Lack of Systemic Colonization of Alfalfa Plants After Inoculation of Uninjured Leaves with Conidia of *Verticillium albo-atrum*

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ABSTRACT

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Healthy uninjured or injured leaves of alfalfa plants were inoculated with a conidial suspension (10^5 or 1.2×10^6 per milliliter) of the alfalfa strain of *Verticillium albo-atrum*. Leaflets were pierced with a sterilized needle, rubbed with Carborundum, or touched with a hot glass rod or a piece of dry ice to injure them. Inoculation consisted of spraying plants or individual leaves with the conidial suspension and placing single drops of the suspension either at the bases or the edges of selected leaflets. Ingress and systemic colonization were determined by attempts to isolate the pathogen from leaves and stems. On uninjured healthy leaves, conidia germinated readily, usually producing two germ tubes, which in turn led to profuse branching of hyphae and development of phialidelike conidiophores and conidia. Some hyphae penetrated and grew within the cuticle. No fungal structures were observed either within epidermal cells or in palisade or mesophyll tissues by 16 days after inoculation, and no deleterious effects were detected. The pathogen remained restricted to the inoculated leaves, and neither symptom development nor systemic colonization by the pathogen occurred. Symptoms characteristic of Verticillium wilt and systemic colonization by the pathogen occurred only when leaves had been injured before inoculation.

Verticillium wilt (VW) of alfalfa (*Medicago sativa* L.) induced by the alfalfa strain of *Verticillium albo-atrum* Reink. & Berth. has been a major disease of alfalfa in Europe for many years (17,25,33). In the United States, the disease was first detected in Washington and Oregon in 1976 (12). It now severely affects alfalfa in several northern states (6,13,14).

The pathogen is introduced into wiltfree regions by infested or infected seeds (4,5,34) and by infested plant material carried with seed lots (22,34). However, how the pathogen subsequently is dispersed within and between fields has not been clearly established. Conclusive information on this aspect should increase understanding of the epidemiology of the disease and provide direction in developing strategies to control it.

Conidia of *V. albo-atrum* form abundantly on infected senescent or necrotic plant parts (22; R. M. Jimenez-Diaz and R. L. Millar, *unpublished*), and they have been trapped above (22,26; R. M. Jimenez-Diaz and R. L. Millar, *unpublished*) and 150 yd distant from affected alfalfa fields (9). Airborne conidia, therefore, appear to have an important role as inoculum for infection of alfalfa foliage. However, whether they serve as inoculum for systemic infections of alfalfa and thus contribute to the incidence of the disease within and between fields has not been investigated.

We report the results of experiments to determine whether conidia of *V. albo-atrum* produced on infected tissue can act as inoculum for infection of leaves and systemic colonization of alfalfa plants.

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MATERIALS AND METHODS

Plants of cultivar Iroquois (susceptible to VW) were grown for 7–8 wk in sterilized Cornell peat-lite mixture in clay pots. The plants were inoculated with conidial suspensions of pathogenic isolates of *V. albo-atrum*. For some experiments, only uninjured tissues were inoculated; for others, both injured and uninjured tissues were inoculated. A monoconidial isolate obtained from affected plants at Freeville, NY, was used for one experiment; for the remainder, a mixture of monoconidial isolates used regularly in other investigations in our laboratory was employed.

Inoculum was obtained from infected alfalfa stems to preclude any deficiencies that might be associated with inoculum produced on a prepared nutrient medium. Lengths (2–4 cm) of stems from plants inoculated with the isolates were surface-disinfested in 0.5% NaClO for 1.5 min and incubated on glass rods in sterile moist chambers at 20–21 C for 3–4 days. Stem pieces determined microscopically to be free of fungal contaminants were placed in 10 ml of sterile distilled water in test tubes and gently agitated in a Vortex mixer to dislodge conidia. Conidial suspensions were obtained by filtering the contents of the test tubes through six layers of cheesecloth. Conidia in the suspension were counted with a hemacytometer and the suspensions adjusted to the desired concentrations. Just before inoculation, Tween 20 (polyoxyethylene sorbitan monolaurate) was added (one drop per 100 ml) to the suspensions.

Inoculation consisted of spraying each plant or leaf individually with the conidial suspension with a DeVilbiss atomizer at 0.4 bar or by placing single drops of the suspension at selected sites. Leaves were sprayed until droplets formed, but runoff was avoided. Contamination of the peat-lite mixture was precluded by wrapping the base of a stem with nonabsorbent cotton, enclosing the pot in a polyethylene bag, and securing the bag tightly over the cotton. The bag was kept in place throughout an experiment, and the plant was subirrigated by adding water to a saucer under the pot. Viability of conidia in the suspensions was assessed after each inoculation by placing drops on, or directing the spray to, ethanol-streptomycin agar (ESA) in petri plates (29). The plates were kept at 20–21 C and observed for growth of *V. albo-atrum*.

Plants were grown at 20–22 C in a clear-plastic chamber (19) in a greenhouse and received about 10 klux of fluorescent light for 16 hr/day. One day before inoculation, the relative humidity (RH) was brought near 100%, which caused water drops to form on the leaves. After inoculation, the chamber was programmed to provide continuous light and RH near 100% to maintain free water on the leaves for 3 or 5 days, then the normal photoperiod was resumed and RH was brought to 60–85%. For most experiments, inoculated plants remained in the plastic chamber; for one experiment, they were kept for 3 days in the plastic chamber, then moved to a growth chamber programmed to 20/18 C (day/night), 70–80% RH, and 16-hr/day photoperiod of about 18 klux of fluorescent and incandescent light. Plants were observed for symptoms for 24 and 30 days.

