Survival of *Verticillium albo-atrum* in Alfalfa Tissue Buried in Manure or Fed to Sheep

H. C. HUANG, Plant Pathologist, and R. HIRONAKA, Animal Nutritionist, Agriculture Canada Research Station, Lethbridge, Alberta, Canada T1J 4B1, and R. J. HOWARD, Plant Pathologist, Alberta Horticultural Research Center, Brooks, Alberta, Canada T0J 0J0

ABSTRACT

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A study was carried out to determine the survival of *Verticillium albo-atrum* in diseased alfalfa tissue when buried in manure or fed to sheep. Alfalfa stems naturally infected with *V. albo-atrum* were buried in indoor cow manure packs and in an outdoor manure pile. Stems were recovered at intervals over 6 wk and examined for viable *V. albo-atrum* in the tissue. The rate of survival of *V. albo-atrum* in the stems buried 10, 30, or 60 cm deep for 1 wk was 0–26%, but it was 54–90% in the stems buried near the surface of the manure pile. In the outdoor experiment, *V. albo-atrum* was viable in 93% of the stems near the surface of the manure pile after 6 wk. When alfalfa hay infected with *V. albo-atrum* was fed to sheep, the pathogen was present in feces collected within 2 days. The maximum number of *Verticillium* propagules in sheep feces collected each day was 19 and 29 per dung ball for the experiments in 1982 and 1983, respectively. *V. albo-atrum* did not persist in the digestive tract and was absent in feces collected two or more days after the animals were returned to a diet free of the pathogen.

Verticillium wilt, caused by *Verticillium albo-atrum* Reinke & Berthold, has long been considered an important disease of alfalfa (*Medicago sativa* L.) in Europe (15). The disease has recently been found in the United States (5) and Canada (1,16). It has become a serious threat in British Columbia, Alberta, and Ontario, Canada, and in many northern states of the United States, especially those in the Pacific Northwest (4). The disease can drastically reduce yield (1,8), quality of the hay (8), and duration of an alfalfa stand (4). Therefore, in addition to affecting alfalfa hay and seed production, Verticillium wilt may also have an impact on the beef and dairy industry, the alfalfa dehydration industry, and the alfalfa leafcutter bee industry.

The pathogen overwinters as dark, thick-walled hyphae in infected alfalfa plants (6). Under cool, moist conditions, numerous spores are produced on the diseased tissue. The pathogen can spread by direct contact between diseased and healthy roots, or it can spread with running water, wind, footwear, harvest equipment, alfalfa hay, alfalfa seed (14), and insects (7,10–12).

Information remains unavailable on the fate of the pathogen in infected alfalfa tissue buried in stored manure or passed through the digestive systems of ruminant animals. The objectives of this study were to determine the survival of the Verticillium wilt pathogen in alfalfa tissue either buried in manure or fed to sheep.

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MATERIALS AND METHODS

Survival of *V. albo-atrum* in infected alfalfa stems buried in manure. Alfalfa stems with symptoms of Verticillium wilt on the leaves were collected from a field near Lethbridge, Alberta, and were air-dried at room temperature. The tip and base of each stem were excised, surface-sterilized in 70% ethanol for 2 min, and placed on V-8 juice agar medium to verify *V. albo-atrum* infection. The remaining portions of stems found infected were cut into segments 8 cm long and sealed in individual compartments of nylon mesh bags (27 cm long × 23 cm wide) with 20 segments per bag. Stems collected in July 1982 were used for the two indoor experiments (A and B), and those collected in July 1983, for the outdoor experiment (C).

For each indoor experiment, four plastic garbage cans (44 cm in diameter × 64 cm high) were filled with cow manure and placed in a calf barn with limited heat. Wood shavings were used to fill the spaces around and between the cans to reduce heat loss and stabilize temperatures. The nylon mesh bags containing diseased alfalfa stem segments were buried in each can at three depths: near the surface (about 1–3 cm) and at 10 and 30 cm. Four bags from each depth were recovered to determine the survival of *V. albo-atrum* in the stem segments 1 and 3 wk after burial in experiment A and 1, 3, and 5 wk after burial in experiment B. The temperature at each depth was recorded daily with a 2176A Digital Thermometer (John Fluke Manufacturing Co. Inc., Everett, WA) with a copper/constantan thermocouple (Omega Engineering, Stamford, CT). At the end of the 3- or 5-wk period, percent moisture of the manure at each depth was determined by drying samples at 100 C for 24 hr. The pH of the manure in experiment B was determined at the end of the 5-wk period.

