Demonstration of *Verticillium albo-atrum* Within Alfalfa Seed

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ABSTRACT

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Plant to seed transfer of *Verticillium albo-atrum* was demonstrated in both naturally and artificially infected alfalfa plants. *Verticillium* was isolated from seed of greenhouse-inoculated alfalfa cultivars: susceptible Apalachee, WQS1 (a Washington line), and resistant Vertus. Colony development from surface-sterilized seed indicated that *V. albo-atrum* was present within the seed. Scanning electron microscopy revealed that mycelia were located within and between osteosclerid cells of the outer integument of the seed coat. Internal seedborne infection was as high as 25% in the small seed fraction (0.91–1.6 mg) of seed produced on plants inoculated within 2 wk following pollination. Infection occurred less frequently in larger seeds.

Verticillium wilt of alfalfa caused by *Verticillium albo-atrum* Reinke and Berth. (dark mycelia type) is widespread in alfalfa fields in the Pacific Northwest (3) and could spread to other parts of North America (3). *V. albo-atrum* has been isolated from alfalfa pedicels and pods, debris carried with seed, and seed (6,7,10), but whether this fungus can be internally seedborne is unknown. Host age at inoculation determines whether internal seed infection caused by *Verticillium* is detected in greenhouse experiments with lupine (9). Because much of the alfalfa seed produced in the Pacific Northwest is planted in other parts of the country, the present investigation was conducted to determine whether *V. albo-atrum* could be internally seedborne in alfalfa.

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MATERIALS AND METHODS

Production of infected seed. Cultivars Apalachee, WQS1 (a Washington line), and Vertus were planted and grown under greenhouse conditions. Vertus is moderately resistant to the alfalfa strain of *V. albo-atrum* but Apalachee and WQS1 are susceptible. A spore suspension (3) of a *V. albo-atrum* isolate with known

pathogenicity to alfalfa was used to inoculate alfalfa stems below each raceme of florets, which had previously been pollinated. Inoculations were made on different stems during the first, second, third, fourth, or fifth weeks after pollination. Seeds from each of the five inoculation periods from each alfalfa were graded into six size classes with the aid of a South Dakota seed blower (Table 1) (11). Forty racemes of seeds were also obtained from plants in a seed production field plot. Plants moderately infected at the time of flowering were chosen.

Detection of *V. albo-atrum* in seeds. Harvested seeds were surface sterilized in a solution of 70% ethanol, 5.25% sodium hypochlorite (Clorox), 9:1 v/v, and a 1:1,000 final dilution of