Whether infection occurred was determined by attempts to isolate the pathogen. In preliminary trials, surface-disinfestation for 1 min was sufficient to kill *Verticillium* on leaves that had just been inoculated. Therefore, entire leaves were dipped in 95% ethanol for a few seconds, then surface-disinfested with 0.5% NaClO for 1 min, blotted dry between paper towels, and separated into petioles and leaflets, which were plated on ESA or placed on moist, sterile filter papers in petri plates. Stems were surface-disinfested with 0.5% NaClO for 1.5 min, blotted dry between paper towels, and cut into lengths of 1–1.5 cm. Care was taken to disinfest the scalpels between sequential cuttings. Stem pieces were arranged in a plate to match their positions in the intact stem, then pushed into close contact with the ESA. Isolations were attempted both from individually inoculated leaves and from uninoculated leaves above or below them. Plates were kept at 20–21 C and observed periodically under a stereoscope for 10–12 days for growth of *Verticillium*. In three experiments, all plants were allowed to regrow for 1 mo or longer and checked again for symptom development and systemic colonization.

Inoculation of uninjured leaves. For one experiment, plants were inoculated by placing one 5-μl drop (10^5 conidia per milliliter) either at the base or at the margin on either side of the midvein of each of the three leaflets of selected leaves or at the junction of the leaf petiole and stem. Three leaves midway up the stem were inoculated for each inoculation site on 17 plants. Five plants that did not receive inoculum but otherwise were treated similarly served as controls for each treatment.

For a second experiment, the three uppermost trifoliolate leaves were inoculated. A drop (10—15 μl, 1.2×10^6 conidia per milliliter) was placed on a central leaflet of each inoculated leaf on 16 plants, or the inoculum was sprayed on the adaxial and abaxial surfaces of comparable leaves on 24 plants. Five plants treated similarly but not inoculated served as controls for each type of inoculation.

Inoculation of injured leaves. For one experiment, healthy leaves either injured or uninjured were inoculated. Each leaflet was injured just before inoculation by either 1) piercing it 10 times with the point of a sterilized needle; 2) dusting the adaxial surface slightly with Carborundum 320 Grit-Powder, then gently rubbing the surface with a piece of foam, which caused no macroscopic lesions; 3) touching the adaxial surface with a hot glass rod, which caused the tissue to become desiccated and necrotic; or 4) placing a small piece of dry ice onto the adaxial surface for about 10 sec, which caused the tissue to become chlorotic, flaccid, and wrinkled.

Inoculation consisted of spraying inoculum (10^5 conidia per milliliter) onto the adaxial surface of each leaf. Nontarget leaves were shielded from inoculation. For individual leaves, the petiole was placed through a narrow slit in the shield so that the leaflets lay flat against it during inoculation. Inoculum was directed at the adaxial surfaces. Three leaves midway up the stem on each of 15 plants were inoculated for each of the treatments. Five plants that did not receive inoculum but otherwise were treated similarly served as controls for each treatment.

Pathogenicity of conidia in the inoculum suspension was tested on 10 Iroquois plants. Stems of a plant were cut with disinfested scissors, then a drop of inoculum suspension was transferred immediately on a camel's-hair brush to the cut surface. Five plants that did not receive inoculum but otherwise were treated similarly served as controls.

For two other experiments, the leaflets were injured and sprayed with inoculum (10^6 conidia per milliliter) immediately or inoculation was delayed 3 or 6 days. The inoculum was prepared from cultures of the isolates grown in Czapek-Dox broth (Difco). The cultures were centrifuged at 2,500 rpm for 10 min, the supernatant liquid was discarded, and the conidia were resuspended in sterile demineralized water. Injury was done by piercing the leaflet with a needle or by touching the adaxial surface with a hot glass rod. Three leaves midway up the stem on each plant were inoculated for each injury treatment. In one experiment, plants in each treatment were kept 3 days (before injuring them) under continous light and RH near 100% as described, or the plants were injured without exposing them to these conditions.

Histology. Inoculation consisted of placing a 5-μl drop of conidial suspension (10^5 conidia per milliliter) at the base of each of the three leaflets of four leaves about midway up the stem. Plants were observed periodically for symptoms. One inoculated leaf from each of 10 plants was removed at 8-hr intervals for the first 24 hr and 2, 3, 4, 6, 10, 13, and 16 days after inoculation and fixed for histological examination. Sampled leaves were examined as whole mounts with a Zeiss Photomicroscope II (×800–2,500) using interference contrast optics. Light photographs were taken at ×204.8–640 on Kodak Ektachrome 400 daylight film. Samples taken for whole mounts at 8–24 hr were placed in open petri plates that were enclosed for 2–4 hr in sealed glass dishes containing paper towels saturated with formalin. The fixed leaflets were floated on chloral hydrate for 24 hr, stained in 0.1% cotton blue or in alcoholic 0.05% aniline blue in lactophenol (35) for several hours, and mounted in 50% glycerin on microscope slides. Samples at 2–16 days were fixed in FAA (95% ethanol/water/40% formalin/glacial acetic acid, 40:14:3:3, v/v) or in a mixture of absolute ethanol and glacial acetic acid (2:1, v/v) for at least 24 hr and stored in screw-capped glass vials at 5 C. Tissue fixed in FAA was subjected to a schedule of dehydration and clearing in methyl salicylate (40), stained in 0.1% cotton blue or 0.1% aniline blue in a mixture of methyl salicylate and absolute ethanol (3:1, v/v) for 7–24 hr, and mounted in methyl salicylate on microscope slides. Tissue fixed in absolute ethanol/glacial acetic acid was cleared in lactophenol for 24 hr, stained in 0.1% acid fuchsin in lactophenol for 8–10 hr, and mounted in 50% glycerin on microscope slides.

Observations and measurements were made on numbers of conidia, germinated conidia, and germ tubes; length, width, and orientation of germ tubes; position, shape, and size of appressoriumlike swellings; degree of penetration; and sporulation of the fungus on the leaf surface. A conidium was considered germinated when the length of the longest germ tube was equal to or greater than the spore width. Quantitative data were obtained for 100 conidia on five leaflets sampled at each of 8, 16, 24 and 48 hr after inoculation. Further observations on prepenetration and penetration activities by the pathogen were made on 10 leaflets at each sampling time.

RESULTS

Inoculation of uninjured tissues. In three experiments, no symptoms were observed on inoculated uninjured healthy leaves that could be attributed to infections by *V. albo-atrum* (Tables 1 and 2). A mild chlorosis or yellowing developed at the apex and edges of some leaflets whether inoculated or not and also on leaves of control plants. Otherwise, the plants remained free of symptoms. No symptoms developed after regrowth of any of the 57 plants.

