Study of Field-Grown Cotton Roots Infected with *Verticillium dahliae* Using an Immunoenzymatic Staining Technique

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ABSTRACT

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Colonization of field-grown cotton roots by *Verticillium dahliae* was studied by using a specific immunoenzymatic staining technique. Colony density of *V. dahliae* increased with distance from the root tip, with the maximal density occurring more than 1 cm from the root apex. Colony densities at distances more than 1 cm from the tip were relatively constant. The mean colony length of *V. dahliae* was 7.3 mm, and increased colony length was correlated with distance from root apices. Hyphae of *V. dahliae* were present through the entire depth of the cortex, and were greatest in the interior of the root cortex at the surface of the vascular cylinder. The colony appearance was consistent with growth of hyphae from the root surface toward the stele. Hyphae of *V. dahliae* also were found within numerous cortical cells. Colonies of *Fusarium oxysporum*, similarly stained, were found to be mostly confined to the root surface and the outer cortex.

In the past, colonization of roots by fungi has been studied only to a limited extent in field soils because of the relative difficulty of such investigations. Many fungi colonize roots growing through field soils and most appear similar when stained by conventional methods, thus making observation of particular fungal species difficult. The development of specific staining techniques allows the direct observation of particular fungi in the presence of other fungal species (8). Previous researchers have used similar techniques to study *Ophiobolus graminis* on the roots of cereals (2), *Phytophthora* spp. in soil (15), *Verticillium lecanii* in rust pustules (17), and *Eutypa armeniacae* in wood (6).

Verticillium dahliae Kleb. has been shown to colonize the roots of a wide range of plant species, which include both those immune and those susceptible to systemic infection (3,4,9,10,12,14). Most of these studies have involved young plants growing in small pots in the greenhouse. Although such a system allows better control of environmental parameters, it does not permit normal root growth over an extended period of time because of the small soil volume in the system. Although there is general agreement that the fungus is able to reside on the surface of the root, there has been some disagreement on the ability of the fungus to colonize the cortical tissue of the root (13,14,20). The ability of the fungus to grow on or in the root tissue and to colonize the vascular tissue through root apices or branch points, and the ability of continual long-term persistence of colonies are important aspects of the host-pathogen relationship.

The following study was undertaken to determine the dynamics of colonization of cotton roots by *V. dahliae* under field conditions. The study used roots obtained from cotton (*Gossypium hirsutum* L.) plants grown under commercial production practices.

MATERIALS AND METHODS

Root isolation. Roots of cotton plants (cultivar SJ2) were obtained throughout the 1983 growing season from plants growing in a field infested with *V. dahliae* (82 microsclerotia per gram soil) at the West Side Field Station in the San Joaquin Valley of California. The field plot consisted of three replications of four rows (102-cm centers) by 30 m. The sampling dates were 26 June (shortly after commencement of flowering), 7 July, 27 July, 10 August, and 25 August (beginning of boll opening). For each sampling date, the sample consisted of a composite of roots collected from each replication. The roots were retrieved from soil samples collected, with the aid of a spade, from the side of the bed (0–20-cm depth), approximately 15 cm from the center of the row. The soil samples were dispersed in water in buckets (20 L), and the roots and other organic debris were collected with a sieve. Final cleaning and separation of roots was done by wet sieving, selective decanting, and physical separation. The isolated roots were fixed and stored in a solution of 1% formaldehyde, 2% glutaraldehyde, in 0.025 M sodium cacodylate buffer, pH 7.2, until needed.

Root staining. The buffers and solutions used in the study were described previously (8). Roots were removed from the fixative solution and rinsed several times with sodium cacodylate buffer and a final time with distilled water. The roots were washed onto a filter paper and with aid of a dissecting microscope were separated into groups of segments with root tips and without tips. Colonies of *V. dahliae* in both types of root segments were stained immunoenzymatically with the methods described previously (8). To begin the staining procedure, the root segments were incubated in the indirect enzyme-linked immunosorbent assay (ELISA) buffer for at least 15 min to prevent nonspecific binding of antibodies to the root tissue. The roots were rinsed (30 sec) three times with ELISA washing solution. The root segments were then incubated at 37 C for 1 hr on a rotary shaker in whole antiserum (1:100 dilution in indirect ELISA buffer) prepared against soluble proteins extracted from *V. dahliae* (8). After incubation, the root segments were washed as above, then incubated for 1 hr at 37 C on a rotary shaker in goat antirabbit IgG (1:1000 dilution in indirect ELISA buffer) conjugated with alkaline phosphatase (Miles Laboratories, Inc., Elkhart, IN). After incubation, the roots were washed as before, incubated in napthol-AS-phosphate plus fast blue BB substrate solution for 1 hr at 37 C in the dark. The root segments were rinsed with ELISA washing solution, cleared in 0.5% NaClO for 10 to 15 min, and then rinsed in distilled water.

