# Mechanisms of Seed Contamination by *Verticillium albo-atrum* in Alfalfa

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## ABSTRACT

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Infection of alfalfa tissue and contamination of seeds by *Verticillium albo-atrum* were investigated in a growth room with root-inoculated plants of cultivars Anchor, Vela, and Vernal, and progeny of Beaver × Lutèce. The frequency of contamination of seeds by *V. albo-atrum* ranged from 0% in Vela to 5% in Anchor following hand pollination of plants showing disease symptoms. Although the pathogen was present throughout the stems, it occurred only sporadically in peduncles, pedicels, pods, and seeds. When the stigmas of healthy plants of Vernal were surface inoculated with spores of *V. albo-atrum* at the time of flower-tripping, infection occurred readily and resulted in a discoloration of the stigma and upper style. The fungus appeared to be latent in the style during all stages of seed development. It was not detected in seeds from pods with infected styles. Under humid conditions, however, the fungus in the remnant style tissue of a mature seed pod was able to colonize the pod and seed coat.

*Additional key words*: *Medicago sativa*.

Wilt of alfalfa (*Medicago sativa* L.) by *Verticillium albo-atrum* Reinke & Berthold has been reported in British Columbia, Alberta, and Ontario in Canada (1) and in Washington, Oregon (7), Idaho (6), Montana (19), Wyoming (9), Wisconsin (8), and Pennsylvania (17) in the United States. Numerous reports indicate that the pathogen can be carried in commercial seed lots. Some workers (5,14,20) observed that the pathogen was carried with seeds on pieces of plant debris such as pods or pedicels (14) or on the surface of the seeds (5,20). Such externally seedborne *V. albo-atrum* was readily eliminated from the seed by surface sterilization. Others (4,15,20) proved the existence of internally seedborne *V. albo-atrum* in commercial seed lots by isolating the fungus from the seed coat. Christen (4) found a high frequency of internal infection in small seeds (0.91–1.6 mg); she observed the fungal mycelium within and between osteosclerid cells of the outer integument of the seed coat.

Although the association of *V. albo-atrum* with alfalfa seeds can be external or internal, how they become infected remains unclear. This paper provides a detailed account of possible mechanisms involved in the infection by *V. albo-atrum* of alfalfa seeds produced from root-inoculated and flower-inoculated plants.

## MATERIALS AND METHODS

**Seedborne *V. albo-atrum* from root-inoculated plants.** Twelve-week-old plants of cultivars Vela, Vernal, and Anchor, and progeny from the cross Beaver × Lutèce were root inoculated with spore suspensions of *V. albo-atrum* collected from 1-wk-old cultures grown on V-8 juice agar. Plants that developed typical symptoms of Verticillium wilt but still produced flowers were selected to produce seed. When these plants were about 10 mo old, they were randomly hand crossed in a growth room by using a wood toothpick to trip the flowers. Pods were harvested only from stems with leaves showing distinct disease symptoms at the time of flower tripping.

To determine the distribution of *V. albo-atrum* in the tissue, the stem and racemes were surface-sterilized in 70% ethanol for 90 sec and then air-dried on paper towels. Three stem segments from each plant, collected at the base, near the first raceme, and at the top of each stem and also the peduncles, pedicels, pods, and seeds produced on the stem, were plated on a selective medium (3) in petri dishes. They were incubated at room temperature for 1 wk and examined for *V. albo-atrum* by using the technique described previously (11).

**Seedborne *V. albo-atrum* from flower-inoculated plants.** Healthy 6-mo-old flowering plants of cultivar Vernal were selected for this test in the greenhouse. The plants were simultaneously pollinated and inoculated with a mixture of alfalfa pollen and spores of *V. albo-atrum* collected on the broad end of a flat, wood toothpick. This mixture was obtained by scraping the toothpick lightly across the surface of a 1-wk-old fungal culture grown on V-8 juice agar in a petri dish. The contaminated toothpick was then used to trip several alfalfa flowers. Fresh spores and pollen were collected on the toothpick at frequent intervals as the plants were pollinated. Flowers tripped with a toothpick bearing only pollen served as uninoculated controls.

Mature pods were harvested from plants inoculated by this method as well as from the control plants and examined for presence of the pathogen in the tissue. The pods from each raceme were surface-sterilized in 70% ethanol for 90 sec and air-dried on paper towels. The stigma and style, the pod tissue near the style, and the seeds were plated on V-8 juice agar in petri dishes, incubated at room temperature for 1 wk, and examined microscopically for *V. albo-atrum*.

For light microscopy, green 2- to 3-wk-old pods and mature 5- to 7-wk-old pods were used. They were surface-sterilized in 70% ethanol for 60 sec and placed in plastic petri dishes containing sterile distilled water. Each pod was supported by a small piece of wax to prevent the stigma, style, and most of the pod tissue from contacting the water during incubation. The pods were examined daily for 1 wk and the growth and sporulation of *V. albo-atrum* from infected tissue were recorded by time-lapse photography (11).