No growth of *V. albo-atrum* occurred from the cut ends of stem pieces plated on ESA that would indicate systemic

colonization of the plants by the pathogen. Visible sporulation of the fungus occurred on individual leaves only after they were excised, surface-disinfested, and incubated on ESA. Apparently, ingress had occurred, but the fungus remained restricted to the penetration sites on inoculated leaves. Spraying the leaves resulted in a higher incidence (>80%) of infected leaflets. When leaflets received drops of inoculum, fewer than 40% of the inoculated leaves per plant became infected regardless of whether the drop was placed centrally on the blade or at the junction of petiole and stem.

When the excised leaves were incubated on ESA, sporulation of *V. albo-atrum* initially was restricted almost entirely to the sites of inoculation on leaflets inoculated with drops of conidia. No growth or sporulation was observed from petioles of these leaves. Growth and sporulation of the fungus also occurred from the epidermis of stems of plants that had been spray-inoculated; fungal colonies developed on plated stem pieces at sites away from the cut ends for 41% of the plants. Occasionally, sporulation of *V. albo-atrum* also occurred on stipules and on leaves newly formed at axils of leaves sprayed with inoculum.

Table 1. Systemic colonization of alfalfa plants after inoculation of injured healthy leaves with conidia of *Verticillium albo-atrum*[a]

Injury method[b]	Symptom development (%)	Systemic colonization[c] (%)
Not injured	0	0
Pierced with needle	33	33
Rubbed with Carborundum	13	13
Heat	27	40
Dry ice	0	27

[a]Three leaves on each of 15 plants were individually sprayed with a suspension of 10^5 conidia per milliliter for each leaf-injury treatment.

[b]Individual leaflets of the leaves were pierced 10 times with a sterilized needle, dusted lightly with Carborundum 320 Grit-Powder and rubbed with a piece of foam, touched with a hot glass rod, or touched with a small piece of dry ice. Leaves were inoculated immediately after injury.

[c]Observations on symptom development were made for 24 days after inoculation. Systemic colonization was assessed by attempts to isolate *V. albo-atrum* on ethanol-streptomycin agar.

Inoculation of injured tissues. All plants inoculated via the surface of a cut stem developed characteristic systemic symptoms of VW (Tables 2 and 3). Average severity of symptoms was 2.3 on a scale of 0–4 (0 = no symptom, 4 = dead plant) by 3 wk after inoculation. Symptoms of VW like those characteristically associated with the systemic colonization of plants that occurs after ingress by *V. albo-atrum* through injured roots or cut stems were observed only when leaves with visible injury were inoculated (Table 1). For these plants, symptoms characteristic of VW developed both on inoculated and uninoculated leaves. The earliest symptoms occurred by 2 wk after inoculation on pierced leaves, which developed interveinal chlorosis followed by yellowing of tissue and dropping of leaflets. On leaves injured with Carborundum, chlorosis and yellowing were associated with necrotic lesions followed by leaflet drop. Necrotic lesions, but no chlorosis or yellowing, developed in uninoculated controls. Leaflets wounded by heat or dry ice underwent early yellowing and senescence of the uninjured tissue and fell off. Leaflet drop was most intense for leaves injured with Carborundum, heat, or dry ice. No leaflet drop occurred for uninoculated controls.

The highest incidence of symptoms characteristic of systemic colonization was associated with inoculated plants whose leaves were pierced or heated (Table 1); the lowest, with plants whose leaves were injured with dry ice. Symptoms were observed first on shoots formed at the axils of inoculated leaves. Later, symptoms developed on newly formed leaves near the tops of stems.

Systemic colonization of plants was indicated by growth of *V. albo-atrum* from stem pieces taken at locations distant from the inoculated leaves. The fungus grew from the cut ends of stem pieces taken from plants on which injured leaves had been inoculated (Table 1). In some instances, systemic colonization occurred in plants that had not developed VW symptoms 24 days after inoculation.

Additional evidence for the importance of injury was obtained by inoculating a group of 32 plants inadvertently injured by exposure to volatiles from a dichlorvos (Vapona 20%) strip for 24 hr. The injury, which appeared as necrotic spots, was most intense on the youngest leaves. Plants with the least injury were

Table 2. Effects of preinoculation conditions, delaying inoculation with *Verticillium albo-atrum* (*Vaa*), and injuring alfalfa leaves on symptom expression and systemic colonization of plants

Inoculation (days after injury)[a]	Preconditioning period[b]	Injury method[b]	Symptom development[c] at harvest 1 (%)	*Vaa* recovery from inoculated leaves[c] (%)	Systemic colonization[c]: Petioles at harvest 1 (%)	Systemic colonization: Stem sections at harvest 1 (%)	Systemic colonization: Plants, Harvest 1 (%)	Systemic colonization: Plants, Harvest 2 (%)
Leaf inoculation								
0	+	Heating	0	100	22	1	7	13
		Piercing	0	100	4	0	0	0
	−	Heating	0	100	17	0	0	0
		Piercing	0	100	8	0	0	0
3		Heating	0	100	21	4	13	0
		Piercing	7	100	18	1	7	13
0	+	None	0	70	0	0	0	0
	−	None	0	9	0	0	0	0
Cut stem inoculation								
0	...	...	100	...	...	...	100	100
Not inoculated								
0	+	Heating	0	0	0	0	0	0
		Piercing	0	0	0	0	0	0
	−	Heating	0	0	0	0	0	0
		Piercing	0	0	0	0	0	0
3		Heating	0	0	0	0	0	0
		Piercing	0	0	0	0	0	0

[a]Three leaves on each of 15 plants were individually sprayed with a suspension of 10^6 conidia per milliliter for each leaf-injury treatment. Six plants were used for each control.

[b]For the plants inoculated immediately, half of the plants (designated +) were kept for 3 days under continuous light and 100% RH before inoculation and half of the plants (designated −) were inoculated without prior exposure to those conditions.

