Infection, Colonization of *Gossypium hirsutum* and *G. barbadense*, and Development of Black Root Rot Caused by *Thielaviopsis basicola*

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ABSTRACT

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On 28 March and 28 April 1986 and on 12 April and 16 May 1988, *Gossypium barbadense* was planted in naturally infested cotton fields containing approximately 600 colony-forming units (cfu) of *Thielaviopsis basicola* per gram of air-dried soil. Soil temperatures ranged from 18 to 20 C and 24 to 26 C at a depth of 15 cm during the first and second planting dates, respectively, during both seasons. During the 1986 and 1988 seasons, 100% of seedlings sampled 1 mo after the first planting were infected, with the severity of cortical root decay ranging from 75 to 100%. Two weeks after the second planting, 89% of the seedlings sampled in 1986 and 92% of the seedlings sampled in 1988 were diseased with 50–75% cortical root decay during both seasons. Cotton stand counts made at 1 and 2 mo after seedling emergence indicated that the stand was reduced by 28% in 1986 and 32% in 1988 in the first planting and 11% in 1986 and 8% in 1988 in the second planting when compared with the original number of seedlings in each planting. In October 1986, 32% of the plants from the March planting and 5% of the plants from the April planting had darkened stelar root tissue near the crown that contained hyphae and aleuriospores of *T. basicola* in xylem and phloem tissue. *Gossypium hirsutum* was grown at 20 and 28 C under controlled conditions in soils containing *T. basicola* at 0, 90, and 600 cfu/g of soil. Seedlings were evaluated 10 days after seedling emergence. Seedling stunting increased with inoculum density at both temperatures when compared with the control plants without the fungus. However, levels of disease increased at the low-temperature or high-inoculum-density treatments. Scanning electron microscopy demonstrated that phialoconidia and aleuriospores germinated and germ tubes penetrated within 12 and 48 hr, respectively, after root inoculation at 24 C. Germ tubes of both spores produced appressoria before penetration. Hyphae colonized cortical tissue 72 hr after inoculation and grew intracellularly and centripetally. Five days after inoculation, infected cells were filled with hyphae and aleuriospores.

Additional keywords: disease histology.

Black root rot of cotton caused by *Thielaviopsis basicola* (Berk. and Br.) Ferr. (= *Chalara elegans* Nag Raj and Kendrick [12]) was reported first in Sacaton, AZ, in 1922. The disease was described as an internal collar rot of mature American-Egyptian cotton, *Gossypium barbadense* L. (5). Later, it was found to cause a seedling root rot of both *G. barbadense* and *G. hirsutum* L. (Upland cotton) (5,13). Historically, this disease has been observed in Arizona at elevations above 900 m where soil temperatures are cooler at planting than at lower elevations. However, in the 1986, 1987, and 1988 seasons, the incidence of black root rot of cotton increased at lower elevations (Mauk and Hine, *personal observation*), which corresponded with an increase in acreage of *G. barbadense* (Pima cotton). Pima cotton requires a longer growing season than Upland cotton and, therefore, is planted earlier when soil temperatures favor disease. *T. basicola* causes a black cortical decay of the tap root, delays the development of the seedling, and can cause seedling death when environmental conditions favor the disease. However, infected plants can recover when conditions favor plant growth. Although the fungus colonizes the vascular tissue, the pericycle remains uninjured (9), thus allowing cortical regeneration and secondary root growth.

The objectives of this research were to study germination, penetration, and development of *T. basicola* on *G. barbadense* and *G. hirsutum*, and the influences of temperature and inoculum density on disease development.

MATERIALS AND METHODS

Fungal isolates, inoculum preparation, and plant material. Two isolates of *T. basicola* were obtained from soil previously planted with cotton: L-3 from Duncan, AZ, and H2-S1 from Coolidge, AZ. A third isolate, Int-5, was obtained from a diseased cotton plant from Coolidge, AZ. Inoculum was prepared by flooding a 4- to 6-wk-old culture of *T. basicola* grown on 10% carrot extract agar containing 1 g/L of $CaCO_3$ with 5 ml of sterile distilled water and mechanically dislodging the spores. Phialoconidia and aleuriospores were counted with the aid of a hemacytometer, and the total concentration was adjusted to 1×10^6 spores/ml. Cotton species and cultivars used were *G. barbadense* 'Pima S-6' and *G. hirsutum* 'Delta Pine 90.' Seeds were surface-disinfested sequentially in 95% ethanol for 1 min, 1.0% sodium hypochlorite for 10 min, followed by four changes of sterile distilled water over a 30-min period.