Root segments without tips also were stained with the above procedure, with antiserum prepared against a soluble protein extract from a nonpathogenic isolate of *Fusarium oxysporum* Schlecht, amend. Snyd. Hans., obtained from cotton roots (8).

Root observations. Processed roots were evaluated for the presence of stained colonies of *V. dahliae*. Segments with colonies were segregated from the rest with the aid of a dissecting microscope and mounted on glass slides in a mixture of glycerol and sodium cacodylate buffer (1:1). Measurements on the colonies were made using an ocular micrometer in the dissecting microscope. The measurements included colony length, distance from the root apex to the colony midpoint, length of the root

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TABLE 1. Occurrence of colonies of *Verticillium dahliae* on cotton root tip segments

	Date	Interval from root tip (mm)								
		0–1	1–2	2–3	3–4	4–6	6–8	8–11	11–17	17–39
No. of colonies	26 June	0	0	0	0	0	0	0	2	1
	7 July	0	2	2	3	4	3	2	2	3
	27 July	0	0	0	0	0	0	1	0	0
	10 August	0	1	2	4	12	10	13	7	9
	25 August	0	1	0	0	0	1	0	0	0
Total no. of colonies		0	4	4	7	16	14	16	11	13
Root length (mm)										
	26 June	70	70	70	68	132	106	106	103	98
	7 July	184	184	184	178	346	278	280	270	258
	27 July	29	29	29	28	55	44	44	43	41
	10 August	261	261	261	251	491	394	397	384	365
	25 August	49	49	49	48	92	74	74	72	69
Total root length (mm)		593	593	593	573	1116	896	901	872	831
Total frequency (colonies per cm root)		0	0.07	0..07	0.12	0.14	0.16	0.18	0.13	0.16

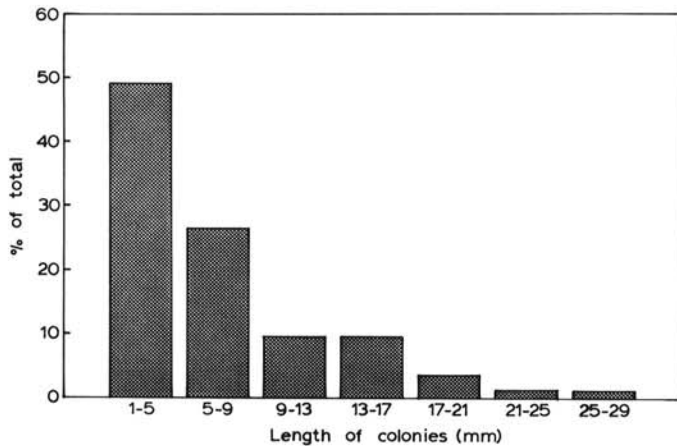


Fig. 1. Distribution of lengths of colonies of *Verticillium dahliae* in cotton roots.

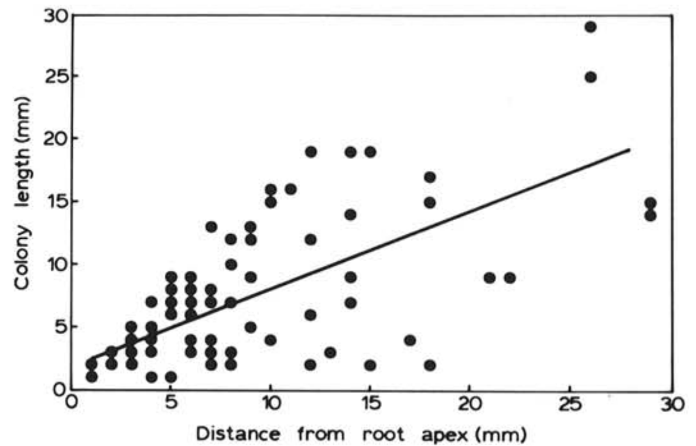


Fig. 2. Relation of lengths of colonies of *Verticillium dahliae* in cotton root to distance of colony from root apex. $R = 0.63$; Prob $\geq R$ 0.0001.