For the outdoor experiment, 64 mesh bags containing diseased stems were buried in fresh cow manure in an outdoor pile 20 m long × 10 m wide × 3 m high in July 1983. The bags were buried at four depths: near the surface and at 10, 30, and 60 cm. Four bags from each depth were recovered 1, 2, 4, and 6 wk after burial. The temperature at each depth was recorded daily. At the end of the 6-wk period, pH and percent moisture of the manure at each depth were determined.

To assess survival of *V. albo-atrum* in the buried stems, each stem segment was air-dried and cut into pieces, which were plated without surface-sterilization on the selective medium (2) in petri plates. Plates were incubated at room temperature for 2 wk and checked daily under a stereomicroscope for growth of *V. albo-atrum*. The *Verticillium*-like colonies derived from buried stems were transferred to V-8 juice agar and incubated at room temperature for 1 wk, then colony morphologies were compared with those of known *V. albo-atrum* cultures from alfalfa.

Survival of *V. albo-atrum* passed through digestive system of sheep. Sheep were used in 1982 and 1983 as a model to study survival of *V. albo-atrum* in ruminants. Sheep were used in preference to cattle to reduce the amount of diseased alfalfa hay required for the experiments. Alfalfa plants with Verticillium wilt symptoms were harvested from the field, then air-dried and used as infected diet. In 1982, one sheep in a metabolism crate was fed alfalfa as cubed hay for 4 days, infected hay for 5 days, then cubed hay for 6 days. Feces (dung balls) from the animal were collected daily, air-dried, and stored in paper bags at −10 C until assayed. To examine for *V. albo-atrum*, 50 dung balls from each date were weighed, mashed, and suspended in 120 ml of sterile distilled water. The suspension was filtered through a single

layer of cheesecloth, and an aliquot was flooded onto selective media in petri plates, 1 ml per plate and 12 plates per sampling date. The plates were incubated at room temperature for 2 wk and examined under a stereomicroscope for colonies of *V. albo-atrum* and other contaminants.

In 1983, two sheep were placed in metabolism crates and fed an all-barley grain diet for 1 day, *V. albo-atrum*-infected alfalfa hay for 2 days, then barley grain for 7 days. Feces from each animal were collected daily, air-dried, and stored in paper bags at −10 C until assayed.

During the initial 5 days of feces collection, 20 petri plates containing the selective medium were used each day to check for air contamination of *V. albo-atrum* in the sheep-feeding area. Sixteen plates were left open for 24 hr in various locations: three above each sheep, two by the feces collection chute of each sheep, four on the counter of the feeding room, and two in an adjoining room where the feces were being air-dried. The remaining four plates were waved in the sheep-feeding area for 1 min.

Ninety dung balls from each sampling date were used to determine the presence of *V. albo-atrum*. They were divided into three subsamples. The 30 dung balls in each subsample were weighed, mashed, and suspended in 60 ml of sterile distilled water. The suspension was filtered through a single layer of cheesecloth, diluted 1:9, v/v (suspension/water), and an aliquot was flooded onto selective media in petri plates at a rate of 1 ml/plate, six plates per subsample. The plates were incubated at room temperature for 2 wk and examined for colonies of *V. albo-atrum* by the technique described previously.

Pathogenicity of *V. albo-atrum* on alfalfa. Eight- to 12-wk-old alfalfa plants (cultivar Anchor) were used to assess the pathogenicity of the cultures of *V. albo-atrum* isolated from alfalfa stems buried in manure or from sheep dung balls. The plants were grown in compartmentalized trays that allowed them to be removed individually. The roots were washed free of soil, and the lower quarter of the root system was clipped off before inoculation. Four- to 6-wk-old cultures on plates of Czapek agar (Difco) were flooded with 5 ml of sterile water and rubbed on the surface with a blunt glass rod to produce a spore suspension. The clipped roots were immersed in the undiluted suspension for about 30 sec, then the plants were transplanted into individual small pots of moistened soil mix. After 4–6 wk of growth in the greenhouse, stems of plants showing wilt symptoms were excised and cut into 18-mm segments. The segments were surface-sterilized for 3 min in 70% ethanol, rinsed once in sterile water, and plated on Czapek agar. After 7–10 days of incubation at 20 C, the plated stem pieces were examined microscopically for *V. albo-atrum*.