In vitro infection, penetration, and colonization of cotton seedlings. Three sheets of 26 × 39 cm germination paper (Anchor Paper, St. Paul, MN) were placed on a 30 × 48 cm sheet of Reynolds Oven Wrap (Consumer Products Division, Reynolds Metal Co., Richmond, VA), saturated with distilled water, rolled lengthwise with the oven wrap to the outside, placed into a 400-ml beaker filled with 300 ml of distilled water, and autoclaved (11). Thirty surface-disinfested cotton seeds were placed 2 cm from the end and 1 cm apart, between the first and second sheet of the autoclaved germination paper. The paper was rolled as before and placed vertically, with the seeds at the top, into a sterile 400-ml beaker containing 200 ml of sterile distilled water and incubated for 7 days at 24 C under 5,200 lx fluorescent lights in a growth chamber with a 12-hr diurnal light cycle. After incubation, seedling roots were dipped for 30 sec in a 0.01% solution of Triton X-100 (Rohm and Haas, Delaware Valley, Inc., Philadelphia, PA) to reduce surface tension and rinsed for 30 sec in sterile distilled water. Seedlings were inoculated by pipetting 200 μl of a 1×10^6 cells/ml suspension of phialoconidia and aleuriospores of an isolate of *T. basicola* onto tap roots. Seedlings were returned to the germination paper and further incubated in the growth chamber for 8, 12, or 24 hr for infection and penetration studies, and 3, 4, 5, or 8 days for seedling colonization studies. After incubation, seedling roots were prepared for microscopic studies. All

histological experiments were repeated three times, with at least 10 samples examined from each treatment. Seedlings were arranged in a completely randomized design.

Influence of temperature and inoculum density on disease development. Field soil from a study plot in Coolidge, AZ, was assayed on a selective medium modified from Specht and Griffin (14) to determine the colony-forming units (cfu) of *T. basicola* per gram of soil. The selective medium contained carrot extract (15% w/v), 10 g/L of agar, 1.0 g/L of $CaCO_3$, 500 mg active ingredient (a.i.)/L of pentachloronitrobenzene (PCNB), 400 mg a.i./L of etridiazol, 50,000 units of nystatin, 500 mg a.i./L streptomycin sulfate, and 30 mg a.i./L of chlortetracycline hydrochloride. Carrot extract was prepared by triturating 150 g of fresh, peeled carrots in 350 ml of distilled water and then straining the extract through four layers of cheesecloth. The medium, without fungicides or antibiotics, was autoclaved for 20 min. PCNB and etridiazol were added immediately after autoclaving, and the antibiotics were added when the medium had cooled to 60 C. Field soil was assayed by placing 10 g of air-dried soil in a 125-ml Erlenmeyer flask containing 90 ml of 0.1% water agar and stirring for 3 min. Aliquots of 1.0 and 0.5 ml were taken from the suspension and spread evenly on the agar surface of the selective medium. After incubation for 8 days at 19 C, colonies of *T. basicola* were counted to determine the number of colony-forming units per gram of soil.

Soil naturally infested with 600 cfu/g of *T. basicola* was diluted volume per volume with sterilized field soil to obtain an inoculum level of 90 cfu/g. The same soil was autoclaved for 1 hr to obtain a fungus-free control field soil. Four surface-disinfested seeds of *G. hirsutum* were planted in 6-cm-diameter pots containing 600, 90, or 0 cfu/g of *T. basicola*; there were five replications per inoculum level. Pots were incubated in growth chambers at 20 and 28 C under 5,200 lux fluorescent light on a 12-hr diurnal light cycle. Ten days after seed germination, seedlings were rated for height and disease index (percent cortical decay) with the rating system described by Tabachnik et al (15). Disease index ratings were as follows: 0 = no blackened cortical tissue; 1 ≤ 25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% cortical decay. Experiments were repeated twice, and data were subjected to an analysis of variance. Least significant difference tests were used for mean separations.

