Survival of Verticillium albo-atrum on Nonsuscept Roots and Residues in Field Soil

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ABSTRACT

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Parasitic colonization of barley roots by *Verticillium albo-atrum* (microsclerotial type), the cotton wilt pathogen, was not a significant survival mechanism under field conditions. Although up to 5.6 colonies per 100 cm of barley root were recorded by plating on selective media during the growing season, 1 to 6 months after harvest buried root residues of immune crops such as alfalfa, barley, corn, and sorghum did not contain *V. albo-atrum* microsclerotia. Barley and cotton stems were not colonized after incubation periods of 1 to 9 weeks in naturally infested soil. No difference was found in inoculum density of *V. albo-atrum* during the growing season in rhizosphere (RS) or nonrhizosphere field soil (NRS) from alfalfa, barley, corn, and sorghum. Few *V. albo-atrum* colonies were recovered from nonsuscept rhizoplanes following a 72-hour air drying period. Nonsuscept rhizosphere and rhizoplane isolates of *V. albo-atrum* were pathogenic to cotton. Symptoms of *Verticillium* wilt never were observed on nonsuscepts growing under field conditions, nor was the fungus isolated from plant tissue above soil line.

Additional key words: crop rotation, *Gossypium hirsutum*.

The value of crop rotation for reducing disease losses from *Verticillium albo-atrum* Reinke & Berth (*V. dahliae* Kleb.) has been evaluated recently by quantitative assessment of microsclerotia (ms) in soils with various cropping histories (11). The inoculum density of *V. albo-atrum* at the time a field is rotated to a nonsusceptible crop might explain the "success" (6, 16) or "failure" (8, 14, 23) of crop rotation. Since as few as 3.5 ms/g of soil are needed for 100% infection in cotton (2), fields put into rotation near this inoculum density should have a decreased rate of infection in a subsequent susceptible crop. Since ms have a relative low attrition rate (11), fields with inoculum densities of 30-60 ms/g at the start of rotation might not benefit from rotations of even 5 or 6 years.

Survival of the pathogen on nonsusceptible crops during rotation periods has been suggested as an alternative explanation for the failure of crop rotation (5, 13, 14). Nonsusceptible crops whose root systems are colonized (superficially penetrated) by *V. albo-atrum,* but whose vascular system is not systemically invaded, are considered immune (5, 13).

Colonization of immune hosts, presumably, arises from ms in the rhizosphere and rhizoplane (20). Agronomically important immune crops that are colonized by *V. albo-atrum* include barley (*Hordeum vulgare* L.), bluegrass (*Poa pratensis* L.), carrot (*Daucus carota* L.), corn (*Zea mays* L.), field pea (*Pisum sativum* L. var. *arvense* L.), mung bean (*Phaseolus aureus* Roxb.), oat (*Avena sativa* L.), onion (*Allium cepa* L.), pea (*Pisum sativum* L.), soybean (*Glycine max* L.), and wheat (*Triticum aestivum* L.) (5, 13). Alfalfa (*Medicago sativa* L.) is a host of *V. albo-atrum* in England (12). In the United States, artificially inoculated alfalfa plants were not colonized by the fungus even though the plants were transplanted to a field with a high level of *Verticillium*-infected cotton (22). Sorghum was a host of *Verticillium* in laboratory tests, but the pathogen never was isolated from plants growing in infested soil in the greenhouse or field (21).

Studies reported herein were conducted to determine the role of immune hosts and their residues on the parasitic and saprophytic survival of *V. albo-atrum* in soils of the San Joaquin Valley of California.

MATERIALS AND METHODS

Barley root samples were taken at monthly intervals from February to June, 1974, from two random locations in each of two commercial fields with a history of Verticillium wilt of cotton. Samples were collected with adhering soil, placed in plastic bags, and transported in ice chests to the laboratory. Roots were washed in running tap water, excised in sterile water, and incubated on ethanol streptomycin agar (5, 18). Ten replicates of fifty 2-cm root segments were plated on each sampling date from each field. A similar experiment using a naturally infested soil (44.6 ms/g) was conducted in 15-cm diameter pots in the greenhouse with barley, sorghum, and corn. Roots were harvested and plated 14 days after plant emergence (5).