[c]Observations on symptom development and systemic colonization were made 4 wk after inoculation and after the plants had regrown for 4 wk. Systemic colonization was determined by incubating stem sections on ethanol-streptomycin agar.

Table 3. Effects of delaying inoculation of injured alfalfa leaves with *Verticillium albo-atrum* (*Vaa*) on symptom expression and systemic colonization of plants

Inoculation (days after injury)[a]	Injury method[b]	Symptom development[c] at harvest 1 (%)	*Vaa* recovery from inoculated leaves[c] (%)	Systemic colonization[c]: Petioles at harvest 1 (%)	Stem sections at harvest 1 (%)	Plants: Harvest 1 (%)	Plants: Harvest 2 (%)
Leaf inoculation							
0	Heating	8	100	19	14	25	17
	Piercing	0	100	37	17	42	8
3	Heating	8	100	25	6	17	8
	Piercing	0	100	42	19	42	33
6	Heating	17	100	14	6	17	8
	Piercing	8	100	9	9	25	8
Cut stem inoculation							
0		100	...	...	...	100	100
Not inoculated							
0	Heating	17	...	...	...	17	17
	Piercing	0	...	...	...	0	0
	None	0	...	...	...	0	0

[a] Three leaves on each of 12 plants per treatment were individually sprayed with a suspension of 10^6 conidia per milliliter for each leaf-injury treatment. The remainder of each plant was shielded from the inoculum.

[b] Individual leaflets of the leaves were pierced 10 times with a sterilized needle or touched with a hot glass rod.

[c] Observations on symptom development and systemic colonization were made 9 wk after inoculation and after the plants had regrown for 4 wk. Systemic colonization was determined by incubating stem sections on ethanol-streptomycin agar.

Table 4. Prepenetration activities of *Verticillium albo-atrum* on alfalfa leaves

Activitiy	Time after inoculation[a] (hr): 8 (%)	16 (%)	24 (%)	48 (%)
Conidia germinating	61.0	88.0	94.0	100.0
Conidia forming one germ tube	80.3	51.1	20.2	8.0
Conidia forming two germ tubes	19.7	47.7	74.5	87.0
Conidia forming three germ tubes	0.0	1.1	5.3	5.0
Conidia forming appressoria	0.0	11.4	15.9	22.0
Germ tubes forming appressoria	0.0	7.6	8.6	11.0

[a] Uninjured alfalfa leaves were inoculated with a 5-μl drop of a suspension of conidia (10^5/ml) of *Verticillium albo-atrum*. One hundred conidia were observed at each sampling time.

selected and individual leaves without visible injury were sprayed with inoculum (10^5 conidia per milliliter). Nontarget leaves were shielded from inoculum as described before. Other plants that showed severe injury were inoculated by spraying the leaves of the uppermost half of the plant.

Leaves without visible injury from exposure to dichlorvos did not develop VW symptoms. Moreover, none of 32 such plants developed symptoms after the plants were cut back and allowed to regrow for 1 mo. Visible sporulation of *V. albo-atrum* occurred only on inoculated leaves and only after they were excised, surface-disinfested, and incubated on ESA, indicating that ingress had occurred but that the fungus remained restricted to the inoculated leaves. Sporulation was abundant in discrete areas of leaflet blades inoculated by spraying individual leaves, but the fungus did not grow from proximal and distal ends of the petioles as would be expected if systemic colonization had occurred. Occasionally, sporulation occurred in small areas on petioles that presumably had been protected from inoculation.

VW symptoms were observed on newly formed uninoculated leaves of only three of 32 severely dichlorvos-injured plants by 17 days after inoculation. Only two of these plants developed severe VW symptoms when they were cut back and allowed to regrow for 1 mo. Systemic colonization of the plants, as indicated by growth of *V. albo-atrum* from stem pieces plated on ESA, occurred only for the three severely dichlorvos-injured plants that had characteristic VW symptoms; the pathogen grew from the cut ends of all stem pieces whether or not they were from inoculated stems.

Effect of delayed inoculation of injured leaves. For one experiment, three leaves per plant on lots of 12 plants were wounded by piercing or by heat and then spray-inoculated (10^6 conidia per milliliter) immediately or after 3 or 6 days. Controls consisted of 12 plants wounded but not inoculated, six plants inoculated via freshly cut stems, and six plants neither wounded nor inoculated. All plants were checked for symptoms and systemic colonization 9 wk after inoculation and for systemic colonization after they were allowed to regrow for 4 wk.

All six plants inoculated via freshly cut stems developed severe VW symptoms (Table 3). However, only five of 72 plants whose injured leaves were spray-inoculated were rated positive for symptoms; these plants were distributed over four treatments regardless of method of wounding or time of inoculation. Of the five plants that developed symptoms, only three were positive for systemic colonization at the first harvest, and only one was positive at the second harvest.

The incidence of systemic colonization was low (20/72 plants) regardless of method of wounding or time of inoculation (Table 3); the incidence was even lower (10/72) by the second harvest. Although the fungus was recovered from 100% of the inoculated leaves, its invasion of associated petioles and stems was markedly less (Table 3).

For a second experiment, leaves on lots of 15 plants were wounded by piercing or by heat and then spray-inoculated immediately or after 3 days. For only those plants inoculated immediately, half of the plants used for each type of injury were kept for 3 days under continuous light and 100% RH before inoculation and half were inoculated without prior exposure to these conditions. Controls consisted of inoculation via freshly cut stems (six plants); not wounded and inoculated at 0 time (six plants); and not wounded and not inoculated (six plants). Symptom development and systemic colonization were determined 4 wk after inoculation and again when they had regrown for 4 wk.

Although *V. albo-atrum* was readily recovered from inoculated leaves, the incidence of systemic colonization was very low regardless of preinoculation conditions, whether inoculation was done immediately or delayed, and injury method (Table 2). On the other hand, all plants inoculated via cut stems became systemically colonized and developed typical wilt symptoms.