Infection and colonization of *G. barbadense* in the field. Seeds of *G. barbadense* were planted into two cotton fields naturally infested with *T. basicola* in Coolidge, AZ, on 28 March and 28 April 1986 and on 12 April and 16 May 1988. The fields had been in monoculture cotton for 4 yr before the initiation of this study. Plant stand densities were determined by counting the number of plants in 10 completely random, replicated, 4-m rows at 0.5 (seedling emergence), 1, and 2 mo after planting. Percent infection was determined on 75 plants randomly collected from each plot 1, 2, 3, and 6 mo after the first planting date and 0.5, 1, 2, and 7 mo after the second planting date. Isolations for *T. basicola* were conducted for sampled plants by a modification of Yarwood's (18) carrot baiting technique. For this, carrots were peeled, cut into 5- to 7-mm-thick cross sections, surface-disinfested for 2 min in 0.5% sodium hypochlorite, rinsed with sterile distilled water, and air dried. A 3-cm root segment from each plant sampled was washed in running water for 5 min, surface-disinfested in 0.5% sodium hypochlorite for 2 min, and placed on a sterile moistened filter paper in a sterile petri dish with a carrot slice positioned on top of the root. These petri dishes were incubated in a moist chamber in the dark at 22 C and were examined with a dissecting microscope for fungal growth after 5–7 days.

Seventy-five roots of cotton plants, sampled after 6 and 7 mo from both planting dates in 1986, were observed macroscopically for infection of stelar root tissue and were prepared for both bright-field and scanning electron microscopy (SEM).

Preparation of root tissues for SEM and light microscopy. Roots from laboratory studies and roots having blackened stelar tissues from field studies were cut into 3- to 10-mm sections with a razor blade and fixed in a mixture of 4% paraformaldehyde-1% glutaraldehyde in a 0.1 M Sorensen's phosphate buffer (pH 7.2) for 3 days. Samples were rinsed, dehydrated in ethanol (10–100% in 15% increments at 30 min per increment), and critical-point dried with carbon dioxide. Samples were mounted on SEM stubs, coated to a thickness of 15 nm with gold/palladium (60:40), and observed on an ISI DS 130 scanning electron microscope (International Scientific Instruments, Santa Clara, CA). Root tissues from seedling colonization studies were fixed as described above, rinsed with distilled water, dehydrated from 10% ethanol to 100% tertiary butyl alcohol in 15% increments, changed at 1-hr intervals, and embedded in paraffin. Embedded roots were cut by hand into longitudinal sections and cross sections with a razor blade. Paraffin was removed from the tissues by washing twice daily with xylene for 3 days. Tissues then were washed in a 1:4, 1:1, and 4:1 anhydrous ethanol:xylene series and rinsed three times in 100% ethanol to remove the xylene. Root sections were critical-point dried with carbon dioxide, mounted on SEM stubs, and coated as described above.

For bright-field microscopy, 20 mature Pima cotton roots having blackened stelar tissues were free-hand sectioned longitudinally with a razor blade. Tissue sections were observed with a Nikon microscope (Nippon Kogaku K.K., Tokyo 100, Japan) at 10 and 40 × and photographed with an AFX Nikon camera with Kodachrome 64 film.

RESULTS

In vitro infection, penetration, and colonization studies. Phialoconidia began germinating 6 hr after inoculation, and mycelial penetration of host tissue occurred within 12 hr after inoculation. Aleuriospores germinated after 24 hr incubation, and host tissue was penetrated within 36 hr. Phialoconidia germinated with one germ tube rupturing the spore wall (Fig. 1A). Aleuriospores generally produced a single germ tube (Fig. 1B) that emerged from between the cylinder wall and the top of individual aleuriospore cells in both intact and separated spore chains. Occasionally, aleuriospores germinated with two germ tubes (Fig. 1C). Germ tubes of both phialoconidia and aleuriospores elongated and often developed an appressorium (Fig. 1D). Penetration was similar for both phialoconidia and aleuriospores (Fig. 1E). Phialoconidia (arrow) and aleuriospores of *T. basicola* were produced on the surface of the root within 72 hr after germination and colonization (Fig. 1F).

Tissue colonization occurred immediately after penetration and was characterized by the presence of both constricted and unconstricted hyphae as described by Christou (3) and Mathre et al (9). Seventy-two hours after inoculation, unconstricted hyphae had penetrated cortical cells and had grown toward the vascular stele (Fig. 2A). After 4 days, cortical cells were colonized by constricted and unconstricted hyphae (Fig. 2B). After 5 days of incubation, infected cells began to fill with constricted hyphae (arrow, Fig. 2C). Frequently, aleuriospores were produced in the cells filled with hyphae. *T. basicola* reproduced by both aleuriospores and phialoconidia on the surface of the root within 72 hr after inoculation (Fig. 2D). These events were the same irrespective of species of *Gossypium* inoculated or the isolate used as inoculum.

