Mix (1). When the first lobed leaves were about half-expanded (about 8 weeks), the plants were inoculated by stem puncture (7, 23) with approximately 50 µl of a conidial suspension (1×10^6 conidia/ml) of *V. dahliae*. Inoculations were also made by gently sliding the entire

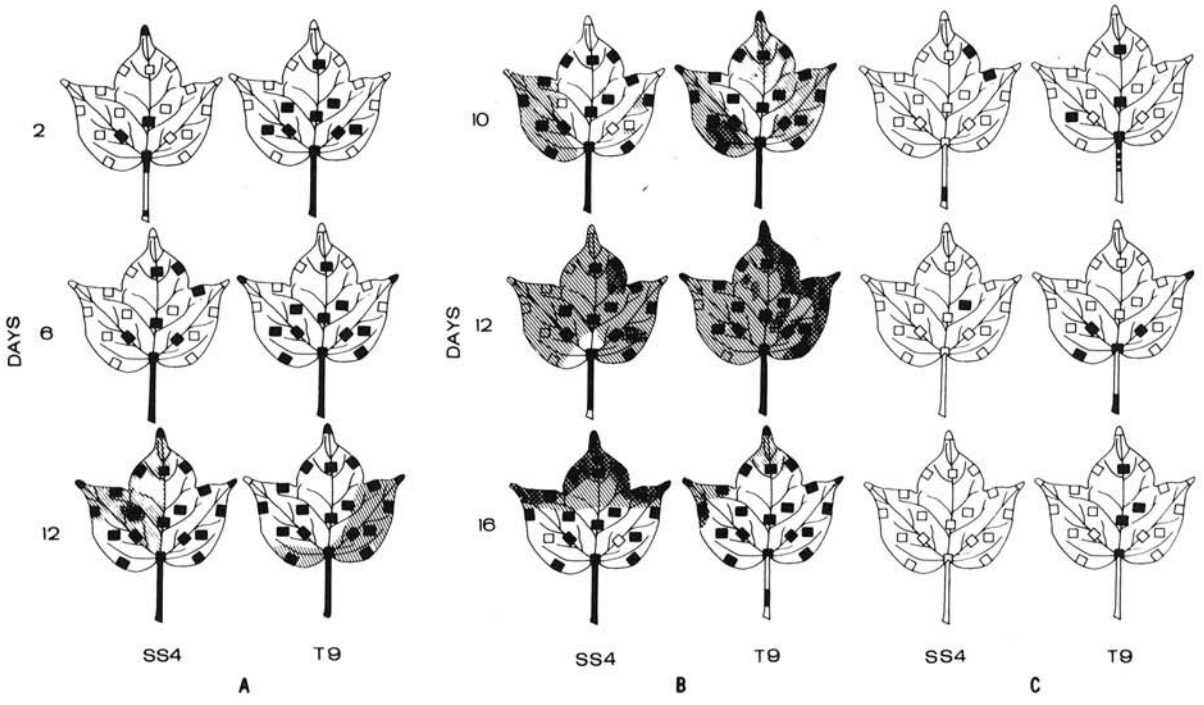

Fig. 1. Patterns of movement and distribution of *Verticillium dahliae* in leaves of 'Acala SJ-1' cotton plants at different times after stem inoculation with conidial suspensions. Black squares and petioles indicate locations where the fungus was recovered, cross-hatched areas indicate chlorotic tissue and checkered areas the tissue which was necrotic. **A)** Plants maintained in the glasshouse at 23 C and inoculated with pathotypes SS-4 and T9. Leaf diagrams are representative of the patteerns of pathogen recovery from leaves harvested at daily intervals for 16 days after inoculation. **B)** Plants maintained in controlled-environment chambers, 26-C day, 20-C night. **C)** Plants maintained in environment chambers, 33-C day, 26-C night.

soil-root ball from the pot and spraying the roots with 10 ml of the conidial suspension (20). Check plants were sprayed with sterile water. Two strains of *V. dahliae* were used: T9, a highly virulent defoliating pathotype; and SS4, a nondefoliating pathotype of intermediate virulence (23).

