Relationship of Cultivar Resistance to Distribution of *Verticillium dahliae* in Inoculated Cotton Plants and to Growth of Single Conidia on Excised Stem Segments

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ABSTRACT

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Verticillium dahliae was recovered, in laboratory cultures, from apical vascular stem and leaf petiole tissues of various cotton cultivars within 3 days of basal stem-puncture inoculation with either a mild (SS-4) or a severe (T-1) strain of the fungus. This movement of *V. dahliae* in cotton stems was observed in young greenhouse-grown plants of all the susceptible, tolerant (*Gossypium hirsutum*), and resistant (*G. barbadense*) cultivars tested. Leaf symptoms appeared after 7–9 days. The pathogen was isolated less frequently from the resistant Seabrook Sea Island than from the tolerant or susceptible cotton cultivars (*G. hirsutum*). Compared with wilt-susceptible and wilt-tolerant cotton cultivars, excised stem segments of resistant plants strongly inhibited mycelial growth of *V. dahliae* when inoculated on cut surfaces with single germinated conidia. Of those stem segments on which the fungus grew, microsclerotial formation was sparse and delayed, whereas susceptible cultivars supported vigorous mycelial growth and abundant microsclerotial formation. The presence of *V. dahliae* in the upper stem and petiole tissues of both susceptible and resistant plants soon after inoculation, as well as the differential growth and microsclerotial formation of the fungus on cut stem surfaces of resistant versus susceptible cottons suggest that resistance is due to the activation of a host chemical response. These results are consistent with the information presented in the subsequent paper on the accumulation of methylated sesquiterpenoid phytoalexins, which appear to be the primary determinants of Verticillium wilt resistance in cotton.

Infection of the cotton plant with *Verticillium dahliae* Kleb. may result either from direct penetration of young uninjured root tissues (6) or through root or stem-puncture wounds (9). The fungus penetrates the epidermis and grows intercellularly and intracellularly through the cortex until it reaches the xylem (11,14). Garber and Houston (6) found no differences in root penetration of resistant and susceptible cotton cultivars by *V. dahliae*; consequently, they related resistance to limited vascular colonization by the fungus (7). Presley et al (11) reported that after reaching xylem elements, the fungus was distributed within the plant chiefly by conidia being carried in the xylem stream and not by actual mycelial growth. Their results also showed no differences in the rates of conidial movement in healthy resistant, tolerant, and susceptible cotton plants.

In a comprehensive program in which many cotton lines were screened for resistance to Verticillium wilt in naturally infested field soil, Wilhelm et al (15,16) showed that none was immune to vascular infection; however, susceptible *Gossypium hirsutum* L. cottons were often defoliated while resistant Seabrook Sea Island (SBSI) (*G. barbadense* L.) and its hybrids were not. The fungus could be consistently isolated from lower vascular stem tissues of both resistant and susceptible cottons even though the symptoms of vascular discoloration were diminished or absent in the area where isolations were made.

Harrison and Beckman (8) reported that Verticillium wilt resistance in cotton involved both physical and chemical responses. The physical host response involved the walling-off of infected vessels immediately above the primary infection site. The chemical response included the accumulation of terpenoid aldehydes (1,10,18) in the infected region, which accompanied or followed localization of the pathogen and the eventual decline in viability of its fungal propagules.

In this investigation, the distribution, growth, and microsclerotial formation of *V. dahliae* in relation to the level of resistance of the host were studied to determine whether resistance was expressed as physical localization of the fungus near the inoculation site or as a chemical response expressed throughout the host. Experiments with single germinated conidia placed in contact with excised stem segments from susceptible, tolerant, and resistant cotton cultivars were done to explore these relationships. A preliminary report has been published (4).

MATERIALS AND METHODS

Fungal strains and cotton cultivars. Two strains of *V. dahliae* were used: mild, nondefoliating SS-4 strain (13) and severe, defoliating T-1 strain (13) (both courtesy of W. C. Schnathorst, University of California, Davis). Cultures were maintained on potato-dextrose agar (PDA) slants (Difco) at 24 C. Conidial suspensions were prepared from 4- to 5-day-old cultures and were filtered through two layers of cheesecloth.