Influence of temperature and inoculum density on disease development. Ten days after incubation, seedlings in growth chambers were stunted and their roots were heavily decayed at both inoculum levels (Table 1). However, plants grown in soil containing 600 cfu/g and incubated at 20 C had a significantly higher disease index than those incubated at 28 C. Height of the control plants (0 cfu/g) was reduced by 29% at 20 C when compared with plants at 28 C. However, plant height were reduced significantly from that of the control plants at both temperatures when the fungus was present.

Infection and colonization of field plants. *T. basicola* was recovered from root tissue of *G. barbadense* throughout the 1986 growing season. However, the percent recovery from both internal and external cortical root tissues was reduced as the season progressed. After 6 wk, external symptoms of blackened cortical tissues appeared as narrow black strips containing aleuriospores on the surface of apparently healthy, white cortical tissue in plants

from both planting dates. Cortical root decay for plants collected 2 wk after planting from the March plot was estimated to be 76–100% and 51–75% for the April plot. Recovery of *T. basicola* from cortical tissues of plants from the March planting date was reduced from 100% (1 mo after planting) to 81% (3 mo after planting); recovery from plants from the April planting was reduced from 89% (2 wk after planting) to 49% (3 mo after planting) (Table 2). Plant stand estimations 2 mo after planting showed a 28% reduction in number of seedlings in the March plot and a 11% reduction in the April plot. Although the plants in both plots had no apparent cortical decay 3 mo after planting, *T. basicola* could be recovered from vascular tissues from 96% of the plants sampled in the March plot and 62% of the plants in the April plot.

At harvest, aleuriospores of *T. basicola* were observed in the central stelar region of roots (Fig. 3A and B) in 32% of the plants sampled from the March plot and 5% of the plants from the April plot. Hyphae frequently filled the individual phloem cells of

