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Sporulation on infected tissues, and presence of airborne *Verticillium albo-atrum* in alfalfa fields in New York

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The phenology of *Verticillium albo-atrum* (*Vaa*) sporulation on infected tissues and the presence of *Vaa* in the air were investigated in alfalfa fields affected by verticillium wilt (VW) during the 1983/84 growing season. Few and scattered *Verticillium* conidiophores developed on stubble by the end of May, but abundant *Verticillium* sporulation was found on necrotic leaflets and petioles by the beginning of June at the first appearance of symptoms. Abundant conidiophores developed also on necrotic stems and leaf tissues sampled later. Sporulation was never found on entirely green stems. Concentration of airborne colony-forming units (cfu) of *Vaa* ranged from 0·0 to 15·3 cfu/m^3 above fields with a VW incidence of 2·2–33·0% in June and August, and from 5·9–25·9 cfu/m^3 during mowing of the crop in August. The ratio of *Vaa* to other airborne fungi above sampled fields was less than 5×10^{-3}. Airborne fungi were even more abundant at the time of mowing. Our results suggest that airborne *V. albo-atrum* may not play an important role in the epidemiology of VW of alfalfa.

INTRODUCTION

Verticillium wilt (VW), induced by a strain of *Verticillium albo-atrum* Reink. & Berth., is a major disease of alfalfa (*Medicago sativa* L.) in Europe (Kreitlow, 1962; Heale & Isaac, 1963; Raynal & Guy, 1977) and in North America (Arny & Grau, 1985). In the USA, the disease was first found in Washington and Oregon in 1976 (Graham *et al.*, 1977). It now affects alfalfa in several of the northern tier of states above 40° latitude (Arny & Grau, 1985), and has been recently detected in Kansas, below 38° latitude (Stuteville *et al.*, 1986).

V. albo-atrum is introduced into pathogen-free regions through infested or infected seeds (Sheppard & Needham, 1980; Christen, 1982, 1983), and by infested plant material carried with seed lots (Isaac, 1957; Sheppard & Needham, 1980). However, the major means by which the pathogen is dispersed within and between fields has not been clearly established.

Heale *et al.* (1979) suggested that infection of freshly cut stems by conidia carried on the cutter blades was the most important cause of rapid spread of the pathogen both within and between fields. Alternatively, the observations that *V. albo-atrum* sporulates abundantly on infected stems under moist conditions (Isaac, 1957; Huang *et al.*, 1983), and that conidia can become airborne (Isaac, 1957; Davies & Isaac, 1958; Lindemann *et al.*, 1982), would suggest that conidia dispersed by air may play on important role in local spread of the pathogen.

To be important in the epidemiology of VW, airborne conidia should be infective, abundant, and readily available throughout the crop season, although dispersal of small amounts of them could be significant in initiating new disease foci if conidia infect susceptible cut stems. In New York state, sporulation by *V. albo-atrum* occurs on dead alfalfa stems in the fall (R. L. Millar, unpublished). However, there is no information as to whether or when conidia are produced on infected tissues and released into the air during the growing season. The objectives of this investigation were to determine whether conidia of *V. albo-atrum* are formed on necrotic stubble and infected tissue, and whether they are present in the air above infected plants during the growing season.

MATERIALS AND METHODS

Four alfalfa fields, designated F1–4, were selected for this study. Fields F1–3 were located near

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Freville, NY. F1 was sown in 1980. F2 was established at least 1 year before F1, and 300 m north of it in the direction of the prevailing winds. F3 was sown adjacent to, and north of, F2 in 1982. In the fall of 1983 incidence of VW was high in F1 and F2 and a trace in F3. F4 was sown near Groton, NY, in 1983, to establish microplots (2×2 m^2) for a study on the interaction between *V. albo-atrum* and *Pratylenchus penetrans*.

Production of conidia of *V. albo-atrum* on stubble and newly infected tissues

Infected plants in selected fields were sampled at several times during the growing season. Symptomatic and necrotic stems were sampled from fields F1 and F2 on 6 and 11 October 1983. On 26 April, 5 and 22 May and 6 June 1984, individual plants were dug and their tap roots examined for the presence of the yellow to brown vascular discoloration which often develops in affected plants (Christen & Peaden, 1981). Nine to 15 plants with root discoloration were selected on each sampling date for further observations. On 15 June 1984 all stems and stubble from nine infected plants were collected at F1 and F2, and on 7 August symptomatic and necrotic stems were sampled at F4. Sampled tissues were placed in plastic bags and kept at 5°C until observations were completed within 24–36 h from the time of sampling.