segment, and width of the root segment at the colony midpoint. Also, a notation was made on whether the colony was whole or fragmented at the end of the segment. If the colony was not whole, the end of the root segment was assumed to be the colony midpoint in the measurement of colony distance from the root apex. Lengths of all root segments with tips but without stained colonies also were measured. Colony densities of *V. dahliae* as a function of distance from the root tip were calculated as follows: Total number of colonies whose centers fell within a given distance interval (measured relative to the root tip) was divided by the sum of all root lengths within the same interval for segments that had gone through the staining procedure. Many of the root segments without tips also were mounted on glass slides, and general observations on colony morphology were made using both a dissecting and a compound microscope.

RESULTS

Colony densities. The colony density of *V. dahliae* on roots varied with distance of tissue from the root tip. A total of 85 colonies was found on the 593 segments (697 cm) with root tips assayed (Table 1). Colony density increased with distance from the root tip. *V. dahliae* only sparingly colonized the root tissue near the apex (Table 1). At distances from the tip greater than 5 mm, colony density was relatively constant and comparable to that observed for bulk roots (without tips). The mean colony length on root tip segments was 7.3 mm (standard deviation 5.8), and the median was 5.5 (Fig. 1). A positive correlation was observed between colony length and distance of the colony midpoint from the root apex ($R = 0.63$; Prob $\geq R$ 0.0001) (Fig. 2). No significant correlation was found between colony length and the root diameter, which ranged from 0.21 to 0.88 mm.

General colony appearance. The stained colonies of *V. dahliae* were easily seen on the root after the tissue was cleared in NaClO. Hyphae were seen on the surface of the root, usually near the center of the colony (Fig. 3). Surface hyphae usually were limited only to this small area near the colony center. The colony appearance was consistent with growth of hyphae from the root surface, through the cortex, and toward the stele. Approximately 1 mm from the center of the colony, the hyphae were located exclusively deep within the cortex near the stele and usually completely surrounded the stele (Fig. 4).

In approximately 20% of the colonies observed, isolated individual cortical cells, completely ramified with hyphae, were found (Fig. 5). These cells usually were found in the outer portion of the cortex and did not extend inward toward the stele. In approximately 5% of the colonies on segments with root tips, the vascular tissue at the site of colonization exhibited mild disorganization (Fig. 6). The red pigmentation (visible before the HOCl clearing step), apparently caused by a reaction between the diazo salt and phenolic compounds in the root (8), was less abundant in these areas. Hyphae were absent from the points of lateral root emergence (Fig. 7). Sometimes hyphae were seen in the root tissue to within a small distance of the point of emergence, but they were never observed in the area of the cortex disrupted by the root emergence. Points of lateral root emergence also developed

higher levels of the red pigmentation during the assays.

Colonies stained with the antisera produced against *F. oxysporum* were noticeably different from those stained with the antiserum prepared with *V. dahliae*. Most hyphae were confined to the root surface and the outer cortex (Fig. 8). Hyphae of *F. oxysporum* were scarce in the inner cortex. Often hyphae grew parallel to the anticlinal wall of the cortical cells. The isolated, heavily stained cortical cells observed with *V. dahliae* were not present in root tissue stained with the antisera produced against *F. oxysporum*, but a few mildly staining cells were observed.