The test cultivars of cotton used were SBSI 12-B-2, *G. barbadense* (highly resistant), and four *G. hirsutum* cultivars: Acala SJC-1, tolerant to SS-4 strain and susceptible to T-1 strain; Acala 4-42, tolerant to SS-4 and susceptible to T-1; and 70-110 and Deltapine 15, susceptible to both strains (Table 1). The first four seed lots were obtained from A. Hyer, U.S. Cotton Research Station, Shafter, CA, and the Deltapine 15 was from W. C. Schnathorst. Plants were grown two per 10-cm pot in a mixture of basic steamed U.C. mix and fumigated greenhouse compost soil (1:1, v/v). During the growing period, daytime greenhouse temperatures were kept at about 25 C and were lowered to about 21 C before inoculation. Plants were fertilized weekly with a balanced liquid fertilizer. All seedlings were about 35 cm tall (5–7 wk old, depending on the season) when they were inoculated.

Inoculation and serial culturing. In two trials, disease resistance was assayed by observing the movement (or distribution) of *V.*

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dahliae in inoculated seedlings. In the first trial, the defoliating T-1 strain was used for inoculating SBSI and Acala SJC-1 cotton cultivars. The second trial used the mild SS-4 strain of the fungus, SBSI, and the very susceptible Deltapine 15. The stem-puncture technique (2) was used to introduce the conidia directly into the vascular tissues. Three stem-puncture inoculations were made in the hypocotyls of test cotton lines with a 20-gauge hypodermic needle that had been dipped in a suspension containing $2–3 \times 10^6$ conidia per milliliter. Depth of the puncture was controlled at 2 mm by a guard on the needle made from Tygon plastic tubing.

A serial sampling procedure and laboratory culturing was used to detect the fungus every day for 7 days after inoculation. This provided a comparison of the development of the fungus in the resistant and susceptible cottons in terms of time of onset of infection and the upward movement of conidia in the stem and into the leaves. Three plants of each cultivar were sampled daily. Samples for culturing were taken from the stem and the leaf petiole at three stations, the first, third, and fifth internodes, which corresponded to basal, middle, and apical stem sections of the plants. The stem and petiole samples were treated in 10% commercial Clorox for 3 min and air-dried. Freehand cross sections were cut aseptically from each sample, 10 from each stem position and five from each petiole. All sections were incubated in petri plates on barley straw agar at 20 C. Plates were examined every 3–5 days for characteristic growth, sporulation, and microsclerotial formation.

TABLE 1. Interactions of the mild (SS-4) and severe (T-1) strains of *Verticillium dahliae* with cotton (*Gossypium* spp.) cultivars

Cotton species and cultivars	*V. dahliae*		References
	SS-4	T-1	
G. barbadense			
Seabrook Sea Island	R[a]	R	13,19
G. hirsutum			
Acala SJC-1	T	S	...[b]
Acala 4-42	T	S	13
70-110	S	S	12
Deltapine 15	S	S	13

[a] R = resistant, T = tolerant, and S = susceptible.
[b] W. C. Schnathorst (*personal communication*) and results of our greenhouse tests.

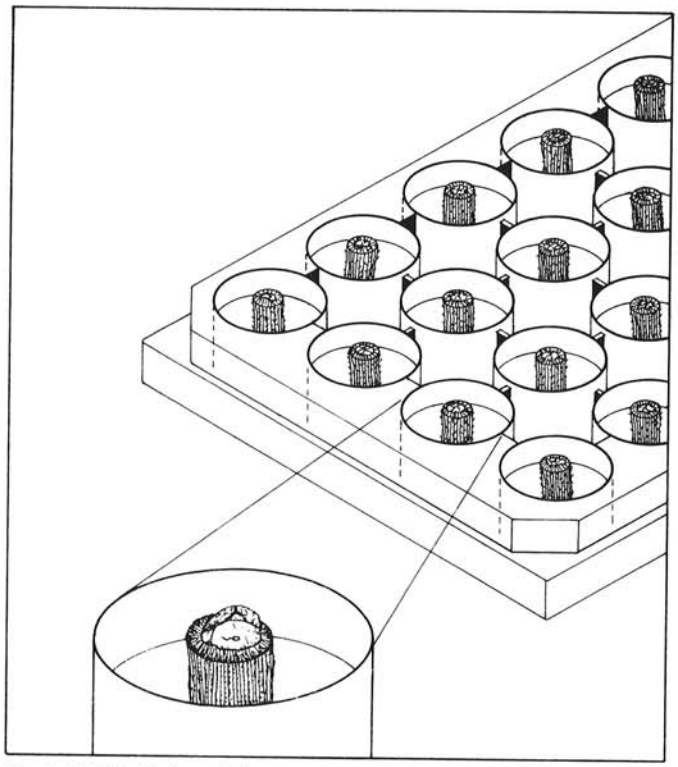

Fig. 1. Multiwell plate with cotton stem segments embedded in water agar (2 ml per well) and a single germinated conidium placed on the cut surface of each segment.