RESULTS

Survival of *V. albo-atrum* in infected alfalfa stems buried in manure. In both the indoor and outdoor experiments, *V. albo-atrum* in the infected alfalfa stems survived well when buried near the surface of the manure pile, but survival was very poor at 10 cm or deeper. Many stems buried 10 cm or deeper for 1 wk or longer showed signs of decomposition and became fragile and porous, whereas stems buried near the surface remained intact and firm. After 1 wk of burial, the percentages of stem segments with *V. albo-atrum* from the three depths (near the surface and at 10 and 30 cm) were 90, 26, and 16% in experiment A; 54, 0, and 0% in experiment B; and 84, 4, and 0% in experiment C, respectively (Table 1). No *V. albo-atrum* was detected in the stems buried for 2 wk or longer at 10 cm or deeper. Although survival of *V. albo-atrum* in stems buried near the surface of the manure pile was drastically reduced after 3 or 5 wk in the two indoor experiments, this phenomenon did not occur in the outdoor manure experiment, where *V. albo-atrum* remained viable in 93% of the stems after 6 wk of burial.

Stems buried in a manure pile were often colonized by nematodes and/or other microorganisms including fungi, bacteria, and actinomyces. Microbial contamination was heavy on stems buried at 10 cm or near the surface of a manure pile. Contamination was found on stems buried at 30 cm in the two indoor experiments (A and B) but was not found on the stems buried at the same depth or deeper in the outdoor experiment (C).

Temperature varied with depth in the manure piles. Results of the indoor experiment showed that the temperature of the manure in garbage cans rose rapidly at all three depths (Fig. 1). It peaked within 2 days and then declined during the rest of the testing period. Maximum temperatures were 38, >50, and 41 C at depths near the surface, at 10 cm, and at 30 cm, respectively. The

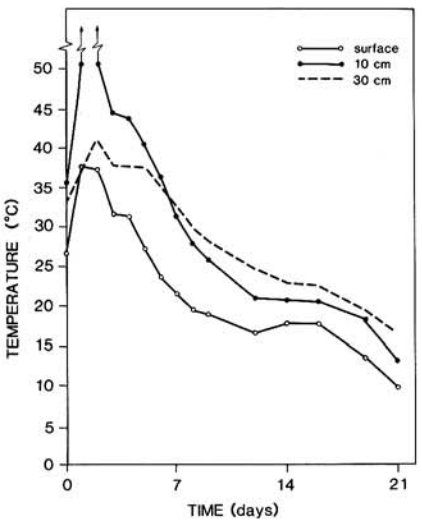

Fig. 1. Temperature changes at various depths of the cow manure stored indoors (1982).

Table 1. Percent viable *Verticillium albo-atrum* recovered from infected alfalfa stem segments buried in cow manure

Experiment[a]	Weeks of burial	Depth of burial (cm) 1–3	10	30	60
A (indoor)	1	90	26	16	···
	3	65	0	0	···
B (indoor)	1	54	0	0	···
	3	36	0	0	···
	5	13	0	0	···
C (outdoor)	1	84	4	0	0
	2	95	0	0	0
	4	89	0	0	0
	6	93	0	0	0

[a]Experiments were begun: A = September 1982, B = February 1983, and C = July 1983.