Histology. No morphologic symptoms were observed. *V. albo-atrum* did not grow from stem pieces plated on ESA. Conidia germinated readily; 61.3% of the spores germinated within 8 hr and almost

90% germinated within 16 hr (Table 4). Most of the germinating conidia produced two germ tubes (Fig. 1A), but occasionally, one or three germ tubes per spore were observed (Table 4). Germ tube walls formed as an extension of the spore wall. Emergence of germ tubes was mostly polar. Of 576 germ tubes from 343 conidia observed in the period 8–48 hr after inoculation, 65.3% were polar, 34.1% were subpolar, and 0.6% were lateral. Germ tubes were 1.6–1.9 μm wide and highly variable in length, ranging 2.5–32.5 μm at 8 hr, 2.5–87.5 μm at 16 hr, and 2.5–135.7 μm at 24 hr. When two germ tubes per spore occurred, one was consistently longer than the other. The long and short germ tubes averaged, respectively, 6.8, 22.1, and 41.8 μm and 3.9, 9.1, 13.6 μm at 8, 16, and 24 hr after inoculation. Direction of germ tube growth showed no orientation. Germ tubes grew along or across anticlinal wall junctures without any change in direction and over stomata without penetrating them. One or two branches 2.5–31.2 μm long had developed in about 8% of the germ tubes by 24 hr, but no septa had formed.

Growth of the germ tube ended with the development of an appressorium on a low percentage of germ tubes (Table 4). Appressoria were first observed 16 hr after inoculation; after 24 hr, about 16% of germinated conidia bore an appressorium (Table 4). Appressoria were hyaline, thin-walled, and not delimited from the germ tubes by a septum (Fig. 1B); they were round or elliptical, averaging 2.9 or 3.9 × 2.5 μm, respectively, and were positioned over anticlinal wall junctures or the lumen of an epidermal cell. Of 47 appressoria observed for 503 germ tubes in the period 16–48 hr after inoculation, 36.2% were positioned over the lumina and 63.8% over anticlinal wall junctures.

Formation of an appressorium did not lead to development of a penetration peg; rather, a hypha developed from the appressorium and grew over the leaf surface.

Septa had formed by 48 hr in germ tubes that had increased slightly in width, and new thinner hyphae 1.0–1.25 μm wide developed from the germ tubes. By 3 days after inoculation, older hyphal cells had become swollen (2.5–3.5 μm wide) and thick-walled, which gave a hypha a torulose appearance. Deep constrictions occurred at the septum and hyphal fragmentation was observed. Numerous thin hyphae developed from the swollen cells. These thinner hyphae were less intensely stained than the older torulose hyphae. At this time, single phialidelike conidiophores also developed from the swollen hyphal cells either laterally on inner cells or longitudinally at the distal ends of the hyphae (Fig. 2A). Conidiophores with a septum at the base were 12.5–25.0 μm long and tapered from 2.0–2.8 μm at the base to 1.2 μm at the apex. Conidia 3.5–5.0 × 2.5 μm were borne singly at the apices of the conidiophores; numerous ungerminated conidia occurred in the vicinity of the conidiophores. Hyphae up to 475 μm long were observed by 6 days after inoculation, and branching was profuse.

Thin hyphae penetrated and grew within the cuticle (Figs. 2B and 3). The frequency of penetrations was low at 48 hr but had increased by 3 days after inoculation; intracuticular hyphae were up to 35 μm long by 6 days. Evidence for cuticle penetration was obtained by examining whole mounts at ×2,500, which allowed for fine differences in focal planes. No fungal structures were observed either within epidermal cells or in the palisade or mesophyll tissues by 16 days after inoculation, and no deleterious effects were detected in the leaf tissues.

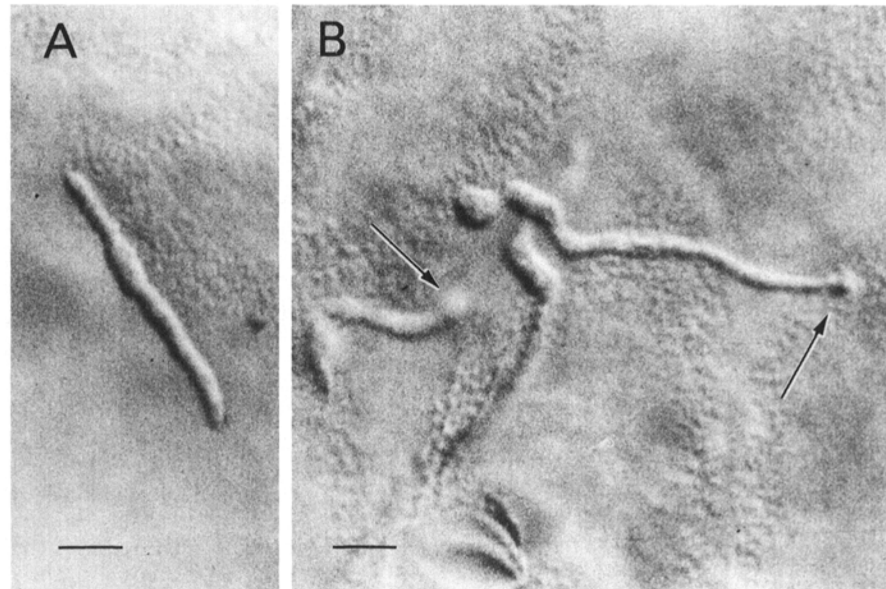

Fig. 1. Germinated conidia of *Verticillium albo-atrum* on alfalfa leaf surface. **(A)** Bipolar germination and **(B)** hyaline appressoria (arrow). Scale bar = 10 μm.