Growth behavior of single *Verticillium* conidia on cut stem segments. In this set of experiments, resistance in cotton to Verticillium wilt was assayed by placing individual germinated conidia on cut stem segments and observing subsequent mycelial growth and sporulation. Six healthy plants of each cultivar were selected for uniform stem size and severed 2 cm above the soil surface, then 5-cm sections were cut from the hypocotyl and the apical portion of each stem. The pieces were surface-sterilized for 2 min in 0.1% mercuric chloride solution and rinsed in distilled water. After air-drying, the pieces were stripped of the cortical tissues to obtain vascular tissues (stele) free of any glands, and four segments, each about 6 mm long, were cut aseptically from each piece.

Each stem segment was transferred to a single well of a tissue-culture multiwell plate (Corning Glass Works, Corning, NY) containing 1.5% solidified water agar, 2 ml per well (Fig. 1). The segments were inserted in a vertical position in the agar so that a single germinated conidium could be placed on the cut surface. The 24-well plates allowed for convenient handling of the six-plant replicates per cotton cultivar and four subreplicates per plant at each location on the stem, thus preserving the integrity of each plant and stem position. The excised segments remained at room temperature in the agar wells overnight (18–20 C). The following day, with the aid of two dissecting microscopes, single germinated conidia were transferred from 1-day-old 1.5% water agar dilution plates to the cut surfaces of the stem segments. Each conidium was placed downward to allow direct contact with the stem tissues. The plates were incubated at room temperature and examined microscopically at 1-wk intervals for 3 wk during which time fungal growth and sporulation were recorded. The rating system for growth of *V. dahliae* on each stem segment was as follows: 0 = no growth, 1 = trace growth, 2 = slight to less than half of cross-section surface covered, 3 = more than half covered, 4 = dense growth of mycelial mat on entire surface, and 5 = dense growth plus microsclerotia. The growth index was obtained by dividing the sum of the growth scores by the number of replicates (six plants × four segments = 24 replicates).

Tests for uniformity and viability of *V. dahliae*. The uniformity of the *V. dahliae* isolates used was tested by transferring single germinated conidia, referred to as sporelings, from the dilution plates prepared for each experiment, to PDA slants. The growth of the sporelings was compared concurrently. This test was essential to ensure the lack of any variability in the conidial population used in each experiment.

Viability of sporelings that showed zero growth after 3 wk of incubation on the cut surfaces of stem segments was tested by retransferring them from the stem segments to PDA slants. Resumption of growth from these sporelings indicated that the failure to grow on cotton stem segments was due to inhibition directly attributable to the cotton tissues and not to any inherent lack of viability.

RESULTS

Distribution of *V. dahliae* in inoculated plants. Serial culturing of cotton plants inoculated with the mild, nondefoliating SS-4 strain of *V. dahliae* showed no difference in the distribution of this strain in stem tissues of the resistant SBSI and the susceptible Deltapine 15 (Fig. 2A). Both cultivars yielded the fungus from basal segments sampled the same day as inoculated, from the middle segments after 24 hr, and from the apical part of the stem after 48 hr. By the third day after inoculation, *V. dahliae* was present in cross sections from all stem segments in all replicates of both cultivars. However, some differences in fungal growth and sporulation from these cross sections were observed. Microsclerotia were formed by the fifth or sixth day of incubation of the susceptible Deltapine 15 stem sections sampled 4–5 days