DISCUSSION

The immunoenzymatic staining assay has several advantages over conventional bioassays in determining the colonization pattern of fungi on roots. It allows direct detection of the hyphae present on the root; the specificity of the staining of hyphae is dependent on the specificity of the serum. The assay does not rely on fungal growth; thus competition between fungi on a growth medium will not restrict detection. With a bioassay, small colonies might be overgrown by other fungi. Colony size and location of fungal hyphae within the root may be observed. No problems were observed with the penetration of cortical tissue by the antibody proteins. The hyphae of *V. dahliae* in the inner cortex were stained to an equivalent degree as those on or near the surface of the root. A disadvantage of the staining assay as it now exists is that only one fungus can be observed at a time. By using antisera specific to other fungi that are produced in different animals, and by using different enzymes, one may be able to visualize several different fungi simultaneously.

Colonization of roots by *V. dahliae* occurs primarily near the root tip (10,13). Fungal propagules probably responded to the high levels of exudates from the zone of elongation. The combination of higher microbial metabolism and lower exudation rates on older root tissue probably minimizes new colonization of older tissue (10). The finding by Huisman (11,12) that the density of colonies of *V. dahliae* on cotton roots was constant throughout the growing season and was unaffected by either root age or the incidence of cortical damage is consistent with this interpretation.

The mean colony length of *V. dahliae* observed in the root tissue (7 mm) is greater than the 2-mm colony length reported by Evans and Gleeson (4) and the 2.3-mm length reported by Huisman (11). This discrepancy could be explained by difference in methodology. Evans and Gleeson (4), using a bioassay, estimated colony length by plating different size root segments on media and determined the number of cut colonies. Huisman (11), using a similar bioassay, based his estimates on the frequency at which random breaks in roots appeared to occur within a colony of the fungus. This study has shown that the hyphae toward the edges of colonies of *V. dahliae* are in the internal portion of the cortex, immediately around the endodermis. In the bioassay, which relies upon growth of *V. dahliae* from the roots onto an agar medium, competition from other fungi located in the outer cortex may have precluded the detection of *V. dahliae* in the inner cortex when root breaks or cuts occurred more than 1 mm from the center of the colony. Consequently the bioassay probably underestimated colony length.

The positive correlation between colony length and distance from the root apex suggests that the fungal colonies continue to expand in size with time. The observations made with the staining technique suggest that this continual growth occurs in the interior of the cortex and not on the root surface. Other fungi and bacteria, on the root surface, may inhibit further growth of *V. dahliae* on that area of the root, or *V. dahliae* may be better adapted for growth in the interior of roots due to other factors. Previous workers have reported that *V. dahliae* was unable to colonize the interior of the cortex (4,14,20). Our data show that *V. dahliae* can establish itself in the inner cortex and that expansion occurs to a significant extent after initial colonization. Beckman and Talboys (1) point out that this is a selective advantage due to the escape from competition from other organisms on the root surface.

The ability of the fungus to penetrate and colonize individual cells is evident from the frequent occurrence of heavily stained and mycelium-packed cortical cells. This phenomenon has been reported earlier for infection of potato roots by *V. dahliae* (18). These cells superficially resemble mycorrhizal arbuscles, but arbuscles occur more frequently and usually in groups. Also, they have a characteristic treelike hyphal branching structure (7), absent from the cells observed in this study. The mechanism by which this selective colonization occurs and its role in the host-parasite interaction is not known. More extensive work on this topic is needed.

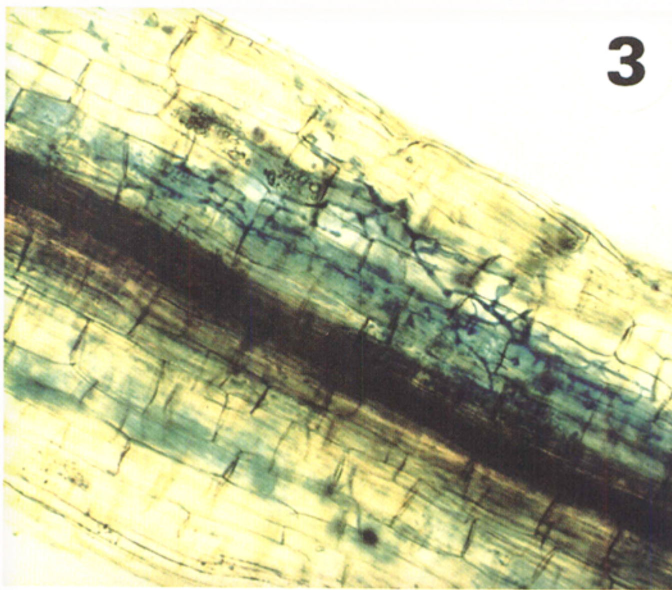
Establishment of cortical colonies does not necessarily lead to vascular infection. Huisman (10) has established that cortical root colonies of *V. dahliae* are in several thousandfold excess of systemic infections for the average cotton plant. Thus the presence of an established cortical colony of this pathogen is not enough of a foothold for gaining access to the stelar tissue. The entry sites for *V. dahliae* into the vascular system are unknown. Garber and Houston (5) suggested that the fungus was able to enter the vascular system through the undifferentiated tissue near the root apex. This undifferentiated area extends 0.25 mm behind the root apex in the roots in this study. The colonies nearest the root apex were more than 1 mm from the apex, an area in which the stelar tissue is developed. Also, this analysis revealed no vascular presence of the fungus, at least in the first few centimeters, though this may be due to inability of the reagents to penetrate the endodermis.