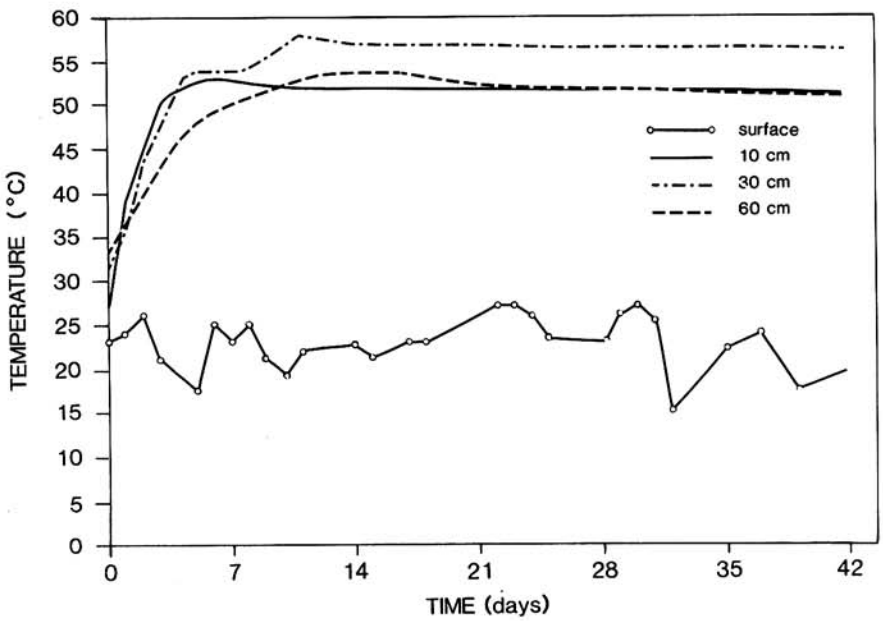

Fig. 2. Temperature changes at various depths of the cow manure pile stored outdoors (1983).

duration of temperature higher than 30 C was more than 7 days in the manure buried at 10 and 30 cm but only 4 days at depths near the surface. Results of the outdoor experiment showed that the temperature in the manure pile rose to >50 C at depths of 10, 30, and 60 cm within 1 wk (Fig. 2). The temperature at the 30-cm depth peaked at 58 C on day 11. The temperature for all three depths remained high, only dropping 2–3 degrees (C) throughout the 6-wk burial period. However, temperatures at the surface of the manure pile were lower than those at the three depths and ranged from 15 to 27 C during the 6-wk period.

The moisture content in the manure ranged from 30.9 to 36.9% near the surface but from 52.4 to 80.3% at 10 cm or deeper (Table 2). The pH of the manure decreased with depth, from 8.95 near the surface to 7.73 at 30 cm in the indoor experiment and from 8.06 near the surface to 7.46 at 60 cm in the outdoor experiment (Table 3).

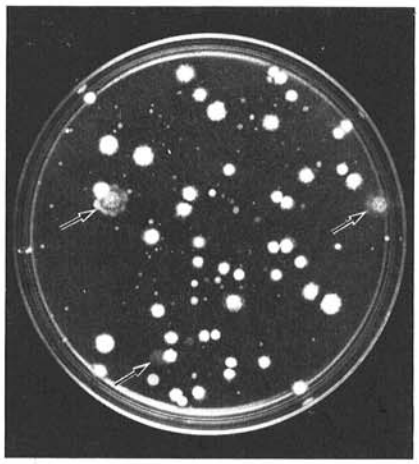

Fig. 3. Colonies of *Verticillium albo-atrum* (arrows) and other fungal contaminants in sheep dung balls collected 1 day after the animal was fed diseased alfalfa hay (incubated on selected medium at room temperature for 2 wk).

Table 2. Percent moisture of the cow manure sampled at various depths

Experiment[a]	Depth (cm)			
	Surface	10	30	60
A (indoor)	31.5	52.4	74.4	···
B (indoor)	30.9	79.3	80.3	···
C (outdoor)	36.9	72.7	76.0	77.7

[a]Experiments were begun: A = September 1982, B = February 1983, C = July 1983.

Table 3. The pH of cow manure sampled at various depths

Experiment[a]	Depth (cm)			
	Surface	10	30	60
B (indoor)	8.95	8.34	7.73	···
C (outdoor)	8.06	8.86	8.69	7.46

[a]Experiment B = manure stored indoors in cans, February 1983; experiment C = outdoor manure pile, July 1983.