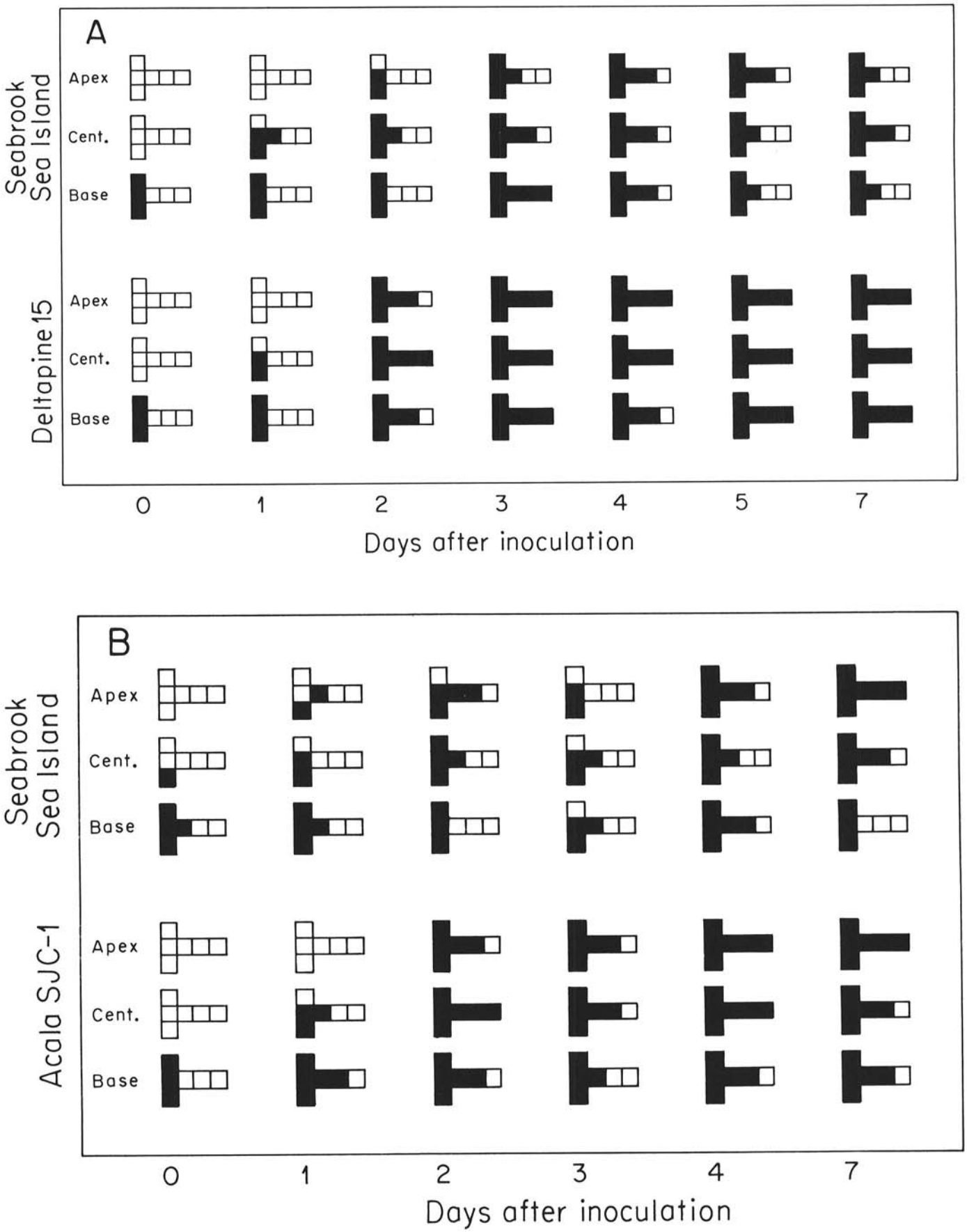

Fig. 2. Distribution of *Verticillium dahliae* on *Gossypium barbadense* and *G. hirsutum* cotton plants after inoculation with **A,** mild SS-4 strain and **B,** severe T-1 strain of the fungus. Each sample consisted of three plants per cultivar per day. For each sample location, stem tissues are arranged vertically with each square representing one plant (10 cross sections per plant), and petiole tissues are arranged horizontally with each square representing one plant (five cross sections per plant). Black squares indicate presence of *V. dahliae*; unshaded squares indicate absence of *V. dahliae*.

after inoculation. Cultures from the SBSI sections required at least five more days to reach this stage. Another varietal difference was observed in stem samples cultured 7 days after inoculation. In an early examination of these sections 3 days after inoculation, *V. dahliae* growth was not detected in any of the SBSI apical stem samples, whereas growth was detected in all Deltapine 15 stem sections. After five more days of incubation, all SBSI stem sections were showing *V. dahliae* growth.

In contrast to the stem, the movement of the SS-4 strain from stem tissues into leaf petioles was much slower in resistant than in susceptible cotton cultivars. Culturing of cross sections of leaf petioles showed clear cultivar differences that appeared primarily as delay in onset of infection and as a reduction in the frequency of infection of the leaf petiole tissues. Two days after inoculation, *V. dahliae* was present in only one petiole sample from SBSI compared with seven petioles of Deltapine 15. During the first 6 days of incubation, petiole pieces from the second through the seventh day after inoculation showed the fungus present in two to eight times as many petiole samples of susceptible compared with resistant cotton cultivars. The same but more moderate trend was expressed in the final reading after the stem pieces had been incubated for 3 wk (Fig. 2A).