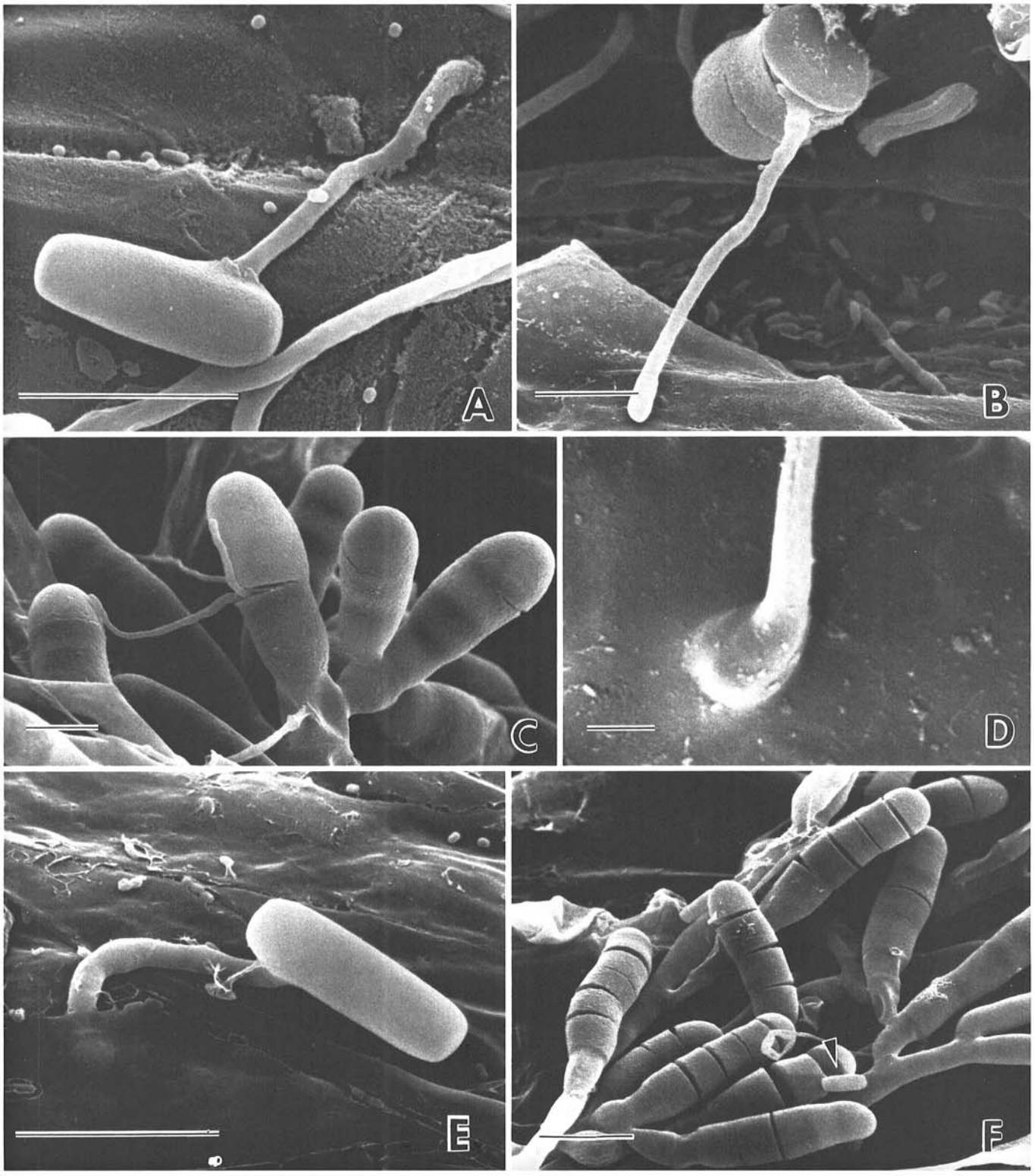

Fig. 1. Germination and penetration of phialoconidia and aleuriospores. **A,** Germinating phialoconidium (bar = 10 μm). **B,** Germinating aleuriospore with one germ tube (bar = 10 μm). **C,** Germinating aleuriospore with two germ tubes (bar = 10 μm). **D,** Appressorium of penetrating hypha (bar = 1 μm). **E,** Penetrating germ tube from phialoconidium (bar = 10 μm). **F,** Aleuriospores on root surface with phialoconidium (arrow) (bar = 20 μm).

mature roots, whereas other cells were completely filled with aleuriospores (Fig. 3C). Thin sections of root tissues from heavily infected roots had aleuriospores in the white cortex and in the blackened vascular tissues. The heaviest spore production occurred within the stelar region of the root (Fig. 3D), with spores forming in the primary and secondary xylem and phloem.

TABLE 1. Influence of temperature and the inoculum density of *Thielaviopsis basicola* on plant height and cortical root decay of *Gossypium hirsutum* 'Delta Pine 90'

Temperature (C)	Inoculum density (colony-forming units per gram soil)	Mean plant height (mm)[x,y]	Mean disease index[z]
20	0	36.8 a	0.0 a
	90	27.2 b	2.2 b
	600	26.2 b	4.0 c
28	0	51.7 c	0.0 a
	90	44.8 d	1.9 b
	600	38.3 e	3.1 d

[x] Mean of 30 replications of one experiment. Results of repeated experiments were similar.
[y] Numbers within each column followed by the same letter are not statistically different at $P = 0.05$ according to least significant difference separation of means.
[z] Disease index is based on percent cortical root decay 10 days after germination: 0 = healthy; 1 ≤ 25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%.

TABLE 2. Percent infection and internal colonization of roots of *Gossypium barbadense* 'Pima S-6' by *Thielaviopsis basicola* from a naturally infested field containing 600 colony-forming units per gram of *T. basicola*

Planting date of cotton[a]	Sampling date	Plant age[b]	Percent infection	Percent internal colonization[c]	Percent stand reduction[d]
March 28, 1986	4/28	1	100	...[e]	13
	6/2	2	97	...	28
	7/17	3	81	43	28
	10/28	7	...	32	...
April 28, 1985	5/8	0.5	89	...	0
	6/2	1	83	...	11
	7/17	2	49	7	11
	10/28	6	...	5	...

[a] Soil temperatures were 18–20 C and 25–27 C at 15-cm depth for March 28 and April 28, respectively.
[b] Plant age in months after planting.
[c] Percent internal colonization of 75 plants sampled for aleuriospores of *T. basicola* in internal root tissues.
[d] Percent stand reduction from initial plant stand.
[e] Not determined.