Lowermost 8–10 cm lengths of stubble, entire necrotic and symptomatic stems, leaflets and petioles, were examined under a stereo-microscope for the presence of verticillate conidiophores. Pieces of stubble without conidiophores were washed thoroughly in running tap water, surface-disinfested in sodium hypochlorite (0·5% available chlorine) for 1·5 min, blotted dry, incubated in moist chambers at 21°C, and examined under a stereo-microscope for sporulation of *Verticillium* after 1 and 2 weeks.

When sporulation occurred on sampled tissues the viability of conidia was checked, and the morphology of conidiophores was confirmed with a compound microscope. Pieces of sporulating tissues were placed in 10 ml of sterile distilled water in test tubes and agitated in a Vortex mixer for 1–2 min to dislodge conidia. The suspension was filtered through a single layer of cheesecloth and 1 ml of it was spread on ethanol-streptomycin agar (ESA) (Nadakavukaren & Horner, 1959) in each of two petri plates per sample. The plates were kept at 21°C for 1 week and examined for growth of *Verticillium*. In some instances *Verticillium* was directly isolated by removing verticillate conidiophores and spore balls with a sterilized fine needle and transferring them to ESA in petri plates.

Presence of airborne *V. albo-atrum* within and above the canopies of VW-affected fields

The presence of propagules of *V. albo-atrum* in the air above fields F1–4 was assessed by means of a six-stage Andersen particle-sizing sampler containing petri plates with 27 ml of ESA. The sampler pump was adjusted to a 2·54-cm vacuum which allowed a flow rate of 1·7 m^3/h. Sampling periods were 10.00–15.00 on 15 June (F1–3) and 10.00–12.30 and 14.00–16.30 on 9 August (F4), 1984. F2 and F4 were also sampled while the crop was being mown, between 10.00 and 15.00 on 19 July and 10 August 1984, respectively. Two samples, each of 30 min duration, were obtained in each field during each sampling period on the standing crop at sites with infected plants. The sampler outlet was located 50 cm above the crop canopy for one sample and within the canopy in the other. For the air sampling at harvesting, two 30-min samples were obtained by placing the Andersen sampler 50 cm above the crop canopy and 10 m downwind from plots being mowed. Data on weather conditions prevailing at the times of sampling periods were obtained from the Meteorology Department, Game Farm Road, Ithaca, NY.

The incidence of disease in fields F1–3 was estimated just after the air sampling was completed. The numbers of healthy and infected stems were counted in 0·1-m^2 random samples of the stand. In each sample, a stem was considered infected if it showed chlorosis, yellowing and 'bunchiness' characteristic of VW. Although many dead stems occurred beneath the canopy that could have been killed by infection of *V. albo-atrum*, they were not counted. Therefore, our estimated VW incidences might have been lower than the actual disease incidence. The sampling for disease incidence was continued until at least 100 stems were counted. Because of differences in stand density among fields, the number of samples varied from four in F3 to seven in F2. The incidence of VW in F4 was determined on 9 August, by counting the actual number of infected plants in microplots, 2×2 m^2, before the air sampling.

Plates from the air sampling were kept at 21°C for 6 days and examined under a stereo-micro-

scope for colonies of *V. albo-atrum* and other fungi.

Pathogenicity of *V. albo-atrum* isolates to alfalfa

Verticillium colonies derived from air sampling, necrotic tissues and stubble, were transferred to prune lactose yeast agar (PLYA) to confirm the identity of *V. albo-atrum* (Talboys, 1960). Pathogenicity of 19 mass isolates of *V. albo-atrum* representative of those obtained from the air (seven), necrotic tissues (seven), and stubble (five) at various sampling dates was tested on 8-week-old cultivar Iroquois plants grown in sterilized Cornell peat-lite mixture in clay pots. Inoculum (10^5 conidia/ml) for each isolate was obtained from 12-day-old cultures grown on V8 agar slants at 21°C. The cultures were flooded with 5 ml of sterile distilled water and agitated in a vortex mixer to dislodge conidia. Conidia in the suspension were filtered through two layers of cheesecloth, counted with a haemocytometer and adjusted to the desired concentration. Stems of a plant were inoculated by cutting just above the first node with disinfested scissors, then immediately applying a drop of inoculum suspension on a camel-hair brush to the cut surface. Five plants were inoculated with each isolate; another five that did not receive inoculum, but otherwise were treated similarly, served as controls. After inoculation the plants were kept in a growth chamber programed to 20/18°C (day/night), 70–80% relative humidity, and 16 h/day photoperiod of about 18 klux. The severity of disease reaction was assessed on a scale 0–4 (0, no symptoms; 4, dead plant), 3 weeks after inoculation.