Survival of *V. albo-atrum* passed through digestive system of sheep. Propagules of *V. albo-atrum* in sheep dung balls were readily detected with the selective medium in petri plates (Fig. 3). When examined under a stereomicroscope, each *V. albo-atrum* colony was seen to contain numerous verticillate conidiophores bearing spore droplets on the whorled branches. Certain types of unidentified fungi varying in colony color and size were found in sheep feces but were distinct from colonies of *V. albo-atrum* in colony appearance (Fig. 3).

In the 1982 trial, *V. albo-atrum* was found in the feces collected during days 5–9 when the animal was fed the diseased alfalfa hay (Table 4). The pathogen was also detected in samples collected during the 3 days before (days 2–4) and 1 day after (day 10) the feeding of diseased hay, when the sheep was fed only alfalfa cubes. A remnant of *V. albo-atrum* was also detected on day 14, 5 days after changing from diseased hay to alfalfa cubes. The number of *V. albo-atrum* propagules in each sample ranged from 0.2 to 19 per dung ball or from 3 to 185 per gram of air-dried fecal pellets (Table 4). In the 1983 trial, *V. albo-atrum* was found only in feces collected during the first 2 days (days 4 and 5) and the first day (day 4) after feeding diseased alfalfa hay to animals A and B, respectively (Table 5). No *V. albo-atrum* was found on any other dates. The numbers of propagules per dung ball were 29 and 6 for days 4 and 5 in sheep A and 9 for day 4 in sheep B.

Results of the 1983 trial showed that *V. albo-atrum* was present in the feeding room and the feces-drying area. The average number of *V. albo-atrum* colonies per plate, from air-borne contaminants trapped during the initial 5 days of feces collection, varied from 0 to 15.7 on days 2 and 3 when the diseased hay was fed (Table 6). The highest counts were in plates placed above both sheep. Before feeding the diseased hay, *V. albo-atrum* was found at low levels (0.3–0.5 colonies per plate) on those exposed above sheep A, by the feces chute of sheep A, and on the counter. On days 4 and 5, when barley grain was fed, no *V. albo-atrum* was found in any locations, except for 0.3 colonies per plate on those above sheep B on day 4 (Table 6).

Pathogenicity of *V. albo-atrum* on

Table 4. Relation between feeding sheep with diseased alfalfa hay and the presence of *Verticillium albo-atrum* in fecal pellets (1982)

Feed	Feeding schedule (day)	Propagules of *V. albo-atrum* in feces[a]	
		No./g feces	No./dung ball
Alfalfa cubes	1	0	0
	2	147	13
	3	124	12
	4	123	12
Diseased alfalfa hay	5	185	19
	6	118	13
	7	93	10
	8	100	8
	9	177	11
Alfalfa cubes	10	27	2
	11	0	0
	12	0	0
	13	0	0
	14	3	0.2
	15	0	0

[a]Based on examination of 50 dung balls from each day.

Table 5. Relation between feeding sheep with diseased alfalfa hay and the presence of *Verticillium albo-atrum* in fecal pellets (1983)

Feed	Feeding schedule (day)	Propagules of *V. albo-atrum* in feces[a]			
		Sheep A		Sheep B	
		No./g feces	No./ dung ball	No./g feces	No./ dung ball
Barley grain	1	0	0	0	0
Diseased alfalfa hay	2	0	0	0	0
	3	0	0	0	0
Barley grain	4	283	29	44	9
	5	54	6	0	0
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0

[a]Based on average of three subsamples from each day at 30 dung balls per subsample.

alfalfa. *V. albo-atrum* isolated from the stems buried in manure and the sheep dung balls was morphologially identical to that isolated from the field-collected stems. All 70 representative isolates tested (31 from manure and 39 from sheep dung balls) were pathogenic to alfalfa cultivar Anchor. Results of reisolation indicated that the pathogen was present in the stems of the root-inoculated plants.

DISCUSSION

V. albo-atrum in infected alfalfa stems was completely destroyed when buried in a manure pile at 10 cm or deeper for 2 wk or longer but survived in more than 90% of the stems buried near the surface. When diseased alfalfa was fed to animals, numerous infected stem segments and leaves remained undigested by the animal and were incorporated into manure. This may pose a great danger of introducing *V. albo-atrum* into alfalfa fields through manure application in the field. To avoid this danger, manure should be collected and piled up for at least 1 wk before it is spread in the field. Because *V. albo-atrum* survives well near the surface of the manure pile, the top 10-cm layer should be gathered and buried for another week before it is spread in the field.