Isolation of V. dahliae from inoculated plants.—Two leaves with attached petioles from each of three Acala SJ-1 plants from the glasshouse or environment chambers were collected at 24-hr intervals up to 16 days after inoculation. Petioles were cut into 0.5-cm sections, surface-sterilized for 2 min in 0.1% NaOCl, transferred to petri plates of potato-dextrose agar (PDA) with 500 ppm of streptomycin sulfate, and incubated at 24-27 C. Leaf sections (0.5 cm^2) were cut according to a pattern (Fig. 1) from 20 locations on each leaf blade. Each section was described as green, chlorotic, or necrotic according to its condition at collection. *V. dahliae* was isolated from leaf sections by the same procedure used for the petioles. The plates were read 7 to 10 days later. The experiment was repeated once.

Histological methods.—Leaves and petioles of glasshouse plants were collected on the 2nd, 6th, 8th, and 12th days after stem inoculation. Chlorosis is usually not evident before the 8th day, is often evident by the 10th day, and may be distinct by the 12th day; necrotic areas in the leaves may also develop by the 12th day. Samples were placed in FAA fixative when collected, and subsequently embedded for sectioning (12). Sections 15 μ thick were cut, mounted, and then stained with a Safranin O and Fast Green (13).

Petioles from field-grown plants inoculated with *V. dahliae* were collected at various stages of wilt development and immersed for 5 min in boiling 70% ethyl alcohol. Freehand sections were cut from the petiole samples and stained with IKI (12) for starch content.

RESULTS.—*Fungal distribution and symptom development in leaves.*—Pathotypes SS4 and T9 of *V. dahliae* were present in various parts of leaves and petioles of stem-inoculated glasshouse plants at different times after inoculation (Fig. 1-A). Since the patterns of distribution were similar in Deltapine Smooth Leaf and Acala SJ-1, only the latter are presented here. Although pathotype T9 was isolated from all sections of petioles of test leaves within 48 hr of stem inoculation, not until the 4th day was SS4 isolated consistently from all sections of petioles. All the petiolar-laminar junctions, however, yielded *V. dahliae* within 48 hr of stem inoculation in all combinations of cotton variety and fungal pathotype. Each of the pathotypes was isolated also from sections of the lower midrib within 48 hr in both varieties of cotton. By the 10th day, both pathotypes were recovered from all sections of infected leaves, including veinal and interveinal tissues. The fungus was always present where symptoms developed; however, it was also present by the 10th day in apparently healthy areas of diseased leaves.

When plants were stem-inoculated with either SS4 or T9 and maintained in controlled-environment chambers, no apparent symptoms of Verticillium wilt developed in plants kept at 33 C during the light period, whereas severe symptoms developed in plants kept at 26 C during the light period (Fig. 1-B, C). The patterns and rates of distribution of both pathotypes in leaves of plants maintained at 26 C during the light period was similar to those observed in glasshouse plants. In plants at 33 C during the light periods, however, the nondefoliating pathotype SS4 could not be recovered by the 16th day after inoculation. Pathotype T9 was more persistent at 33 C, being recovered on the 16th day from 20% of leaf sections but not from the petiole sections.

Histology of leaves and petioles.—Leaves and petioles of Acala SJ-1 and Deltapine Smooth Leaf plants grown in the glasshouse were collected on the 2nd, 6th, 8th, and 12th days after stem inoculation with pathotype SS4 or T9. Conidia were observed in sections of petiole vessels from material collected on the 2nd day from inoculated plants; only seldom were gel-like deposits observed in vessels of these sections. The gel-like deposits that commonly occlude the vessels in tissues affected by Verticillium wilt were never observed in sections from healthy plants. In cross sections of petioles collected on the 6th, 8th, and 12th days many of the vessels, especially in T9-infected plants of both cotton varieties, contained high concentrations of conidia and branching hyphae (Fig. 2-A). Usually, however, less than half the vessels contained fungal structures even in the most severely diseased leaves. Hyphae commonly grew from one vessel into adjacent vessels and tracheids through pit pairs, along the lumen of vessels and tracheids (Fig. 2-B), and into xylem parenchyma (Fig. 2-C, D). Tyloses were seen occasionally (Fig. 2-E), but most common were gel-like deposits (Fig. 2-F). Gel-like deposits were also observed frequently in pith and cortical parenchyma cells of infected tissues.

Fungal structures were commonly observed in sections of leaf tissues collected six days or more after stem inoculation of plants. Vascular discoloration usually preceded wilting and chlorosis of leaves. Tyloses (Fig. 3-A) were seen occasionally in sections of leaf tissues, but the most important single factor in causing the blockage of vascular tissues appeared to be the gel-like deposits in vessels and tracheids.