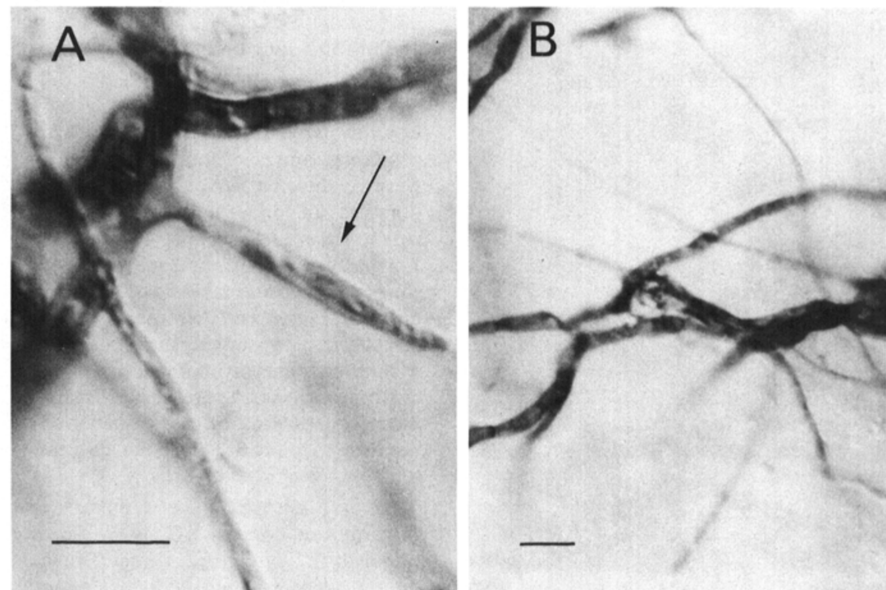

Fig. 2. Mycelium of *Verticillium albo-atrum* on alfalfa leaf surface. Swollen, thick-walled cells of torulose hyphae. **(A)** Single phialidelike conidiophore (arrow) and **(B)** thin hyphae developed from swollen, thick-walled cells and growing within cuticle. Scale bar = 10 μm.

DISCUSSION

Conidia of the alfalfa strain of *V. albo-atrum* can penetrate uninjured healthy leaves and stems, but the fungus remains localized at the penetration site (Tables 1 and 2). This occurred even at conidial concentrations higher than might be expected in the field. Plants whose leaves were not visibly affected by exposure to dichlorvos volatiles also did not become systemically colonized even though a high frequency of leaf penetrations occurred. Systemic colonization occurred

only in plants with leaves visibly injured at inoculation (Table 1). Even though the pathogen remained viable in association with inoculated leaves (both injured and uninjured) for several weeks, the frequency of plants systemically colonized was markedly lower (Table 2, 0–13%; Table 3, 17–42%) than occurred if inoculation was via freshly cut stems (100%). Moreover, on the basis of the numbers of stem sections positive for *V. albo-atrum*, the frequency of colonization was very low (Table 2, 0–4%; Table 3, 6–19%).

The holonecrosis or plesionecrosis of tissues induced by the several injury treatments presumably facilitated ingress and growth of the fungus and enabled it to gain access to vascular bundles (30). Alternatively, such severe injuries may have interfered with defense mechanisms that restrict development of the fungus in uninjured nonvascular leaf and stem tissues.

Conidia of *V. albo-atrum* germinated readily, and there was extensive mycelial growth on intact alfalfa leaves. However, neither penetration of epidermal cells nor mycelial growth in palisade or mesophyll tissue occurred. Rather, thin hyphae penetrated and grew inside the cuticle but did not cause any detectable deleterious effects on the underlying epidermal cell. Our results disagree with those of Flood and Milton (11), who reported penetration of alfalfa leaves either directly through or between the epidermal cell walls and mycelial growth in the palisade and mesophyll tissues associated with little cell death at 6 days after inoculation. Flood and Milton (11) used excised leaves and an inoculum concentration (10^7 conidia per milliliter) 10- to 100-fold higher than those we employed. Detachment of leaves, even though they were floated on water, might have caused some stress, initiated senesence, or otherwise modified the physiology of the tissues that could enhance penetration and colonization by the pathogen.

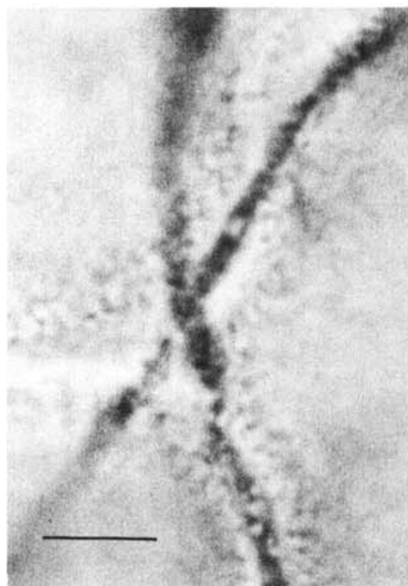

Fig. 3. Granulation of cuticle associated with intracuticular growth of thin hyphae of *Verticillium albo-atrum* after penetration of alfalfa leaf. Scale bar = 10 μm.

V. albo-atrum or *V. dahliae* gained ingress into potato leaves mainly by direct penetration of germ tubes through epidermal cell walls and occasionally by penetration pegs developed from appressoria (21,38). On the other hand, *V. dahliae* failed to penetrate intact tomato leaves (7), even if conidia were supplied with a carbohydrate source (15). Intact tomato leaves, however, were penetrated by hyphae of *V. tricopus* that developed from mycelium supplied with a carbohydrate source and had become modified to form resting structures (15). In our studies, hyphae of *V. albo-atrum* also became markedly modified; they developed a toruloselike appearance but did not appear darkly pigmented as is characteristic of the resting mycelium. Also, contrary to observations reported by Griffiths and Isaac (15), thin hyphae arising from the modified mycelium of *V. albo-atrum* did not penetrate the epidermal cell walls of alfalfa leaves.

Germination of *V. albo-atrum* conidia is strongly influenced by environmental conditions, including temperature and water and nutrients available for growth (10,27,28). Environmental conditions in our studies appeared suitable for conidial germination and germ tube growth. The rate of germination was higher than that on leaf surfaces reported by Flood et al (10), and the rate of germ tube growth was comparable to that reported by others (11,24). Germinating conidia produced two germ tubes that appeared to emerge in sequence, as indicated by their differences in length and increase in number over time. Such a sequence would correspond to the synchronous pattern between germ tube emergence and mitotic division of the nucleus in the germinating conidia observed by Typas and Heale (39).