RESULTS

Production of conidia of *V. albo-atrum* on stubble and newly infected tissues

Verticillium conidiophores were not found on 68 and 45 pieces of stubble from each of 10 plants sampled on 26 April and 5 May respectively, but a few, scattered conidiophores occurred in two of nine plants sampled (7·5% of 52 pieces) on 22 May. When pieces of stubble not bearing *Verticillium* conidiophores were incubated in a moist chamber, a few scattered verticillate conidiophores formed on one piece from one of the plants sampled on 5 May and on 30·3% of 33 pieces from four of those plants sampled on 22 May. Abundant fungal contaminants developed on the stubble pieces.

To relate the phenology of *Verticillium* sporulation on stubble to the availability of newly developed alfalfa tissues, observations were made on the crop development stage at the time of sampling infected tissues. New growth from alfalfa crowns was insignificant on 26 April, but new stems were 5–10 cm tall on 5 May. On 22 May, when healthy stems were 25–35 cm tall, a number of plants were stunted, with poor growth and small, yellowing leaves, but neither *Verticillium* sporulation nor necrotic tissues were found on them.

Symptoms characteristic of VW were first discernible by 6 June. At this time, many green stems had necrotic leaves. While no sporulation occurred on green tissues, *Verticillium* conidiophores and conidia entirely covered necrotic petioles in 10 of 15 sampled plants, but were absent or scarce on necrotic leaflets. By 15 June *Verticillium* was sporulating abundantly on all the infected plants that were sampled. Out of nine plants sampled in each of fields F1 and F2, five and four, respectively, were necrotic and entirely covered by verticillate conidiophores with conidia. In the remaining infected plants, four from F1 and five from F2 had necrotic leaves with intense *Verticillium* sporulation on leaflets, petioles or both. At this time a few verticillate conidiophores were found on stubble of two and three plants, respectively, out of the nine plants sampled in each of F1 and F2. Otherwise, pieces of stubble without signs of *Verticillium* sporulation occurred in crowns bearing symptomatic stems and sporulating tissues.

In field F4, observations and sampling for *Verticillium* sporulation were made on 7 August, about 7 weeks after the crop had first been mowed. Beneath the canopy, numerous dead stems and stubble from the first harvest were entirely covered by *Verticillium* conidiophores with spore balls. Also, abundant *Verticillium* sporulation was found on necrotic petioles, stipules and leaflets from green stems, but no sporulation occurred on green tissues from infected plants. Similar results occurred for observations and samplings made in fields F1 and F2 on 6 and 11 October. *Verticillium* conidiophores and spore balls were abundant on all necrotic tissues and covered dead stems up to the uppermost petioles. However, *Verticillium* sporulation was never found on entirely green stems bearing symptomatic or necrotic leaves.

Table 1. Concentration of airborne *Verticillium albo-atrum* (*Vaa*) within and above the canopy of alfalfa crops affected by verticillium wilt[a]

Sampling location[b]	Colony-forming units per m^3			Disease incidence[c] (%)
	Vaa	Contaminant fungi	Ratio ($\times 10^{-3}$)	
Field 1				32·3
Above the canopy	1·2	363·5	3·3	
Within the canopy	5·9	—[d]	—	
Field 2				33·0
Above the canopy	2·4	568·2	4·2	
Within the canopy	2·4	—	—	
Field 3				2·2
Above the canopy	1·2	—	—	
Within the canopy	0·0	—	—	

[a] A six-stage Andersen particle-sizing sampler was used in three fields near Freeville, NY between 10.00 and 14.00 on 15 June 1984.
[b] At each location, the sampler was run for 30 min with a flow of 0·85 m^3. The sampler outlet was located 50 cm above or within the crop canopy.
[c] Determined as number of infected stems in 100 stems randomly sampled just after air sampling.
[d] No data.

Presence of airborne *V. albo-atrum* within and above the canopies of VW-affected fields

Verticillium colonies, which later were identified as *V. albo-atrum*, formed in small numbers (0–13) on ESA plates exposed to air sampled from undisturbed VW-affected alfalfa crops. Most colonies developed on sampler stages that collected particles 4·7 μm in diameter, although a few colonies formed from particles with diameter in the range 2·1–4·7 μm. Airborne *V. albo-atrum* (1·2–5·9 cfu/m^3) was detected in five out of six sampling sites at three fields with severe or trace incidences of VW on 15 June 1984 (Table 1), and in five out of eight sites (1·2–15·3 cfu/m^3) sampled at two microplots with 0 or 18 infected plants on 9 August 1984 (Table 2). Comparatively, other airborne fungi (*Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium*) were abundant, with a ratio of *V. albo-atrum* to their combined frequency ranging from $0{\cdot}2 \times 10^{-3}$ to $4{\cdot}2 \times 10^{-3}$ (Tables 1, 2). Colonies of these fungi did not grow fast on ESA and they did not interfere in recovering all the *Verticillium* colonies which formed.