Leaf areas in advanced stages of chlorosis differed greatly in cellular structure from areas that were still green or were from healthy plant leaves. In chlorotic tissue, the palisade layer of cells had begun to collapse, chloroplasts were difficult to locate, and the tissue was badly disorganized (Fig. 3-B, C). When the leaf tissues became necrotic and desiccated, the cellular organization was difficult to define. A decrease in starch content in leaves of diseased plants was most evident in leaf areas that had developed noticeable disease symptoms.

In additional studies on field-grown plants, the starch granules of petiolar sections of both Acala SJ-1 and Deltapine Smooth Leaf cotton varieties were relatively more numerous in healthy plants than in infected plants. In healthy plants, the concentration of starch granules in sections taken near the petiolar-laminar junction was greatest in the first layer of cortical cells outside the endodermis but also high in other cortical parenchyma cells. In sections of petiole taken 2.5 cm from the base of the leaf, starch granules were common in the first layer of cells outside the endodermis but seldom seen in the remaining cortical parenchyma cells.

A main difference in petiolar sections between infected and healthy plants of Deltapine Smooth Leaf was the

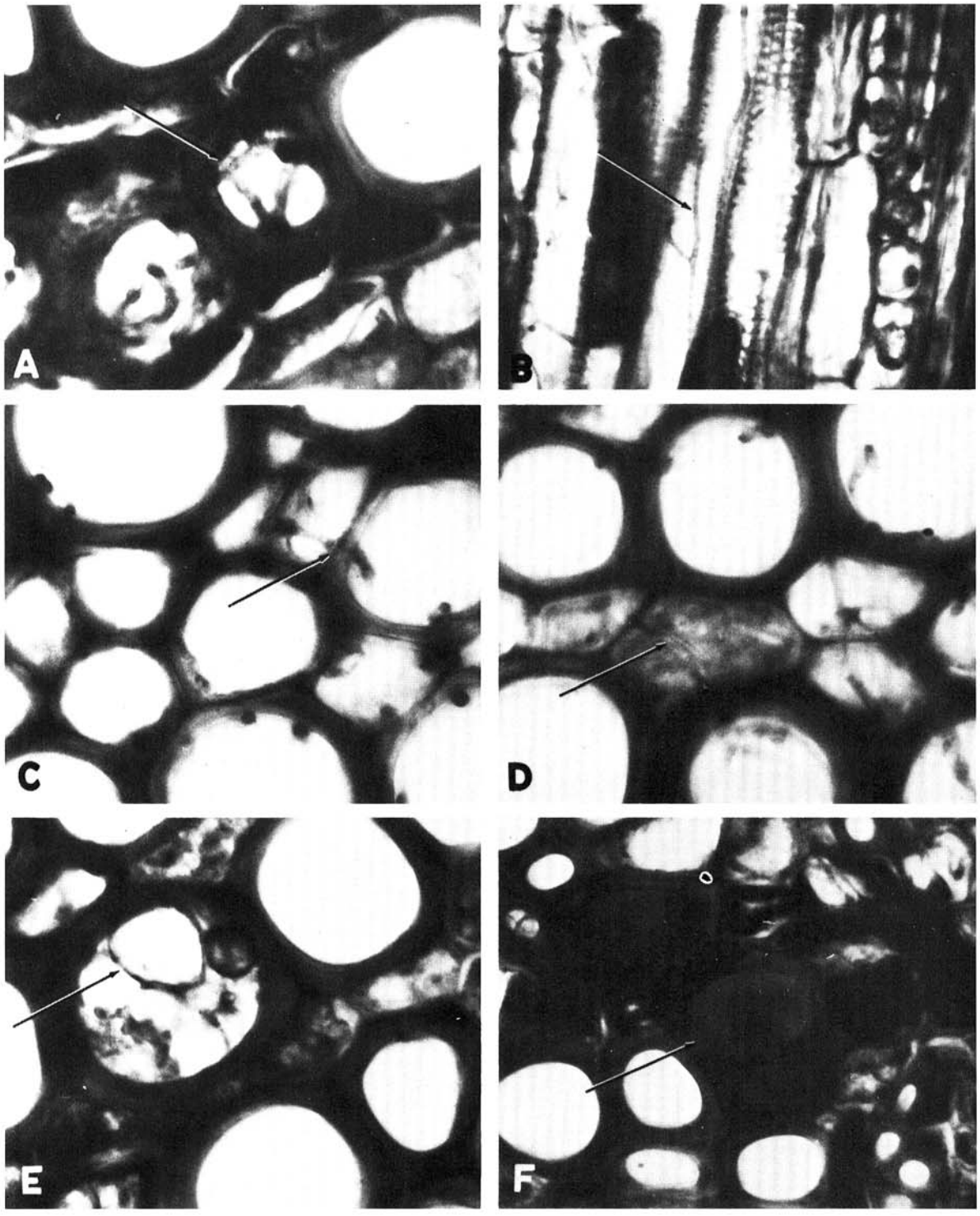

Fig. 2. Sections of tissues from petioles of leaves infected with *Verticillium dahliae* collected on the 6th to 12th day after stem inoculation of 'Acala SJ-1' cotton plants. **A)** Cross section showing branching hyphae in xylem elements. Conidia were numerous but difficult to show in tissue sections. **B)** Longitudinal