A low percentage of germ tubes formed a simple appressorium, most of which were positioned over anticlinal wall junctures of epidermal cells as reported for some leaf-infecting fungi (8,31). Formation of an appressoium by germ tubes of *V. albo-atrum* has been reported only by Hung and Whitney (21), who occasionally observed them associated with direct penetration of epidermal cells of potato leaves. Appressoria formed on alfalfa leaves did not lead to penetration but instead formed hyphae that continued to grow over the leaf surface.

Several authors have reported that germinating conidia of *V. albo-atrum* (3,36) or *V. dahliae* (2) directly formed phialides and smaller secondary conidia. We did not observe phialides on germinating conidia. Rather, phialides and conidia formed abundantly on hyphae growing extensively on the leaf surface. Thus, even in the absence of cell penetration and tissue colonization, the development of *V. albo-atrum* on alfalfa leaves allows the pathogen to reproduce, which might be ecologically and epidemiologically significant.

Sporulation of *V. albo-atrum* was not macroscopically apparent on inoculated, uninjured, nonsymptomatic leaves. However, when these leaves were excised, surface-disinfested, and incubated on selective medium, sporulation occurred within 5 days. Conidiophores appeared on small discrete areas of green tissue regardless of inoculation site. These areas gradually increased in size and in some instances became mildly hydrotic, but they did not become macroscopically necrotic. This observation for leaves differed from that for stems on which sporulation did not occur until the tissues were in advanced stages of senescence or had died (22; R. M. Jimenez-Diaz and R. L. Millar, *unpublished*).

Injured tissues allowed ingress and establishment systemically of *V. albo-atrum* for at least 6 days (Table 3). However, the frequency of successful colonizations was much lower than that for inoculations made immediately after injury to the tissues. A delay of 24–48 hr in inoculating cut stems also results in a marked reduction of systemically infected plants (R. L. Millar, *unpublished*).

Insect dispersal of *V. albo-atrum* has been reported by Huang et al (20) and by Harper and Huang (16). Both pea aphids (*Acyrthosiphon pisum* Harris) and alfalfa weevils (*Hypera postica* Gylenhal) served as vectors. Since these are sucking and biting and chewing insects, respectively, it seems probable that ingress and systemic colonization occurred via wounds caused by the insects. This interpretation is supported by our findings that systemic colonization occurred only on wounded leaves.

The ability of *Verticillium* spp. to infect leaves and systemically colonize suscepts varies with the pathogen and suscept involved. Foliar infection by *V. albo-atrum* resulted in systemic colonization of a low proportion of tomato plants (32) and of a high proportion of potato plants (21,37,38). *V. dahliae* induced types and frequencies of infections of potatoes similar to those induced by *V. albo-atrum* regardless of tissue wounding (38). On the other hand, pathogenic strains of *V. dahliae* induced localized infections in wounded but not unwounded tomato leaves (7). Piercing tomato leaves did not lead to infection by conidia of *V. tricorpus*, but localized infections and subsequently chlorosis and leaf drop occurred for unwounded leaves when conidia were supplied with a carbohydrate source (15). We found that conidia of *V. albo-atrum* produced on infected tissue can accomplish localized infections of uninjured healthy leaves and stems. How-

ever, even under optimal conditions for the pathogen, the efficacy of such inoculum apparently depends greatly on the amount that reaches the penetration court.

The fact that penetrations of uninjured healthy leaves and stems do not give rise to systemic colonization of plants may have important implications in our understanding of the epidemiology of the disease and on measures to control it. The observations that *V. albo-atrum* sporulates abundantly on infected stems under moist conditions (22; R. M. Jimenez-Diaz and R. L. Millar, *unpublished*), that conidia become airborne (9,22,26; R. M. Jimenez-Diaz and R. L. Millar, *unpublished*), and that disease incidence is greater in alfalfa crops under moist conditions or irrigation (1,6,14), which presumably favor sporulation, suggest that conidia dispersed by air may serve as primary and/or secondary inoculum for pathogen spread within and between fields. However, our results suggest that airborne conidia of *V. albo-atrum* may not play an important role in determining disease incidence within and between adjacent alfalfa fields. In support of this interpretation, we have observed that contiguous fields often have widely disparate disease incidences (e.g., 0 and >50%), and we and others (22,23) have observed 1) fields in which the greatest incidence of diseased plants was near the field entrance or oriented in the direction that equipment moves during harvesting (22,23) and 2) that pathogen propagules are readily recovered from the mower bar. We believe, therefore that inoculum carried on the cutter bar is the most important means of dispersal of the pathogen within and between fields (18) and that a significant degree of control should derive from measures developed to eliminate or reduce conidia on the cutter bar.