For the standing crop, the concentration of airborne *V. albo-atrum* was higher within the crop canopy than 50 cm above it (Tables 1, 2). Although the highest concentration of airborne *V. albo-atrum* occurred in fields severely affected by VW (Tables 1, 2), that concentration did not appear to be greatly influenced by the incidence of the disease.

When the sampling took place at harvesting, the concentration of airborne *V. albo-atrum* was also low (5·9–25·9 cfu/m^3) in one field with a range of incidence of VW, but the fungus was not detected in the air above another which had a severe attack of the disease (Tables 2, 3). However, the ratio of *V. albo-atrum* to other airborne fungi (Table 3) was very low ($0{\cdot}9 \times 10^{-3}$–$4{\cdot}8 \times 10^{-3}$), similar to that which occurred with the sampling of standing crops (Tables 1, 2).

Average temperature, relative humidity and windspeed during the hours that spore trapping was taking place ranged, respectively, from 20·2 to 23·6°C, 26 to 44% and 7·7 to 10·9 m/s on 19 July; from 25·3 to 28·7°C, 21 to 28% and 8·8 to 10·4 m/s on 9 August; and from 23·7 to 28·3°C, 25 to 30% and 4·7 to 8·3 m/s on 10 August. Neither rain nor thunderstorms occurred during the sampling periods.

Table 2. Influence of time of day and incidence of the disease on the concentration of airborne *Verticillium albo-atrum* (*Vaa*) within and above the canopy of alfalfa crops affected by verticillium wilt[a]

Sampling time and location		Colony-forming units per m^3			Number of infected plants[c]
		Vaa	Contaminant fungi	Ratio ($\times 10^{-3}$)	
Plot A					0
Morning	Above the canopy	1·2	1976·5	0·6	
	Within the canopy	0·0	2829·4	—	
Afternoon	Above the canopy	0·0	1600·0	—	
	Within the canopy	2·4	4650·6	0·5	
Plot B					18
Morning					
	Above the canopy	0·0	3414·1	—	
	Within the canopy	4·7	3997·6	1·2	
Afternoon	Above the canopy	1·2	5578·8	0·2	
	Within the canopy	15·3	4987·1	3·1	

[a] A six-stage Andersen particle-sizing sampler was used close to two microplots, each 2×2 m^2, in a field near Groton, NY.
[b] For each location and time the sampler was run for 30 min with a flow of 0·83 m^3 from 10.00 to 12.30 and 14.00 to 16.30 on 9 August 1984. The sampler outlet was located 50 cm above or within the crop canopy. Average temperature, relative humidity and windspeed ranged from 25·3 to 28·7°C, 21 to 28% and 8·3 to 10·4 m/s, respectively, during the morning sampling; and from 28·3 to 29·7°C, 23 to 25% and 8·9 to 9·4 m/s, respectively, during the afternoon sampling. Neither rain nor thunderstorms occurred.
[c] All symptomatic plants in the microplots were counted.

Table 3. Concentration of airborne *Verticillium albo-atrum* (*Vaa*) above alfalfa crops being harvested[a]

Sampling location and time[b]	Colony-forming units per m^3		
	Vaa	Contaminant fungi	Ratio ($\times 10^{-3}$)
Field 2			
July 19	0·0	+++[c]	—
Field 4			
August 10			
Morning	25·9	5381·2	4·8
Afternoon	5·9	6345·9	0·9

[a] A six-stage Andersen particle-sizing sampler was used while the fields were harvested. Fields 2 and 4 were located near Freeville and Groton, NY, respectively.
[b] For each location and date the sampler was run for 30 min twice, from 10.00 to 12.00 in field 2 and from 10.00 to 11.00 and 14.00 to 15.00 in field 4. The sampler was located 50 cm above the crop canopy and 10 m downwind of the plots. Average temperature, relative humidity and windspeed at the times of spore trapping ranged from 20·2 to 23·6°C, 26 to 44% and 7·7 to 10·9 m/s, respectively, on 19 July; and from 23·7 to 28·3°C, 25 to 30% and 4·7 to 8·3 m/s, respectively, on 10 August. Neither rain nor thunderstorms occurred.