sections of xylem elements showing fungal hyphae. **C)** Cross section showing the growth of fungal hyphae through pit pairs in vessel and tracheid walls. Sections of fungal hyphae are along the walls of the vessels and tracheids. **D)** Cross section showing fungal hyphae in cells of xylem parenchyma. **E)** Tyloses in cross section. **F)** Gel-like deposits filling xylem elements seen in cross section.

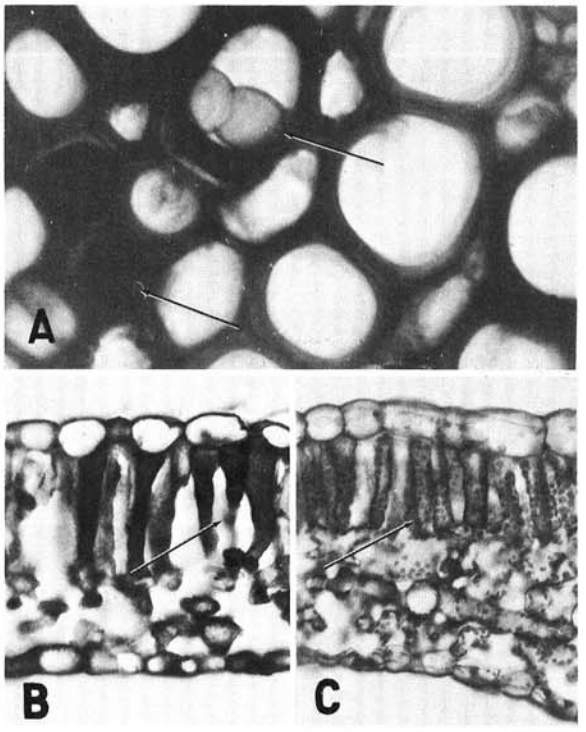

Fig. 3. Sections of tissues from healthy leaves or leaves infected with *Verticillium dahliae* collected 6 or more days after stem inoculation of 'Acala SJ-1' cotton plants. **A)** Tyloses and gel-like deposits in xylem elements of a cross section of the leaf midrib. In small veins, total occlusion of the xylem elements often occurs. **B)** Section through chlorotic leaf tissue (× 258). Note the crumpling of the palisade cells and the disappearance of chloroplasts in these cells. **C)** Section through healthy leaf tissue showing the various cell layers and numerous chloroplasts in the palisade cells (× 220).

relatively high concentration of granules (similar to starch granules) in infected petioles that did not stain a blue-black color with IKI. In petiolar sections of Acala SJ-1, collected on the 19th and 23rd day after inoculation with pathotype SS4, starch granules were observed only in the first two layers of cells outside the endodermis at the petiolar-laminar junction; granules which did not stain with IKI were also present in the cortical cells. Acala SJ-1 infected by pathotype T9 was devoid of starch granules in the petiolar tissues by the 23rd day except in irregular groups of cells near the epidermis.