Inoculum density of *V. albo-atrum* was compared in the rhizosphere soil (RS) and nonrhizosphere soil (NRS) portions of alfalfa, barley, corn, and sorghum growing in fields with a history of Verticillium wilt on susceptible crops. Alfalfa was in the second growing season during sampling, whereas barley and sorghum were sampled from plant emergence to maturity. Corn in the tassel stage

at initial sampling was sampled to irrigation cut off in August. Root systems for rhizoplane and RS samples were removed to a depth of 25-30 cm by careful excavation so that adhering soil could be assayed. Soil samples collected with a push probe to 25-30 cm in areas away from plant crown were considered NRS samples. To differentiate RS and NRS, total root length per gram of soil was determined for the respective samples (19). An inverted horseshoe sampling pattern was used in each field starting 10-m from point of entry. Ten RS and NRS samples were taken at 10-m intervals and bulked so that three bulked samples were obtained. Fields were resampled in the same general area at 2-, 4-, or 6-week intervals depending on the experiment. Temperature and moisture of soil were determined on each sample date. Although two-to-five fields were studied for each immune crop, only representative data from one field per crop are reported.

Samples of RS and NRS were air-dried (3-5% moisture) at 24-26 C for 48 hours prior to assay for *V. albo-atrum* (3). Rhizosphere soil was removed from roots by placing preweighed roots with adhering RS in 300 ml of 1% Calgon solution and mechanically shaking for 20 minutes. After rinsing, the roots were air-dried 24 hours and reweighed; the difference in weights represented RS. The sampled RS and NRS were assayed for ms of *V. albo-atrum* by the procedures of Ashworth et al. (3) and Huisman and Ashworth (9) using sodium hypochlorite to reduce the copper-induced fungistasis associated with ms of *V. albo-atrum* in cold, wet soils (1).

Air-dried roots from RS samples were given 20 serial washings in tap water prior to cutting triplicate samples of fifty 2-cm segments and plating (five per plate) on either ethanol streptomycin agar (18), fortified with 0.2 g sodium polypectate and 5 μg/ml tetracycline (5), or sodium polypectate agar (9). The plates were incubated at 26 C for 7-10 days and examined for *V. albo-atrum* colonies.

After commercial harvest of the immune crops, saprophytic colonization of root residues by *V. albo-atrum* was followed during the autumn and winter by collecting soil samples containing alfalfa, barley, corn, or sorghum residues (decaying roots 5-20 cm in length) as described for RS and NRS. Residues were washed and either (i) observed directly for ms, or (ii) air-dried, milled through the 425-μm (40-mesh) screen of a Wiley mill and incubated (three replicates of 0.5 g tissue divided among 15 plates) on sodium polypectate agar.

Barley and cotton stems were collected from senescent plants standing in the field for studies of saprophytic colonization in the laboratory. Fifty 2-cm segments of cotton stems (*Verticillium*-free) were either autoclaved, or added directly to 200 g of a naturally infested soil at 17% moisture content. Segments were incubated at 26 C for up to 56 days. The segments were recovered on a 3-mm sieve, washed in tap water for 10 minutes, and then soaked in 95% EtOH for 1-2 seconds followed by 3-5 minutes in 0.5% NaClO. After being rinsed in sterile water, the stem segments were plated on ethanol streptomycin agar. Since *V. albo-atrum* never was recovered under these conditions even though the inoculum density of the test soil was 35 ms/g soil, a second experiment was conducted.

Cotton and barley stems were milled to pass a 495-μm sieve and mixed into a naturally infested air-dried soil that had been passed through a flail type mill (3) and a 250-μm sieve. Milled stem pieces larger than 250 μm were added at the rate of 0.05 g tissue per 50 g soil and incubated in tared flasks at 26 C. Soil moisture was kept between 15 to 18% (w/w) by adding tap water as needed. Three replicated stem-piece samples were recovered after various incubation periods by wet-sieving on a 250-μm sieve. Washed stem pieces were plated on sodium polypectate agar. Three flasks of infested soil without stem pieces were assayed to determine inoculum densities after each incubation period.