The distribution of *V. dahliae* in SBSI and Acala SJC-1 after inoculation with the more virulent T-1 strain (Fig. 2B) was similar to that of plants inoculated with the SS-4 strain. The results showed minor differences in the movement of T-1 within the stems of both cultivars, with only slightly less in the most resistant cotton during the first 3 days after inoculation. Both T-1 and SS-4 were present equally in all sections prepared from stems of both cultivars four or more days after inoculation.

In the T-1 strain test, as in the SS-4 test, significantly less fungus was detected in the leaf petiole tissues of the resistant than in those of the susceptible cultivar. In the second and third days after inoculation, the ratios of the total number of infected petioles in SBSI to Acala SJC-1 were favoring the resistant cultivar by 3:7 and 2:5, respectively. This ratio tapered off to 5:7 in the sample taken 7 days after inoculation.

Growth of single conidia on stem segments. Single germinated conidia of the SS-4 strain after incubation on stem segments showed growth responses that differed depending on the cotton cultivar and the location on stems from which segments were cut (hypocotyl or upper 10 cm). The mean growth index (MGI) of the fungus on each cultivar was the average growth scored in six experiments, each involving six plants. In each experiment, four hypocotyl segments and four upper 10-cm segments were prepared from each plant. Stem segments from the most susceptible cotton, 70-110, supported the highest level of fungal growth, whereas segments from the most resistant cultivar, SBSI, supported the least amount of growth (Fig. 3). The growth of the fungus on hypocotyl segments of the two Acala cultivars tolerant to SS-4 was intermediate between that of the most resistant and the most susceptible cultivars tested (Fig. 3A). However, the MGI of the fungus on the upper stem segments of SBSI contrasted markedly with that of *G. hirsutum* cotton cultivars. Almost an entire index point separated the resistant (SBSI) from the *G. hirsutum* cultivars, which were grouped closely together (Fig. 3B). In both hypocotyl and apical stem segments, the differences in mycelial growth from single conidia attributable to the different cotton cultivars remained constant after 7, 14, and 21 days.