DISCUSSION.—Since leaves are the first plant organs to exhibit external symptoms of Verticillium infection, this study related histological changes in leaves and petioles to the sequence of events leading to symptom expression and death of diseased leaves. The disease syndrome was the same in stem-inoculated and root-inoculated plants except that the former developed symptoms 10-14 days earlier. The varieties were alike in morphology and their leaves were infected by the fungus in a comparable fashion. The recovery of *V. dahliae* from apparently healthy (green) and non-wilted areas of diseased leaves confirms the observations of Garber & Houston (8) who also recovered the fungus frequently from green areas of diseased leaves.

Cells of pathotypes of *V. dahliae* studied were observed mainly in vessels and tracheids, although the xylem parenchyma was commonly colonized. Conidia moved rapidly to all parts of infected leaves, but symptoms of chlorosis and necrosis developed in patches and were usually preceded by an actual wilting of tissues in these restricted leaf areas. The sequence of phenomena in the development of leaf symptoms other than epinasty or early abscission (23) involves the colonization of leaf tissue by *Verticillium* and the sporadic induction of a host response that results in the filling of infected vascular tissues with a gel-like substance. The occlusion of xylem elements causes an acute water stress and wilting in isolated parts of leaves, resulting in chlorosis and eventually necrosis of these tissues. The amount of mycelium observed in xylem tissues did not appear extensive enough to occlude or greatly inhibit water conduction in the xylem. The view that the main phenomenon preceding chlorosis is a wilting of leaf tissue is supported by the following (5, 6): Verticillium wilt is inhibited for extended periods when plants are placed in a mist chamber; early wilting and chlorosis are reversible when discs of infected leaf tissues are floated on water; the relative water content is the same in both healthy and diseased leaves when leaf tissues first appeared wilted; and leaf solute potential did not change in the amount of solute per cell which could contribute to wilting.

Structural changes in leaf tissues involving the disappearance of chloroplasts and a collapse of cell structure were apparently caused by the gradual desiccation of tissue during development of Verticillium wilt. Associated with these changes were marked decreases in the starch content of petioles of diseased leaves compared with healthy leaves. Decreases in starch content of cotton leaves with Verticillium wilt has been reported (16); however, the decreases in starch content in petioles in time course experiments appear to follow the development of chlorosis in leaf tissues (22). The starch content of infected petiole tissue increased until about 5 days after stem inoculation and then declined in comparison with petioles of healthy cotton plants (22).

A fungal toxin has been assigned a possible role in the development of wilt symptoms by several workers (15, 21). If a toxin is involved, it would not be required to act over distances greater than the width of several parenchyma cells and its action would be either to induce formation of the gel-like substance by host cells or to cause the occlusion of vascular elements itself. The latter is doubtful since the patterns of chlorosis and necrosis are relatively restricted compared with the general distribution of the fungus in diseased leaves. The findings on water relations of diseased leaves (5, 6) cast further doubt that a fungal toxin has any direct effects on host cells.

The possible role of pectic enzymes also has been reported to play a role in pathogenesis. Wiese et al. (24), however, in studies on 29 isolates of *V. dahliae* and tracheal fluids from healthy and infected cotton plants, found evidence which indicated pectic enzymes were not involved in pathogenesis. This was later confirmed by Keen & Erwin (14).

In the present study, high air temperature (33 C) apparently alleviated the disease primarily though suppressing growth and survival of the pathogen. The greater persistence of the defoliating pathotype T9 in cotton plants at 33 C compared with nondefoliating pathotype SS4 is reflected in cultural studies (26) where T9 was able to grow and withstand higher air temperature than SS4.