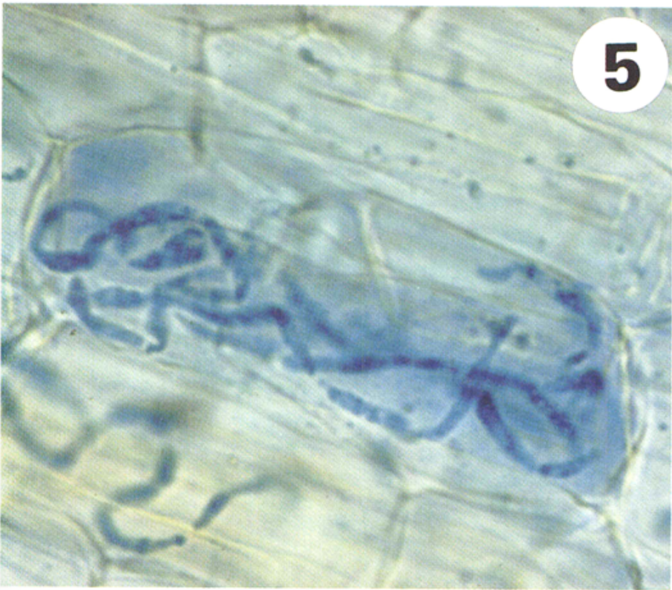
The areas of the root where hyphae of *V. dahliae* surrounded the endodermis could be entry points for the fungus into the stele. Disruption of vascular tissue along with an absence of red pigmentation was observed in these areas, whereas neither of these features was present on either side of the colonies. Although the separation and disorganization of the vascular bundle ultimately may have been caused by external pressure from the cover glass, they suggest the possible degradation of the middle lamella and a general weakening of cell adhesion because they were observed only in association with hyphae of *V. dahliae*. This disruption could provide a possible entry point into the vascular system. The 5% frequency in which it occurred would be above the number of vascular infections expected from the total colony frequency of the roots (10); however, the fungus could be limited in the vascular system after entry (1).