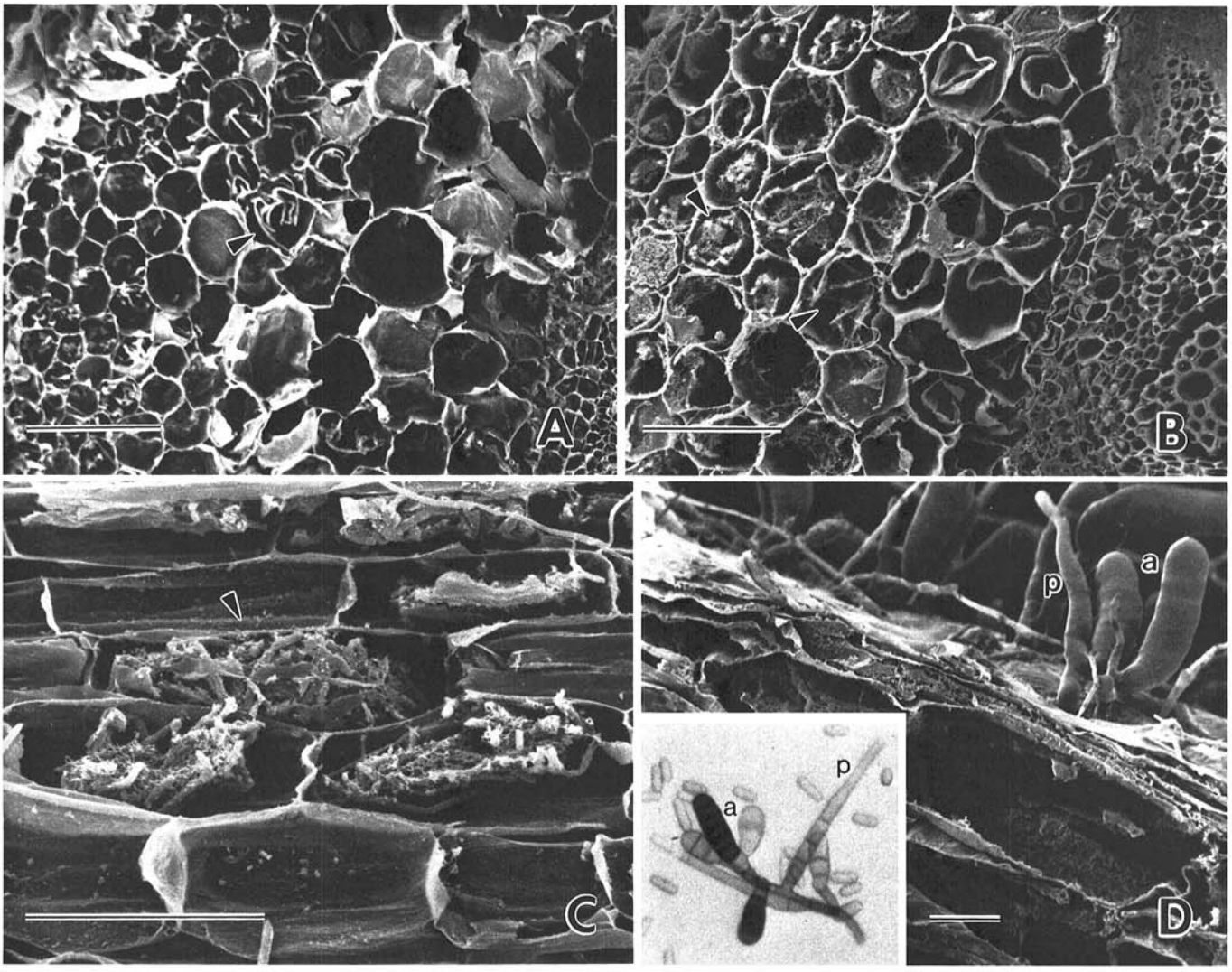

Fig. 2. Colonization of seedlings of *Gossypium hirsutum* by *Thielaviopsis basicola*. **A,** Cross section of cotton seedling root showing colonization by unconstricted hyphae 3 days after inoculation (bar = 100 μm). **B,** Colonization of seedling roots by constricted and unconstricted hyphae 4 days after inoculation (bar = 100 μm). **C,** Longitudinal section of cortical cells filled with constricted hyphae 5 days after inoculation (bar = 100 μm). **D,** Production of aleuriospores (a) and phialoconidiophores (p) 3 days after inoculation (bar = 10 μm). Inset of light micrograph of aleuriospores (a), phialoconidia, and phialoconidiophore (p).

Longitudinal sections of mature roots of *G. barbadense* (Fig. 3D) revealed prolific aleuriospore production within the central stele. These spores had three wall layers, and the interior wall surface had numerous depressions (Fig. 3E).

Results from infection and colonization studies of seedlings of *G. barbadense* for the two planting dates in 1988 were similar to data collected in 1986. Higher levels of infection and colonization occurred in plants from the first planting date (12 April 1988) when soil temperatures ranged from 18 to 20 C as compared with those from the second planting date (16 May 1988) when soil temperatures ranged from 24 to 26 C. The infection level in plants from the first planting date was 100%, with a 32% reduction in stand, compared with a 92% infection level and an 8% stand reduction for the second planting.