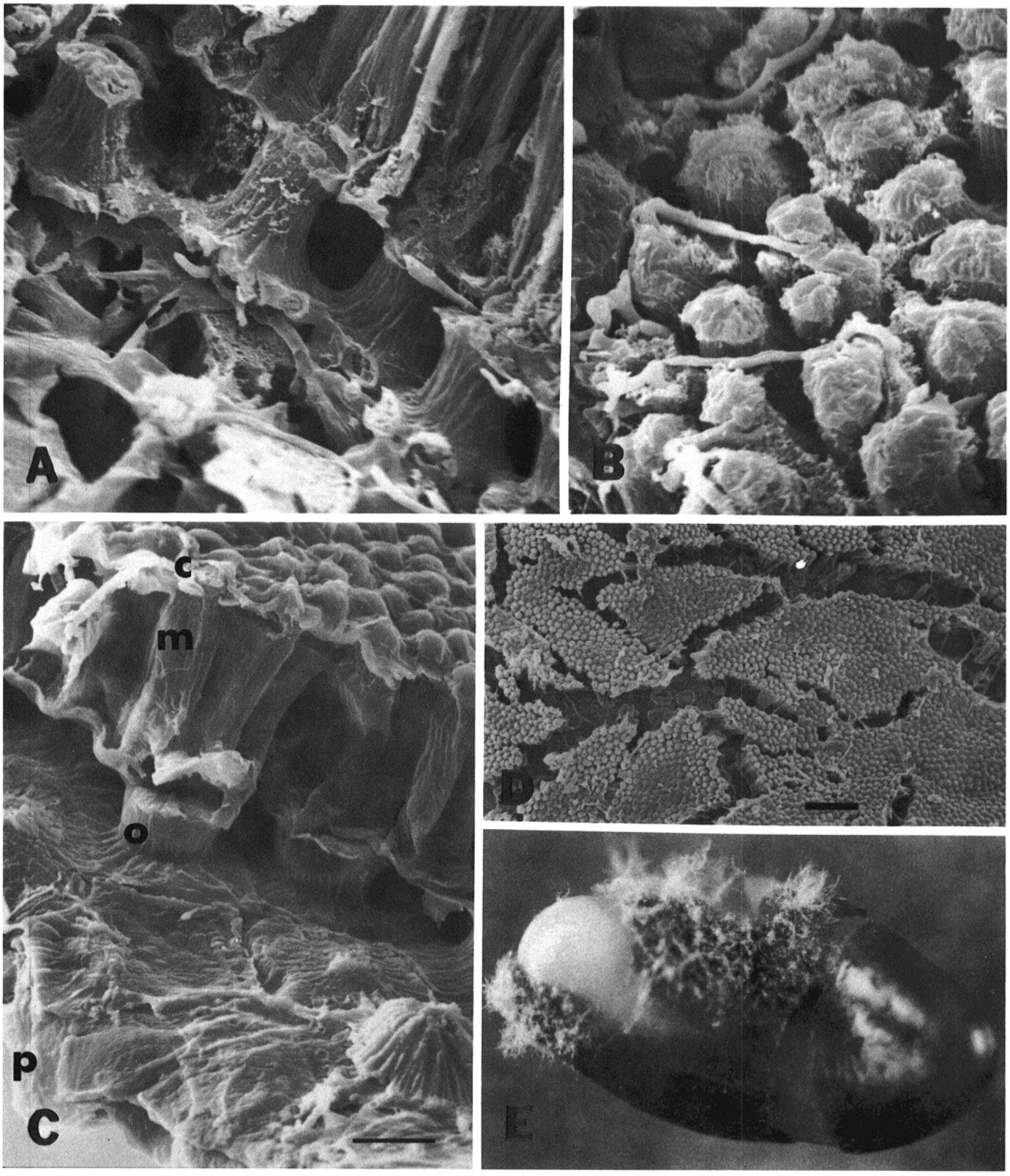

Fig. 1. Scanning electron and light micrographs of *Verticillium albo-atrum* infected and control seeds. **A,** Freeze fracture of seed revealing mycelia within osteosclerid cells (arrow) of seed coat (×3,210) and **B,** between osteosclerid cells (×3,092). **C,** Anatomy of outer seed coat: cuticle (c) and outer integument, which consists of malpighian (palisade) cells (m), osteosclerid cells (o), and parenchyma cells (p) (bar = 7 μm). **D,** Surface view of imbibed severely infected seed (bar = 40 μm). **E,** Conidiophore and mycelial growth from seed.

TABLE 1. Recovery of *Verticillium albo-atrum* from seed produced on greenhouse-inoculated alfalfa plants

Blower setting	Seed weight (mg/seed)	No. of infected seed per no. of tested seed for which inoculation followed pollination by			Mean percent infection
		1 wk	2 wk	3 wk	
≤50	≤0.90	19/198	39/221	10/55	15 a[y]
51–60	0.91–1.60	3/28	34/131	10/91	15 ab
61–70	1.61–1.80	8/110	15/202	1/126	6 abc
71–80	1.81–2.25	9/227	14/227	3/212	4 bc
81–90	2.26–2.35	1/187	3/194	1/203	1 cd
91–100	2.36–2.67	0/107	0/157	0/136	0 d
Mean percent infection		6 ab[z]	10 a	5 b	

[y]Means within a column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range test.
[z]Means within a row followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range test.

Tergitol, NPX (Sigma Chemical Co., St. Louis, MO 63147), a wetting agent. Preliminary studies had shown that a 45-sec sterilization time was adequate to eliminate all *V. albo-atrum* on the seed surface. After disinfestation, seeds were rinsed twice in sterile distilled water, blotted dry on sterile filter paper, and placed on moistened, sterile filter paper for 1 day to imbibe water. They then were transferred to a selective agar medium (2) that also contained 60 μg of 2,4-dichlorophenoxyacetic acid and 1 mg of naphthaleneacetic acid per liter (4). Numbers of seeds from which *V. albo-atrum* colonies grew were recorded after incubation for 1 wk at 20–25 C. Data expressed as percentage of infected seeds were transformed to arc sine (sqr (x)) and analyzed according to a three-way analysis. No significant differences were found between cultivars, so the data were combined in Table 1 within each size and time class.