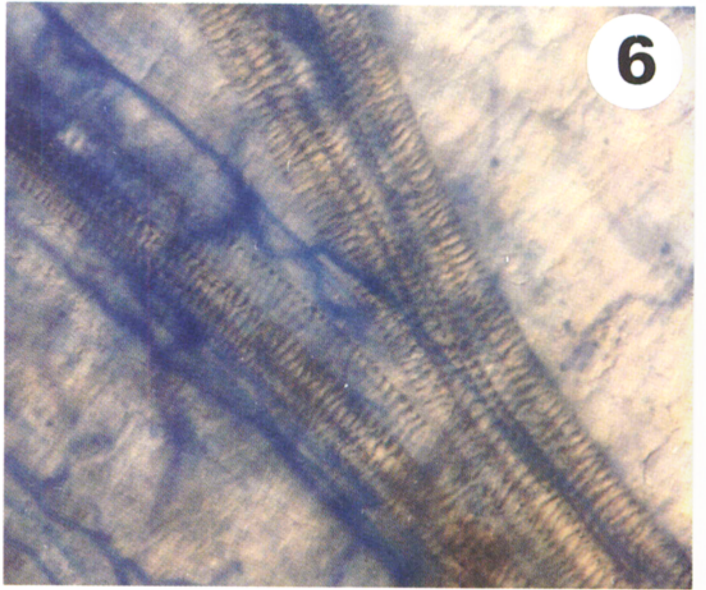
Areas of lateral root emergence have been viewed as potential sites for root entry by *V. dahliae* (5) as well as other fungi (19,21). These sites are zones of extensive cell disruption and presumably nutrient release and thus could allow fungi to become well established. The staining assay revealed an apparent absence of hyphae in these areas of the root. The absence of *V. dahliae* in these areas has been noted before by Garber and Houston (5). These observations suggest that points of lateral root emergence are highly unlikely avenues for infection into the root or the vascular system for *V. dahliae*. Mace et al (16) have reported high concentrations of gossypol-like compounds at sites of lateral root emergence in cotton roots. We observed that in fresh roots these areas developed increased levels of pigmentation in the presence of our substrate solution (Fig. 7). This suggests the presence of phenolic moieties which couple with diazo dyes. Perhaps the presence of such compounds inhibits fungal activity at these sites and renders the tissue unsusceptible to invasion by soil fungi. Mace et al (16) suggested that these compounds could be the reason for the failure of *V. dahliae* to colonize the areas of lateral root emergence.

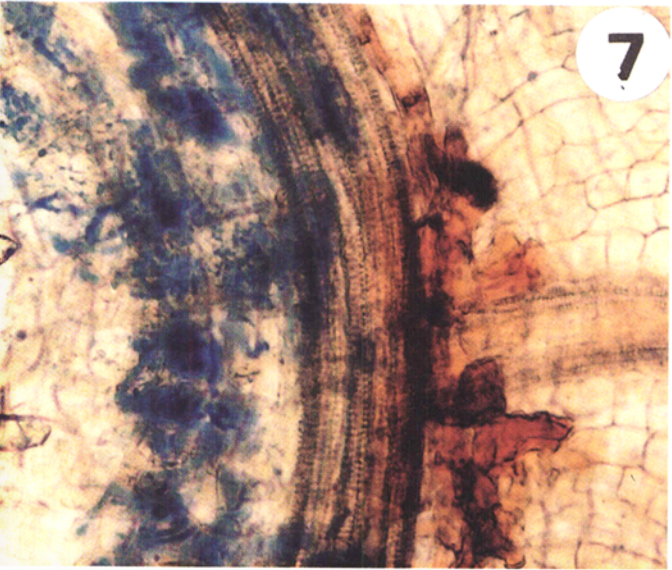
The limited observations on *F. oxysporum* made with the staining technique suggest that the fungus is mostly an epiphytic colonizer. No colonies were observed in the interior of the cortex. Previous data have established that *F. oxysporum* can colonize the root closer to the tip than *V. dahliae* (Gerik and Huisman, *unpublished*). This rapid colonization may enable *F. oxysporum* to be a successful root colonizer although it lacks the ability to colonize the interior of the cortex.

The results obtained with the immunoenzymatic techniques indicate that initial colonization by *V. dahliae* only occurs near the root tip. The fungus grows only to a limited extent on the root









Figs. 3–8. Stained hyphae of fungi in cotton roots. **3,** Hyphae of *Verticillium dahliae* on the root surface and within the cortex near the center of a colony. **4,** Hyphae of *V. dahliae* in the interior of a root around the stele. **5,** Cortical cell colonized by hyphae of *V. dahliae*. **6,** Apparent vascular disruption associated with some (about 5%) of the colonies of *V. dahliae* in roots of cotton. **7,** Hyphae of *V. dahliae* near the site of lateral root emergence. Tissue was partially destained with sodium hypochlorite. Note the absence of hyphae (blue) from the side of the root where branch has emerged but the presence of hyphae on the opposite side of the stele. An increased level of red pigmentation, resulting from nonspecific staining with diazo dyes (see text), can be seen in this area. **8,** Hyphae of *Fusarium oxysporum* on the root surface.

surface, and most growth appears to be in the cortical tissue next to the stele. The fungus was not observed in the root cap area, zone of elongation, or the area of lateral root emergence.

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