DISCUSSION

Hyphae from phialoconidia of *T. basicola* infected roots of cotton within 12 hr, whereas hyphae from aleuriospores of the fungus infected within 36 hr after inoculation of root tissues under optimal temperature (24 C). Examination of germinating aleuriospores with the scanning electron microscope showed germ tube emergence from between the lateral spore wall and the end of the cell (Fig. 1B). This area was considered to be an "operculum" by Tsao and Tsao (16). Individual aleuriospore cells were cylindrical with a flat top. This is contrary to the report by Christias and Baker (2) who indicated that the top of the cell was centrally depressed. This depression could have been caused by improper fixation techniques and, therefore, could be an artifact. We did confirm the presence of a pore in the center of the top of some aleuriospore cells (2). The function of this pore is unclear, but, apparently, it does not function in the germination process. In addition to these morphological features, the aleuriospore wall was examined under SEM and was found to have three layers, with the interior surface having numerous depressions. Further, there was an apparent weakening in the internal junction between the septal and lateral spore wall of each cell where the germ tube emerges.

In contrast to aleuriospore germination, the germ tube of the

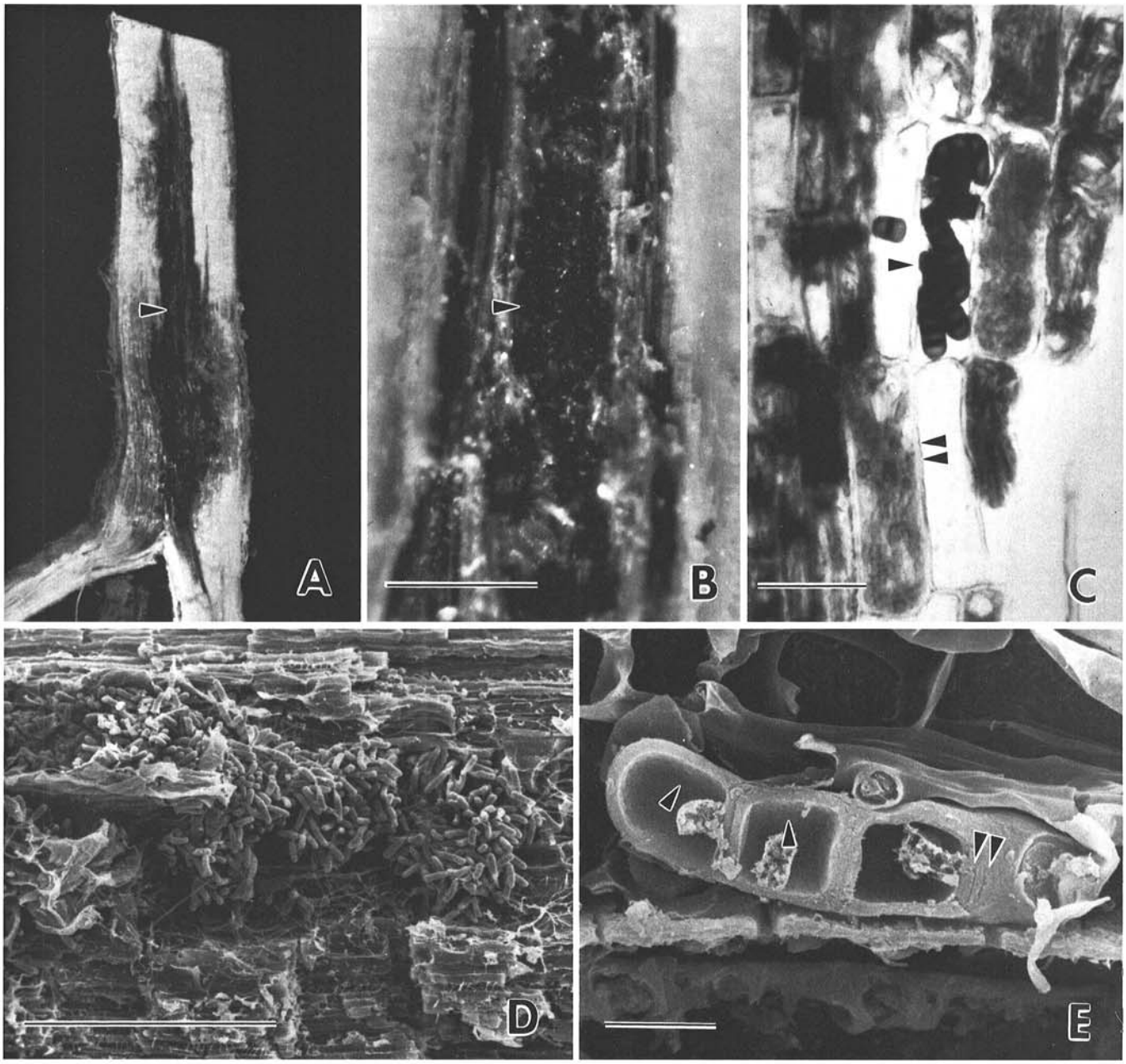

Fig. 3. Internal colonization of mature *Gossypium barbadense* roots. **A,** Longitudinal section of blackened stelar root tissue as a consequence of aleuriospore production. **B,** Aleuriospore production (arrow) in stelar region of the root (bar = 500 μm). **C,** Aleuriospores (arrow) and hyphae (double arrow) within a root cell (bar = 50 μm). **D,** Scanning electron microscopy of blackened stelar root tissue with aleuriospores within the stele (bar = 500 μm). **E,** Longitudinal section through aleuriospore showing three cell wall layers (double arrow) with depressions on the inner wall (arrow) (bar = 10 μm).

phialoconidium ruptures through the spore wall near one end of the spore. After germination of both phialoconidia and aleuriospores and germ tube elongation, an appressorium was produced but disappeared after penetration. Christou (3) did not find appressoria when *T. basicola* infected *Phaseolus vulgaris* L., and Mathre (9) did not report appressorial formation of *T. basicola* infecting *G. hirsutum*.

Colonization of the cortical root tissues occurred immediately after infection, and *T. basicola* began to actively reproduce on the surface of the cortex within 72 hr after infection. Colonization occurred with intracellular penetration by unconstricted hyphae into the cortical cells. The cells initially infected became filled with constricted hyphae and were frequently filled with aleuriospores. Unconstricted, intracellular hyphae apparently are responsible for initial invasion of cortical tissue, whereas constricted hyphae generally are present in cells filled with hyphae and in cells where aleuriospores are produced. Previous histological studies with the light microscope (3,9,17) indicated that in the infection process each cell becomes filled with hyphae before the next cell is invaded.