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LITERATURE CITED

1. Atkinson, T. G. 1981. Verticillium wilt of alfalfa: Challenge and opportunity. Can. J. Plant Pathol. 3:266-272.
2. Buckley, P. M., Wyllie, T. D., and DeVay, J. E. 1969. Fine structure of conidia and conidium formation in *Verticillium albo-atrum* and *V. nigrescens*. Mycologia 61:240-250.
3. Chaudhuri, H. 1923. A study of the growth in culture of *Verticillium albo-atrum* R. & B. Ann. Bot. 37:519-539.
4. Christen, A. A. 1982. Demonstration of *Verticillium albo-atrum* within alfalfa seed. Phytopathology 72:412-414.
5. Christen, A. A. 1983. Incidence of external seedborne *Verticillium albo-atrum* in commercial seed lots of alfalfa. Plant Dis. 67:17-18.
6. Christen, A. A., and Peaden, R. N. 1981. Verticillium wilt in alfalfa. Plant Dis. 65:319-321.
7. Cirulli, M. 1974. Infezioni locali da *Verticillium dahliae* Kleb. su foglie di pomodoro. Phytopathol. Mediterr. 13:23-26.
8. Clark, C. A., and Lorbeer, J. W. 1976. Comparative histopathology of *Botrytis squamosa* and *B. cinerea* on onion leaves. Phytopathology 66:1279-1289.
9. Davies, R. A., and Isaac, I. 1958. Dissemination of *Verticillium albo-atrum* through the atmosphere. Nature 181:649.
10. Flood, J., Khan, F. Z., and Milton, J. M. 1977. The role of phytoalexins in Verticillium wilt of lucerne (*Medicago sativa* L.). Ann. Appl. Biol. 89:329-332.
11. Flood, J., and Milton, J. M. 1982. Studies on the incompatible and compatible interactions between lucerne leaves and *Verticillium albo-atrum*. Physiol. Plant Pathol. 21:97-104.
12. Graham, J. M., Peaden, R. N., and Evans, D. W. 1977. Verticillium wilt of alfalfa found in the United States. Plant Dis. Rep. 61:337-340.
13. Grau, C. R., Delwiche, P. A., Norgren, R. L., O'Connell, T. E., and Maxwell, D. P. 1981. Verticillium wilt of alfalfa in Wisconsin. Plant Dis. 65:843-844.
14. Gray, F. A., and Roth, D. A. 1982. Verticillium wilt in alfalfa in Wyoming. Plant Dis. 66:1080.
15. Griffiths, D. A., and Isaac, I. 1963. Reaction of tomato leaves to species of *Verticillium*. Ann. Appl. Biol. 51:231-236.
16. Harper, A. M. and Huang, H. C. 1984. Contamination of insects by the plant pathogen, *Verticillium albo-atrum* in an alfalfa field. Environ. Entomol. 13:117-120.
17. Heale, J. B., and Isaac, I. 1963. Wilt of lucerne caused by species of *Verticillium*. IV. Pathogenicity of *V. albo-atrum* and *V. dahliae* to lucerne and other crops; spread and survival of *V. albo-atrum* in soil and in weeds; effects upon lucerne production. Ann. Appl. Biol. 52:439-451.
18. Heale, J. B., Isaac, I., and Milton, J. M. 1979. The administrative control of Verticillium wilt of lucerne. Pages 71-78 in: Plant Health: The Scientific Basis for Administrative Control of Plant Diseases. D. L. Ebbels and J. E. King, eds. Blackwell Scientific Publications, Oxford, England.
19. Horst, R. K., and O'Neil, R. T. 1974. High humidity and controlled temperature chamber. Plant Dis. Rep. 58:814-818.
20. Huang, H. C., Harper, A. M., Kokko, E. G., and Howard, R. J. 1983. Aphid transmission of *Verticillium albo-atrum* to alfalfa. Can. J. Plant Pathol. 5:141-147.
21. Hung, K. S., and Whitney, N. J. 1982. Reaction of two potato cultivars to leaf infection by *Verticillium albo-atrum*. Can. J. Bot. 60:554-556.
22. Isaac, I. 1957. Wilt of lucerne caused by species of *Verticillium*. Ann. Appl. Biol. 45:550-558.
23. Isaac, I., and Lloyd, A. T. E. 1959. Wilt of lucerne caused by species of *Verticillium*. II. Seasonal cycle of disease; range of pathogenicity, host-parasite relations; effects of seed dressings. Ann. Appl. Biol. 47:673-684.
24. Khan, F. Z., and Milton, J. M. 1978. Phytoalexin production and the resistance of lucerne (*Medicago sativa* L.) to *Verticillium albo-atrum*. Physiol. Plant Pathol. 13:215-221.
25. Kreitlow, K. W. 1962. Verticillium wilt of alfalfa. A destructive disease in Britain and Europe not yet observed in the United States. Crop Res. ARS 34-40, 1-15.
26. Lindemann, J., Arny, D. C., and Delwiche, D. A. 1982. Detection of *Verticillium albo-atrum* in the air over infected alfalfa fields in Wisconsin. (Abstr.) Phytopathology 72:1382.
27. Mozumder, B. K. G., and Caroselli, N. E. 1974. Influence of microelements in a sporulation medium on subsequent water requirements for germination of *Verticillium albo-atrum* conidia. Indian Phytopathol. 27:537-541.
28. Mozumder, B. K. G., Caroselli, N. E., and Albert, L. S. 1970. Influence of water activity temperature and their interaction on germination of *Verticillium albo-atrum* conidia. Plant Physiol. 46:347-349.
29. Nadakavukaren, M. J., and Horner, C. E. 1959. An alcohol agar medium selective for determining *Verticillium* microsclerotia in soil. Phytopathology 49:527-528.
30. Pennypacker, B. W., and Leath, K. T. 1983. Dispersal of *Verticillium albo-atrum* in the xylem of alfalfa. Plant Dis. 67:1226-1229.
31. Preece, T. F., Barnes, G., and Bayley, J. M. 1967. Junctions between epidermal cells as sites of appressorium formation by plant pathogenic fungi. Plant Pathol. 16:117-118.
32. Provvidenti, R., and Schroeder, W. T. 1959. Foliage infections of tomato and eggplant by *Verticillium*. Plant Dis. Rep. 43:821-826.
33. Raynal, G., and Guy, P. 1977. Repartition et importance des naladies de la lucerne en France et en Europe. Fourrages 71:5-13.
34. Sheppard, J. W., and Needham, S. N. 1980. Verticillium wilt of alfalfa in Canada: Occurrence of seed-borne inoculum. Can. J. Plant Pathol. 1:159-162.
35. Shipton, W. A., and Brown, J. F. 1962. A whole leaf clearing and staining technique to demonstrate host pathogen relationship of wheat stem rust. Phytopathology 52:1313.
36. Smith, H. C. 1965. The morphology of *Verticillium albo-atrum, V. dahliae* and *V. tricorpus*. N.Z. J. Agric. Res. 8:450-478.
37. Thanassoulopoulos, C. C., and Hooker, W. J. 1968. Factors influencing infection of field grown potato by *Verticillium albo-atrum*. Am. Potato J. 45:203-216.
38. Thanassoulopoulos, C. C., and Hooker, W. J. 1970. Leaf and sprout infection of potato by *Verticillium albo-atrum*. Phytopathology 60:196-203.
39. Typas, M. A., and Heale, J. B. 1980. DNA content of germinating spores individual hyphal cells and resting structure cells of *Verticillium* measured by microdensitometry. J. Gen. Microbiol. 121:231-242.
40. Young, B. A., Sherwood, R. T., and Bashaw, E. C. 1979. Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixes in grass. Can. J. Bot. 57:1668-1673.