Seed preparation for scanning electron microscopy. Seeds from greenhouse-inoculated stems were surface sterilized, and part of the seed coat was chipped off each seed and plated directly on the medium. Surface-sterilized seeds from the field were plated directly on the medium. Based on this test, eight of the greenhouse seeds and one field seed that contained the *V. albo-atrum* organism were chosen for further examination under scanning electron microscopy (SEM). The eight seeds were produced on stems inoculated within 3 wk after pollination and represented five of the six size classes. The one seed from the field collection was prepared for SEM as soon as conidiophores emerged from the seed coat. Greenhouse-produced seeds were allowed to imbibe water for 2–5 hr and were then fixed 1 hr in osmium tetroxide vapors, dissected to expose different parts of the seed, vacuum desiccated over drierite or fixed in 2% gluteraldehyde and 1% osmium tetroxide in 0.07 M phosphate buffer (pH 6.8 [1]), ligand bound (8), dehydrated with ethanol, freeze fractured with a razor blade after transfer through ethanol and liquid nitrogen (5), and critical-point dried with carbon dioxide. Specimens were gold coated and examined in a Novascan 30 SEM.

RESULTS

Mycelia of *V. albo-atrum* were present on the surface of some discolored seeds before they were surface sterilized. *V. albo-atrum* was recovered from surface-sterilized seeds produced in the greenhouse and in the field. Greatest frequency of recovery was from greenhouse-grown seeds produced on stems inoculated 1–3 wk after pollination (Table 1). The fungus was rarely found in seeds produced on stems inoculated more than 3 wk after pollination, with only 2 of 489 and 0 of 230 seeds infected from stems inoculated in the fourth and fifth weeks, respectively. Infection occurred most often in seeds that were smaller than 1.8 mg. Seeds larger than 2.35 mg were free of *Verticillium* (Table 1). *V. albo-atrum* grew from three of the field-grown seeds. SEM examinations of the eight greenhouse-infected seeds and the one field-infected seed showed that hyphae of *V. albo-atrum* were found in (Fig. 1A) and between (Fig. 1B) osteosclerid cells of the seed coat (Fig. 1C). Growth of *V. albo-atrum* originated from the vicinity of the hilum and/or "cotyledon end" (Fig. 1E) or from random areas of seeds. The extent of colonization was indicated by emergence of conidiophores from the outer and inner surfaces of the seed coat. Underlying mycelia were revealed in a severely infected seed where the malpighian cells separated during imbibition of water (Fig. 1D). Surface-sterilized embryos removed from 10 infected seeds were free of *Verticillium*.

DISCUSSION

This research shows that internal infection by *V. albo-atrum* in alfalfa seed can be induced by artificial inoculation of the stems and that it also occurs in the field. The results indicate that the transfer or growth of the fungus into the seed appears to be restricted to the seed coat. Reports of seedborne *V. albo-atrum* are not well documented and are in some cases contradictory (9).

A low percentage of the alfalfa seeds produced in *Verticillium*-infected commercial seed fields probably are infected because moderately diseased plants provide the opportunity for transfer of the organism from stem to raceme and are still vigorous enough to mature the seed. The average weight (2.03 mg) of seeds in the five upper size classes in this test corresponds with the generally accepted average of 2 mg (11) in commercially cleaned seed. If the average 4.4% infection in this seed could be accepted as maximum because of the controlled conditions, then commercially grown seed from infected fields would be expected to have a very low percent of infected seed that would not be disinfected by currently recommended seed treatments. The occurrence of infected seeds in sizes up to 2.35 mg indicates that removing all infected seeds during the seed screening process would be impractical. Development and use of an effective heat or systemic fungicide seed treatment to disinfect alfalfa seed produced in areas known to be infested with *Verticillium* (alfalfa strain) could limit the spread of the disease.

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