The lower MGIs of SS-4 strain on both hypocotyl and upper

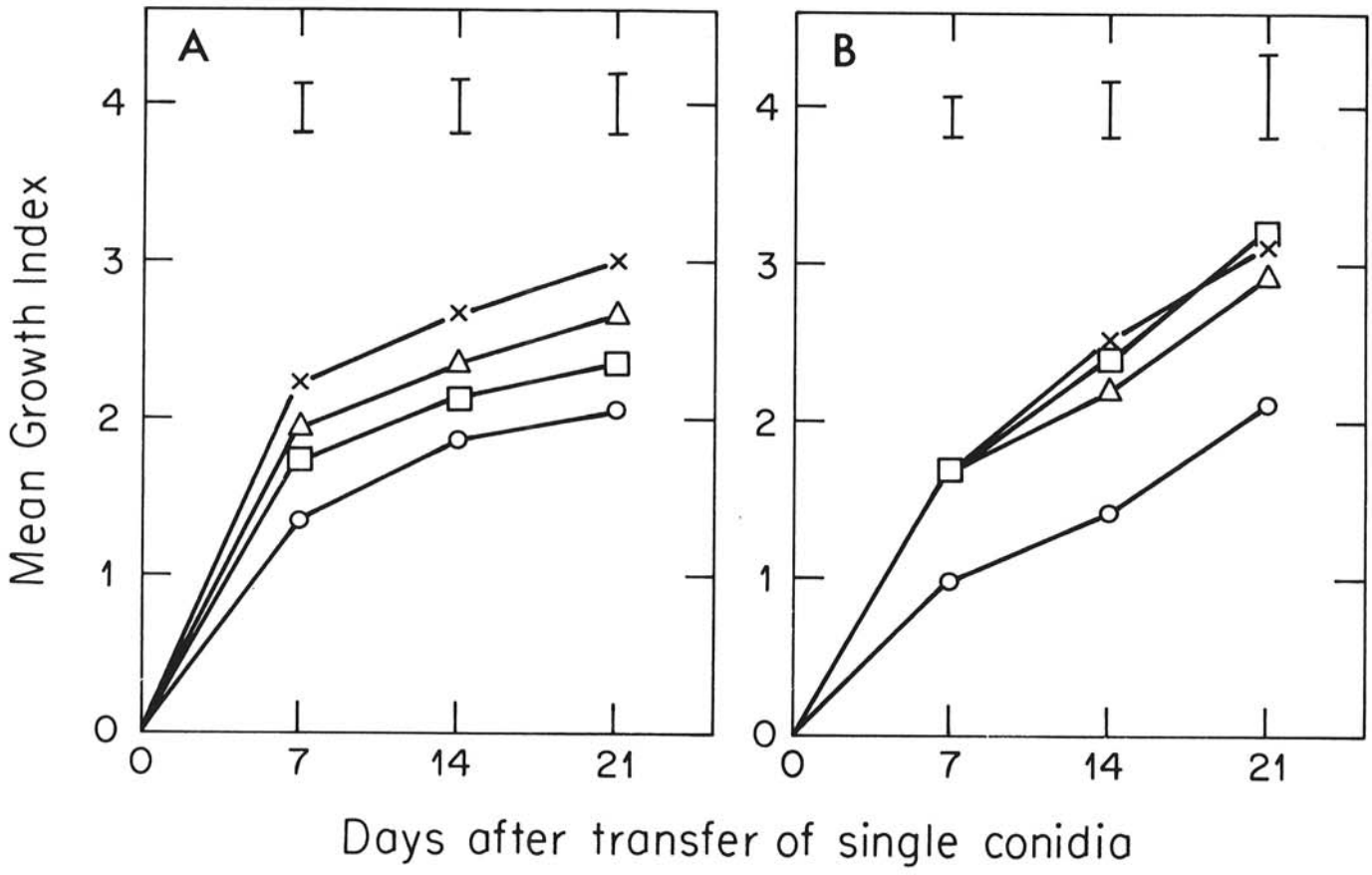

Fig. 3. Mycelial growth from single germinated conidia of mild SS-4 strain of *Verticillium dahliae* after incubation on cotton stem segments from different cotton cultivars: o = Seabrook Sea Island, □ = Acala SJC-1, Δ = Acala 4-42, and × = 70-110. **A,** Segments prepared from the hypocotyl and **B,** segments prepared from the upper 10 cm of the stem. A single germinated conidium was transferred to the cut surface of the stem segments, which were partially embedded in wells of water agar in tissue-culture multiwell plates. The plates were examined microscopically every 7 days after incubation at room temperature, and mycelial growth was assessed. The mean growth index represents the average of six experiments, each consisting of six plants per cultivar and four segments per plant per stem location. Vertical bars indicate the pooled standard error for data collected at each observation date.

10-cm segments of SBSI were due in part to the significantly higher percentage of conidia that scored zero growth during the entire 3 wk of incubation. However, when the viability of these sporelings was tested by transferring each to a PDA slant, 85% of the conidia germinated and developed into typical SS-4 strain cultures within 8 days.

The growth of single conidia of the T-1 strain on different stem segments showed a response different from that of the SS-4 strain. The hypocotyl stem segments of SBSI were inhibitory to the mycelial growth of the T-1 strain the same as they were to the SS-4 strain. However, there were no differences between all three *G. hirsutum* cotton cultivars tested (Fig. 4A). The MGIs on the upper 10-cm stem segments were grouped tightly together during the 3 wk of incubation, with no comparative inhibition of growth by SBSI. Apparently, the severe T-1 strain of the fungus, when placed in direct contact with the vascular stem tissues, was able to colonize excised apical stem cross sections of the resistant as well as susceptible cotton cultivars (Fig. 4B).

Microsclerotial formation on stem segments. The formation of microsclerotia on mycelial mats developed from single germinated conidia of SS-4 was significantly less on stem segments of the resistant SBSI (*G. barbadense*) than on the more susceptible *G. hirsutum* cultivars (Fig. 5). In general, formation of microsclerotia was more extensive on the immature upper stem segments than it was on the hypocotyl segments. In terms of percentage of segments on which any microsclerotia were formed, the development of these structures on the upper stem segments of SBSI, though erratic, was less frequent than on other cultivars. The fungus developed microsclerotia on fewer than 27% of the total number of upper stem segments of SBSI; percentages for the others were 37 for Acala 4-42, 58 for Acala SJC-1, and 57 for 70-110.

DISCUSSION

In greenhouse-grown plants, both SS-4 and T-1 strains of *V. dahliae* were isolated from the vascular tissues of the upper stems and petioles of resistant and susceptible cotton plants in fewer than 3 days after stem-puncture inoculation of the hypocotyl. The presence of the fungus in these tissues preceded the first appearance of symptoms by 4–6 days. The rate of movement of *V. dahliae* in the xylem exceeded that which can be accounted for by mycelial growth. While providing no evidence that conidia form in all cotton cultivars after infection, these results agreed with previous reports (5,7,14) indicating that the fungus advanced this distance by rapid upward movement of conidia in the transpiration stream of resistant and susceptible cotton cultivars. However, after systematic culturing of leaf petioles of resistant and susceptible cultivars above the point of inoculation, both T-1 and SS-4 were detected in more of the susceptible petioles than in those of resistant cultivar. There was no indication that *V. dahliae* was contained or confined at the point of initial inoculation in the resistant cotton, SBSI. These results agree with earlier observations by Garber and Houston (6) and focused our attention on resistance factors that involved the inhibition of conidial formation in the xylem.