Pathogenicity of *V. albo-atrum* isolates to alfalfa

All colonies of *Verticillium* obtained from stubble, necrotic tissues and the air above or within crop canopies were identified as *V. albo-atrum* (Smith, 1965). All 19 representative isolates tested were pathogenic to alfalfa culfivar Iroquois and induced symptoms characteristic of VW. Average severity of symptoms ranged from 2·1 to 2·7.

DISCUSSION

Under field conditions in New York, *V. albo-atrum* formed viable conidia on stubble from the previous alfalfa crop early in the season. However, because only a few conidiophores formed on a small proportion of stubble pieces of a small proportion of plants, even under controlled conditions suitable for sporulation, it seems that the pathogen has a very limited potential to survive and/or sporulate on stubble after 1 year. Under similar field conditions in New York, Keinath & Millar (1986) have shown that *V. albo-atrum* persists in a small proportion of infected alfalfa stems buried 15 cm or left on the soil surface. Contrary to the scarce sporulation on old stubble, *V. albo-atrum* formed abundant conidiophores and viable conidia on newly infected leaflets, petioles, stipules and stems, all of which became necrotic, as well as on stubble from previous harvests during the season, but not on infected green tissue. Isaac (1957) and Huang *et al.* (1983) reported that abundant sporulation by *V. albo-atrum* covered necrotic stems beneath the canopy of infected plants. Our results indicate that the pathogen can also sporulate abundantly on newly necrotic foliar tissues before the stems became necrotic.

Sporulation by *V. albo-atrum* on newly necrotic alfalfa tissues was abundant by 6 June, when first systemic symptoms of VW were clearly discernible, and it was found to form extensively on tissues which became necrotic throughout the crop growing season. Intense sporulation by *V. albo-atrum* was usually associated with the very moist conditions within the crop canopy which prevail during the growing season in New York. Under moist conditions at 20–22 °C, *V. albo-atrum* sporulates abundantly on infected senescing or necrotic alfalfa stems, in 24–48 h, and it seems therefore that large numbers of conidia of *V. albo-atrum* could be available for airborne dispersal during the crop season.

Nevertheless, in contrast to the high concentrations of other airborne fungi, airborne *V. albo-atrum* occurred at low concentrations within and above the canopies of standing crops severely affected by VW (Tables 1, 2). This occurred at times at which sporulation on infected tissues was abundant (Tables 1, 2). Furthermore, airborne concentrations of the pathogen did not significantly increase when severely affected crops were being harvested (Table 3). This could be because spore balls and conidia of *V. albo-atrum* were not readily carried away by air disturbances during mowing of the crop, although they are easily detached when sporulating stems are gently tapped. Being wet-spored, it is probable that the spore balls can only be 'naturally' picked up by a moving water droplet in an air current. If this is so, only a combination of wet conditions plus air currents is likely to result in a significant air dispersal of the pathogen, and this would only be relevant during and for a very short period after harvesting, before healing of cut stems occurs. However, these weather conditions did not prevail at the times of spore trapping. Although the concentrations of airborne *V. albo-atrum* found were low, they were sometimes larger than those reported (highest concentration 4·7 cfu/m^3) by Lindemann *et al.* (1982), and within the range of some of the volumetric trappings (1, 6, 12 propagules/150 l) reported by Davies & Isaac (1958).

Our results, together with evidence that conidia of *V. albo-atrum* are not able to give rise to systemic infections through penetration of uninjured leaf and stem tissues (Jiménez Díaz & Millar, 1986), suggest that airborne propagules of *V. albo-atrum* do not play an important role in the epidemiology of VW of alfalfa. The abundant sporulation by the pathogen on necrotic tissues throughout the crop growing season may facilitate some dispersal of *V. albo-atrum* by insects (Huang *et al.*, 1983; Harper & Huang, 1984; Huang *et al.*, 1986). However, the observations that contiguous fields often have widely disparate disease incidence (e.g. 0 and >50%) (R. M. Jiménez Díaz & R .L. Millar, unpublished), that infected plants occur with highest incidence near the field entrance or oriented in the direction of harvesting (Isaac, 1957; Isaac & Lloyd, 1959; Roberts & Large, 1963; R. L. Millar, unpublished), and that pathogen propagules are readily recovered from the mower bar (R. L. Millar, unpublished), suggest that inoculum carried on the cutter bar is the most important means of dispersal of *V. albo-atrum* within and between fields (Heale *et al.*, 1979).

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