Pathogenicities of single-spored isolates of *V. albo-atrum* from each immune crop were determined by inoculating stems of 8-week-old cotton plants (*Gossypium hirsutum* L. 'SJ-2') with a turbid spore suspension. Twenty to 30 plants were inoculated with each isolate. Control plants received sterile water. Plants were grown in a mixture of sand:soil (*Verticillium*-free):peat moss (1:1:1, v/v) in the greenhouse under natural lighting. Fourteen days after inoculation, surface-disinfested (3 minutes, 0.5% NaClO) leaf and petiole segments with typical Verticillium wilt symptoms were plated on ethanol streptomycin agar.

RESULTS

Parasitic colonization of barley roots in the field.—Roots of 6-week-old barley plants averaged only 0.7 colonies of *V. albo-atrum* per 100 cm of root, even though inoculum densities were 22.4 and 17.2 ms/g, respectively in the two fields investigated (Table 1). Soil temperatures at this time were near 8 C. As plants approached maturity (15 weeks old) and soils warmed,

TABLE 1. Colonization of barley roots in the field by *Verticillium albo-atrum*

Date[y]	Field 78		Field 5	
	Inoculum density (microsclerotia/g soil)	Colonies/100 cm of roots	Inoculum density (microsclerotia/g soil)	Colonies/100 cm of roots
2/15/74	22.4 a[z]	0 a	17.2	0.7 a
3/6/74	23.0 a	0.1 a	13.8	0.1 a
4/17/74	20.2 a	1.7 b	12.5	5.6 b
5/16/74	29.4 a	2.5 b	33.1	1.2 a
6/11/74	4.5 b	1.4 ab	...	...
Tukey's HSD ($P = 0.05$)	13.3	1.5	N.S.	2.5

[y]Barley plants were 6 weeks old at the first sampling date.
[z]Means followed by the same letter are not significantly different, $P = 0.05$.

extent of colonization of barley roots by *V. albo-atrum* increased to 1.7 and 5.6 colonies per 100 cm of root in the two fields. The respective inoculum densities of 20.2 and 12.5 ms/g soil on 17 April were similar to the inoculum densities on 15 February. As roots matured and died at harvest time, only 1.4 and 1.2 colonies per 100 cm were found on barley roots from the two fields, respectively. Most *V. albo-atrum* colonies came from areas along the root surface, although a few fan-shaped colonies appeared at the cut end of the root segment. Colonized root segments usually had only one colony associated with them, but two to three colonies per root segment occasionally were observed. All isolates from barley roots were pathogenic to cotton.

Parasitic colonization of barley, sorghum, and corn roots in the greenhouse.—Nonsuscept roots were colonized to about the same extent in the greenhouse (approximately four colonies per 100 cm root) as in the field. This level of colonization was reached in only 14 days in the greenhouse compared to 15 weeks in the field. However, the naturally infested soils that were used had inoculum densities two- to threefold greater (40 to 65 ms/g) than those in the fields. As in the field, the nonsusceptible plants did not exhibit symptoms of wilt. *Verticillium albo-atrum* never was isolated above soil level from the nonsuscepts.

Comparison of rhizosphere and nonrhizosphere inoculum densities.—Rhizosphere soil samples had 11-22 times greater root length than NRS samples depending on the immune crop. Except for a seasonal peak, RS and NRS inoculum densities were not different ($P > 0.05$) regardless of immune crop (Fig. 1). During this period of sampling, soil temperature ranged from 26-30 C at 15 cm and soil moisture (crops were irrigated) ranged from 13-25% (w/w). There was no apparent relationship between either soil temperature or moisture and inoculum density.

Isolates of *V. albo-atrum* from nonsuscept RS and NRS were pathogenic to cotton. Only the rhizoplane isolate from alfalfa failed to infect cotton. *Verticillium albo-atrum* was isolated from less than 0.001% of nonsuscept rhizoplanes when roots were air-dried prior to plating.