When single germinated conidia of *V. dahliae* were transferred in a small block of plain, washed agar to the cut surfaces of stem segments of cotton cultivars differing in susceptibility, the vegetative growth and sporulation that ensued reflected the level of susceptibility in each cultivar. For instance, when conidia of the mild SS-4 strain were transferred to stem segments cut near the hypocotyl region, growth was slight, moderate, or abundant on resistant, tolerant, and susceptible cultivars, respectively. On stem

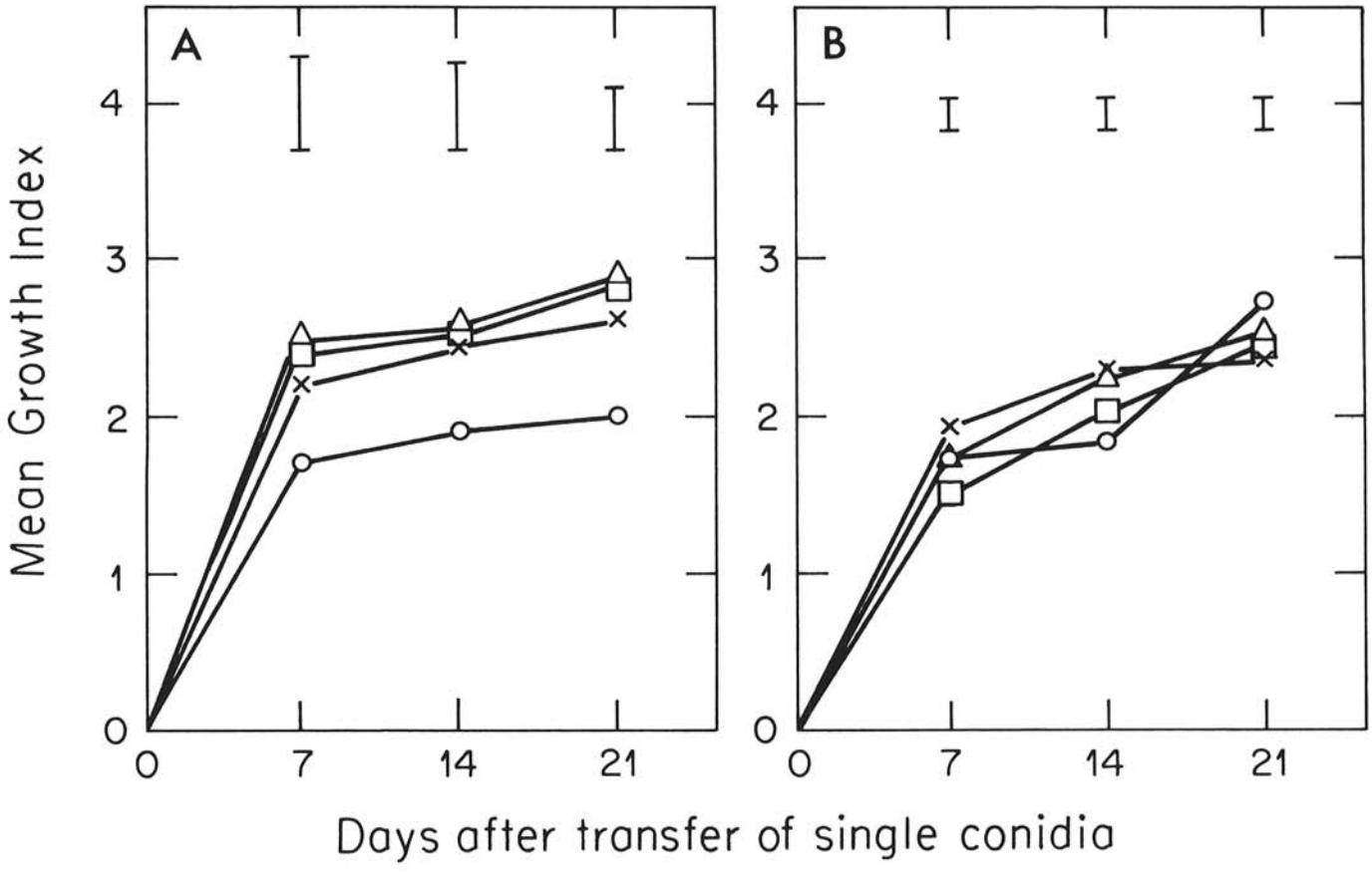

Fig. 4. Mycelial growth from single germinated conidia of severe T-1 strain of *Verticillium dahliae* after incubation on stem segments from different cotton cultivars: o = Seabrook Sea Island, □ = Acala SJC-1, Δ = Acala 4-42, and × = 70-110. **A,** Segments prepared from the hypocotyl and **B,** segments prepared from the upper 10 cm of the stem. Mean growth index represents the average of two experiments, each consisting of six plants per cultivar and four segments per plant per stem location. Vertical bars indicate the pooled standard error for data collected at each observation date.

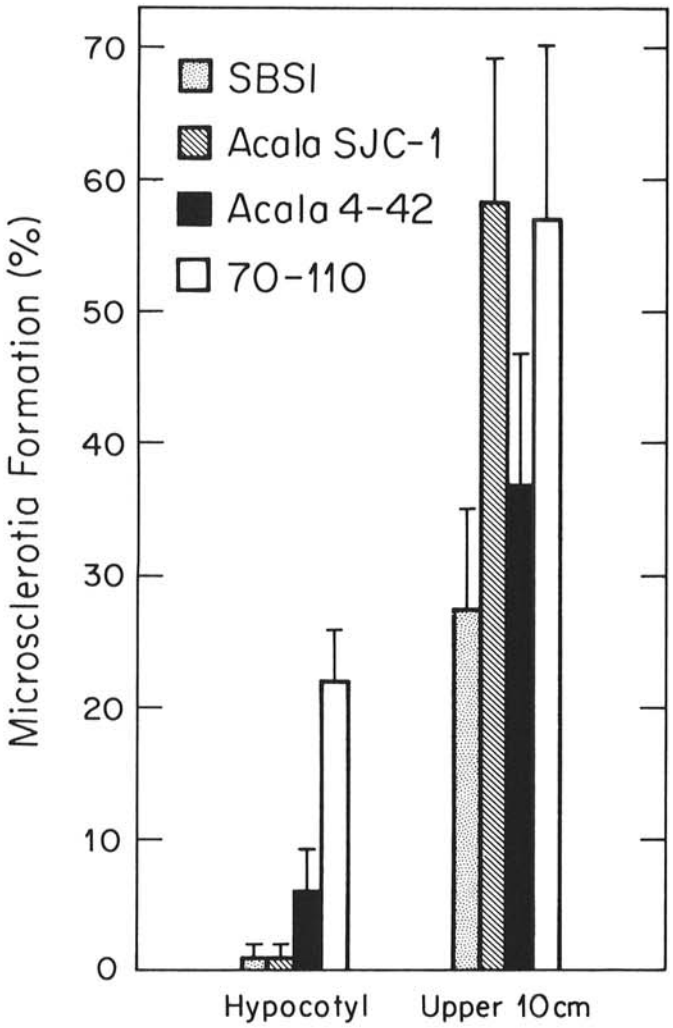

Fig. 5. Formation of microsclerotia on mycelia that developed from single germinated conidia of mild SS-4 strain of *Verticillium dahliae* transferred to cotton stem segments. Data are presented as percentage of segments on which any microsclerotia were formed after 21 days of incubation. Vertical bars represent the means and standard errors of three experiments, each consisting of six plants and four segments per plant per stem location.

segments cut within 10 cm of the apex, only the resistant SBSI cultivar strongly inhibited fungal growth. Germinated conidia of the severe T-1 strain grew poorly on hypocotyl segments of resistant SBSI but grew well on all stem segments above the hypocotyl. This does not suggest that the defense mechanism of the resistant host is inoperative in the upper parts of the plant but rather that under the test conditions, T-1 was able to overcome host resistance at those locations. In any case, the T-1 strain in general showed significantly more aggressiveness than SS-4. The aggressiveness of T-1 is probably responsible for the development of mild symptoms in SBSI under greenhouse and field conditions. However, plants of SBSI normally recovered with little or no adverse effects on productivity.

On the basis of these results and the fact that cotton cultivars in the field generally are all susceptible to penetration and some stem vascular infection regardless of their level of tolerance to strains of *V. dahliae* (6,17), our attention was directed toward attempts to define active defense mechanisms that resistant plants are likely to initiate soon after infection. The paper that follows (3) discusses the induction, accumulation, and chemistry of compounds that may be the primary determinants of resistance in cotton to Verticillium wilt.

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