More severe cortical decay and plant stunting occurred at the highest inoculum level and lower temperature in plants inoculated in growth chambers studies. These results corresponded to field data obtained when soil temperatures were varied by planting date. In both 1986 and 1988, there was a higher incidence and severity of disease and a greater plant stand reduction in the early plantings as compared with the later plantings. Isolations from seedlings and observations of prolific occurrence of aleuriospores suggested that seedling death was caused by *T. basicola*. *Rhizoctonia solani* Kühn was isolated from less than 1% of all plants sampled throughout this study and was not considered to be a factor in seedling death. *Pythium ultimum* Trow was not isolated from any seedlings. Recovery of *T. basicola* from field plants decreased as the season progressed. Because the pericycle is uninjured by *T. basicola*, the root cortex can be regenerated (9). Therefore, as the season progresses, the cortical disease symptoms apparently disappear as the diseased cortical tissue is sloughed off and replaced by new healthy tissue. However, plants that were heavily colonized by the fungus during the seedling stage may contain aleuriospores in the stelar tissue of mature plants.

Although the optimum temperature for growth of the pathogen is between 25 and 28 C (7,8), *T. basicola* caused the greatest amount of damage when soil temperatures were cool (16–20 C) and not favorable for plant growth. This response also has been demonstrated in tobacco (4), peas (7), and cotton (1,10). Low temperatures may stress the growing plant and favor the pathogen. This may explain why a significant amount of cortical decay, 76–100%, occurred at both low and high temperatures, yet more severe stunting occurred only at the lower temperatures.

In addition to the influence of temperature on disease expression in seedlings, we have found that, under conditions of high inoculum levels (600 cfu/g) and low soil temperatures (18–20 C), internal colonization of root tissues by *T. basicola* is favored. Histological examination of mature Pima cotton roots from field plants showed that aleuriospores, but not phialoconidia, are actively produced in the stelar region of the root as well as in the phloem and cortical tissues. King and Presley (5) reported that this condition resulted in an internal collar rot resulting in death of mature American-Egyptian cotton. Leyendecker et al (6) also reported that internal collar rot occurred in *G. barbadense* under conditions where seedlings were heavily infected with *T. basicola* coupled with cool late-season weather. Internal collar rot and plant death may occur only under cool conditions where growth of the fungus is favored over growth of the plant. Internal collar rot with plant death was not observed during our studies. However, internal colonization and aleuriospore production in Pima cotton was found in 32% of the plants when the soil temperatures were between 18 and 20 C. Aleuriospores produced from internal colonization increase soil populations and may act as an additional source of inoculum for the following season.

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