Saprophytic colonization of nonsuscept root residues following crop harvest.—Preliminary observations of corn root residues in January 1973 indicated low numbers of ms of *V. albo-atrum* present in the pith. One of two isolates tested for pathogenicity to cotton was virulent. Systematic collections of corn root residues were made in five fields from October 1973 to March 1974. No ms of *V. albo-atrum* were found in 1,049 corn roots. Soils were moist throughout this period, but soil temperature averaged only 9.7 C at 15 cm. Mean inoculum densities in the five fields ranged from 3.3 to 41.6 ms/g soil. When root residues were ground in a Wiley mill and plated, four colonies of *V. albo-atrum* developed from a total of 13.5 g (approximately 5.4×10^6 pieces) of residue. Isolates from milled corn root residues were pathogenic to cotton. Similar results were found when over 150 root residue pieces each of barley, alfalfa, and sorghum were examined microscopically for *V. albo-atrum* microsclerotia. Inoculum densities during the 6-month sampling period averaged 13.5, 9.2, and 14.6 ms/g for the alfalfa, barley, and sorghum fields, respectively.

Saprophytic colonization of cotton and barley stems in naturally infested soil in the laboratory.—Milled cotton and barley stems added to naturally infested soil (86.6 ms/g) were not suitable substrates for *V. albo-atrum*

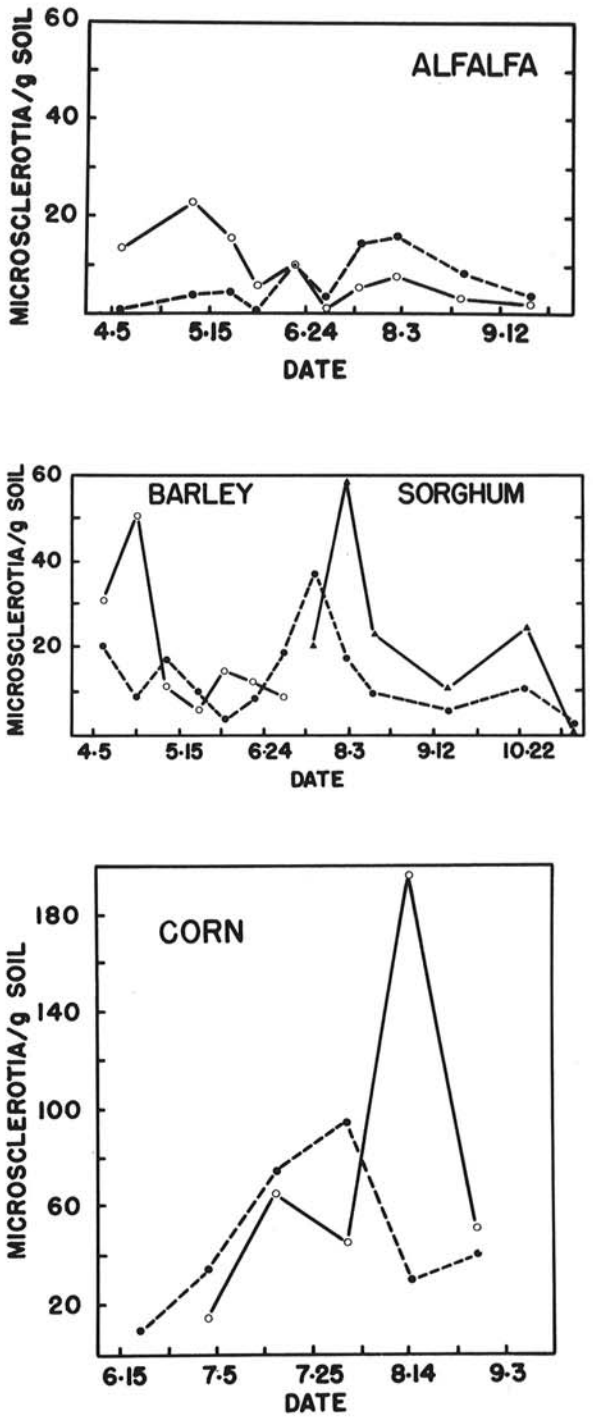

Fig. 1. Microsclerotia population of *Verticillium albo-atrum* in the rhizosphere (open circles or triangles) and nonrhizosphere (solid circles) of alfalfa, barley-sorghum, and corn. Rhizosphere populations were significantly greater than nonrhizosphere populations ($P < 0.05$) on 4 May for alfalfa, 22 May for barley, 3 August for sorghum, and 14 August for corn.