The indoor experiments showed that the temperature of a manure pile rose to >35 C at all depths, but such high temperatures were maintained longer at depths of 10 and 30 cm than at the surface. Similarly, in the outdoor experiment, the temperature of manure at 10 cm and deeper rose to >50 C and remained high throughout the entire 6-wk period of testing. Christen and French (3) found that the optimum temperatures for growth of alfalfa strains of *V. albo-atrum* in culture were 20–25 C and found no growth at >33 C. Isaac (13) reported that the optimum temperatures for *V. albo-atrum* were 22–22.5 C and found no growth at 30 C. The combined effect of high temperature and high moisture in the manure pile may cause rapid decomposition of alfalfa stems, resulting in poor survival of *V. albo-atrum* in infected tissues. On the other hand, *V. albo-atrum* survived well near the surface of the outdoor manure pile, maybe because the manure was relatively dry and the temperature never exceeded 27 C during the 6-wk period of testing.

Optimum pH is 8–8.6 for growth of *V. albo-atrum* (13). Although pH values varied with manure depths, the ranges of variation from 7.73 to 8.95 in the indoor experiment and from 7.46 to 8.86 in the outdoor experiment were still close to the reported optimum pH. Therefore, pH is probably not an important factor affecting survival of the pathogen in a manure pile.

In the sheep-feeding study in 1982, *V. albo-atrum* was found on dung balls when the animals were fed diseased alfalfa hay. Nevertheless, the experiment failed to prove that the pathogen survived passage through the digestive tracts because it did not preclude the possibility of contamination of feces by airborne spores released from old, infected alfalfa plants (11). Moreover, the use of cubed alfalfa hay as the regular diet for the sheep might have interfered with the experiment, because the cubing process is unable to destroy *V. albo-atrum* in alfalfa (9). In 1983, however, the sheep-feeding experiment was modified by using barley grain as the regular diet and monitoring for spores of *V. albo-atrum* in the air of the feeding area. Results showed that *V. albo-atrum* survived passage through the digestive tracts of sheep because there was only trace contamination of the pathogen in the air on days 4 and 5, when the maximum number of *V. albo-atrum* propagules was detected on feces (Tables 5 and 6). The number of airborne spores of *V. albo-atrum* in the feeding area was high only on days 2 and 3, when diseased alfalfa hay was fed. *V. albo-atrum* produces dark, thick-walled, resting mycelia in diseased tissues, serving as overwintering propagules (6). The duration of 1–2 days that infected hay remains in the digestive tracts of ruminant animals appears to be insufficient to destroy the resting mycelia in infected tissue before they are discarded with feces.

The finding that *V. albo-atrum* survives passage through animal digestive systems poses a serious problem if an alfalfa crop is used for grazing. The animal may eat diseased plants and thus spread the pathogen within the field, or to other fields if moved, via contaminated feces. Therefore, we recommend that an alfalfa crop with incidence of Verticillium wilt should not be used for grazing, because the feces may become an important source of inoculum for the development of new infection loci.

The sheep-feeding study showed that all the diseased tissue passes through the digestive system within 2 days (Table 5). It may be possible to prevent the transfer of *V. albo-atrum* from the feeding area to the field by feeding the animals for at least 3 days on a diet such as barley grain before releasing them into an alfalfa field to graze.

Table 6. *Verticillium albo-atrum* collected in sheep-feeding area (1983)

Sampling location	Propagules of *V. albo-atrum* per plate				
	Day 1 (barley)	Day 2 (diseased hay)	Day 3 (diseased hay)	Day 4 (barley)	Day 5 (barley)
Room 1 (feeding room)					
Above sheep A	0.3	15.7	2.7	0.0	0
Feces chute A	0.5	2.5	1.0	0.0	0
Above sheep B	0.0	5.0	3.7	0.3	0
Feces chute B	0.0	2.5	0.5	0.0	0
Counter by sheep	0.3	1.8	2.3	0.0	0
Feeding room (waved 1 min)	0.0	0.3	1.8	0.0	0
Room 2 (feces drying area)	0.0	0.5	0.0	0.0	0

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