LITERATURE CITED

1. BAKER, K. F. 1957. The U.C. system for producing healthy container-grown plants. Calif. Agric. Exp. Stn. Manual 23. 332 p.
2. BELL, A. A. & J. T. PRESLEY. 1969. Temperature effects upon resistance and phytoalexin synthesis in cotton inoculated with Verticillium albo-atrum. Phytopathology 59:1141-1146.
3. BRINKERHOFF, L. A., E. SAMAYOA, & J. C. MURRAY. 1967. Verticillium wilt reactions in cotton as affected by post-inoculation temperatures. Phytopathology 57:805 (Abstr.).
4. BUCKLEY, P. M., T. D. WYLLIE, & J. E. DE VAY. 1969. Fine structure of conidia and conidium formation in Verticillium albo-atrum and V. nigrescens. Mycologia 61:240-250.
5. DE VAY, J. E., J. M. DUNIWAY, SUSAN H. TEMPLE, & LINDA L. FORRESTER. 1972. Temperature, humidity, water stress, and pathogen distribution in cotton leaves as factors in the development of Verticillium wilt. p. 20. *In* Proc. 1972 Beltwide Cotton Production Research Conference, Memphis, Tenn. National Cotton Council, Raleigh, N.C. 105 p.
6. DUNIWAY, J. M. 1972. Pathogen-induced changes in host-water relations. Phytopathology 63:458-466.
7. ERWIN, D. C., W. MOJE, & I. MALCA. 1965. An assay of the severity of Verticillium wilt on cotton plants inoculated by stem puncture. Phytopathology 55:663-665.
8. GARBER, R. H., & B. R. HOUSTON. 1966. Penetration and development of Verticillium albo-atrum in the cotton plant. Phytopathology 56:1121-1126.
9. GARBER, R. H., & B. R. HOUSTON. 1967. Nature of Verticillium wilt resistance in cotton. Phytopathology 57:885-888.
10. GARBER, R. H., & J. T. PRESLEY. 1971. Relation of air temperature to development of Verticillium wilt on cotton in the field. Phytopathology 61:204-207.
11. HALISKY, P. M., R. H. GARBER, & W. C. SCHNATHORST. 1959. Influence of soil temperature on Verticillium hydromycosis of cotton in California. Plant Dis. Reptr. 43:584-587.
12. JENSEN, W. A. 1962. Botanical histochemistry: principles and practices. W. H. Freeman & Co., San Francisco. 408 p.
13. JOHANSEN, D. A. 1940. Plant microtechnique. McGraw-Hill Book Co., New York. 523 p.
14. KEEN, N. T., & D. C. ERWIN. 1971. Endopolygalacturonase: evidence against involvement in Verticillium wilt in cotton. Phytopathology 61:198-203.
15. KEEN, N. T., MARGARET LONG, & D. C. ERWIN. 1972. Possible involvement of a pathogen-produced protein-lipopolysaccharide complex in Verticillium wilt of cotton. Physiol. Plant Pathol. 2:317-331.
16. MATHRE, D. E. 1968. Photosynthetic activities of cotton plants infected with Verticillium albo-atrum. Phytopathology 58:137-141.
17. MISAGHI, I., J. E. DE VAY, & T. KOSUGE. 1972. Changes in cytokinin activity associated with the development of Verticillium wilt and water stress in cotton plants. Physiol. Plant Pathol. 2:187-196.
18. PRESLEY, J. T., H. R. CARNS, E. E. TAYLOR, & W. C. SCHNATHORST. 1966. Movement of conidia of Verticillium albo-atrum in cotton plants. Phytopathology 56:375.
19. RANNEY, C. D. 1972. Cultural control of Verticillium wilt. *In* Verticillium wilt of cotton. ARS-34-137, USDA-ARS Publication Division, Beltsville, Md. 398 p. (In press)
20. SCHNATHORST, W. C., & D. E. MATHRE. 1966. Host range and differentiation of a severe form of Verticillium albo-atrum in cotton. Phytopathology 56:1155-1161.
21. STODDART, J. L., & A. J. H. CARR. 1966. Properties of wilt toxins produced by Verticillium albo-atrum Reinke and Berthold. Ann. Appl. Biol. 58:81-92.
22. TEMPLE, S. 1971. Location of Verticillium albo-atrum and histological changes in cotton leaves during Verticillium wilt development. M. Sc. Thesis, University of California, Davis. 58 p.
23. WIESE, M. V., & J. E. DE VAY. 1970. Growth-regulator changes in cotton associated with defoliation caused by Verticillium albo-atrum. Plant Physiol. 45:304-309.
24. WIESE, M. V., J. E. DE VAY, & A. V. RAVENSCROFT. 1970. Relationship between polygalacturonase activity and cultural characteristics of Verticillium isolates pathogenic in cotton. Phytopathology 60:641-646.
25. WILHELM, S., J. E. SAGEN, H. TIETZ, & A. G. GEORGE. 1970. Waukena white—a new cotton breeding line resistant to Verticillium wilt. Calif. Agric. 24:8-12.
26. WYLLIE, T. D., & J. E. DE VAY. 1970. Growth characteristics of several isolates of Verticillium albo-atrum and V. nigrescens from cotton. Phytopathology 60:907-910.