colonization. With regular culturing during a 10-week incubation period only six colonies of *V. albo-atrum* (0.03%) were recovered from the 17,408 cotton stem pieces plated. No barley stem pieces yielded *V. albo-atrum* under similar conditions.

DISCUSSION

Few colonies of *V. albo-atrum* were found on active barley root systems during the growing season. This level of parasitic survival was reduced even lower as the crop matured and soils dried out. When roots were air-dried for 72 hours prior to plating, only two colonies of *V. albo-atrum* were recovered. Evans and Gleeson (5), working with Australian soils containing two to three times the inoculum density we encountered, found many immune host root systems with up to 10 colonies per 100 cm of root, about twice the level we encountered. Barley, corn, sorghum, and wheat were included among those immune crops. Since there is a direct relationship between inoculum density and disease in susceptible crops (1, 2), this same relation may hold for inoculum density and rate of colonization of immune crops, other things being equal.

Formation of overwintering ms on nonsuscept roots seems unlikely. Three lines of evidence support this: (i) ms are not observed in systemically invaded hosts until tissues become senescent; (ii) ms would survive a 72-hour air-drying period, as air-dried soils stored for up to one week maintain a uniform inoculum density (1); and (iii) colonies of *Verticillium* only were produced when roots were not air-dried prior to plating. The root cortex where colonization by conidia and hyphae of *V. albo-atrum* takes place may be killed by the air-drying procedure. The sensitivity of *Verticillium* in the root cortex has been demonstrated by the inverse relationship between the number of *Verticillium* colonies recovered from nonsuscept roots and the concentration of mercury in a mercuric chloride solution (5). Thus, it is concluded from our data that parasitic colonization of immune crop roots by *V. albo-atrum* is not a significant survival mechanism under field conditions in central California.

Microsclerotial populations did not increase in the rhizosphere or rhizoplane of alfalfa, barley, corn, or sorghum during the growing season. Rhizosphere populations were greater than nonrhizosphere populations on certain sampling dates, but these differences were not consistent. Seasonal ms peaks may be explained by: (i) a reduction of the copper-induced fungistasis of *V. albo-atrum* (1) or (ii) formation of secondary microsclerotia (4). Other scientists have found 2,800 to 6,800 propagules/g soil for rhizosphere populations of *V. albo-atrum* in wheat and corn. The RS populations were maintained for up to 8 weeks under laboratory conditions (13). The nature of the *Verticillium* propagule ms or conidia, was not clear, however (13). Transient increases in *Verticillium* populations have been attributed to sporulation of ms in survival experiments in which carbon and nitrogen amendments were added to soil (17). However, unless an infection court was present, increases in conidial populations would not increase survival of the fungus from year to year (7), since ms account for nearly 100% of the propagules of *V. albo-atrum* in field soils (10).

Because *Verticillium* does not grow through natural soil (14, 24), saprophytic colonization of root residues after crop harvest (or stem pieces added to soil) must result from germination of ms adjacent to the organic substrate. Under field conditions, few ms were recovered from nonhost root residues buried in soil even after 6 months. Under laboratory conditions, saprophytic colonization of barley and cotton stem pieces also was low ($< 0.3\%$) when a naturally infested soil was used. Other investigators (15) have reported somewhat higher rates of colonization (0.5 - 3.5%) of barley, oat, and alfalfa stem pieces when soil was artificially infested with over 4,000 ms/g. Rate of colonization by *V. albo-atrum* was reduced to about half of the original level in 6 months. Thus, neither saprophytic colonization of nonsusceptible crop residues nor parasitic colonization of immune crops by *V. albo-atrum* are likely to be important survival mechanisms under field conditions.

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