For scanning and transmission electron microscopy (SEM and TEM), the stigmas and styles were collected from 4-, 12-, and 21-day-old pods originating from florets that had been tripped with a mixture of alfalfa pollen and spores of *V. albo-atrum* as described above. Healthy stigmas and styles were used as controls. The material was prefixed in 4% gluteraldehyde in 0.2 M sodium cacodylate buffer, pH 7.0, at 4 C for 16 hr; postfixed in 2% osmium tetroxide in cacodylate buffer; and dehydrated in an ethanol series. Specimens for SEM were then critical-point dried, mounted, sputter coated with gold, examined, and photographed on a

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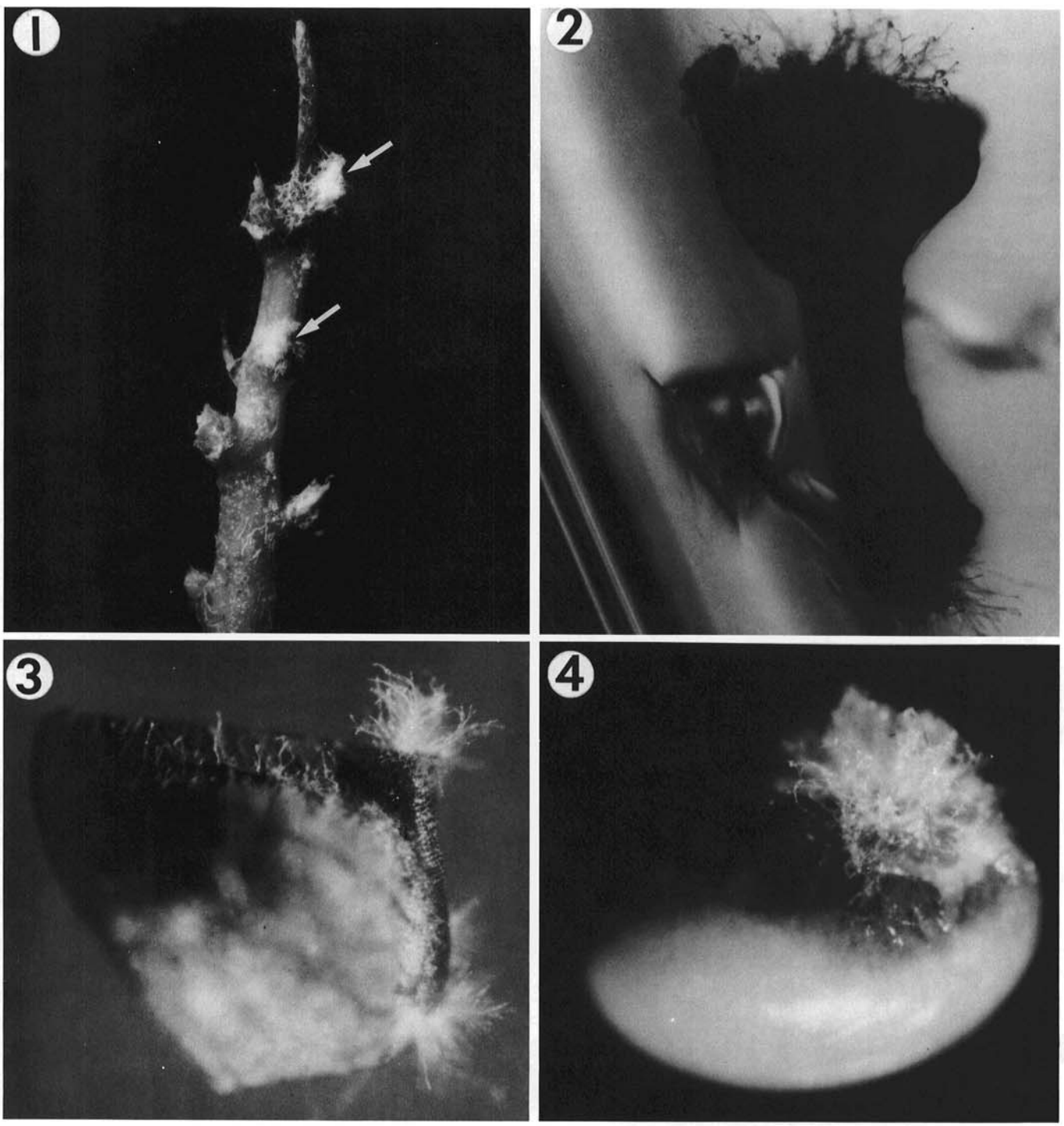
Hitachi S500 scanning electron microscope. Specimens for TEM were infiltrated and embedded in Spurr's medium (22), sectioned, stained with lead citrate and uranyl acetate, examined, and photographed on a Hitachi H-500 transmission electron microscope.

## RESULTS

**Seedborne *V. albo-atrum* from root-inoculated plants.** Data were obtained from a total of 31 stems from plants of Anchor, Vela, Vernal, and the hybrid Beaver × Lutèce. Isolations from leaflets with wedge-shaped lesions at the time of pollination showed that *V. albo-atrum* was present in all 31 stems (Table 1). The fungus was detected at the base, near the first raceme, and at the top of each stem when examined after the seeds were harvested. Some diseased stems produced seeds on only two or three racemes and others on as many as 10 racemes. The number of pods that formed on each raceme and the number of seeds in each pod also varied widely.

*V. albo-atrum* was detected in the tissue of peduncles (Fig. 1), pedicels (Fig. 2), pods (Fig. 3), and seeds (Fig. 4), but its distribution in these tissues was sporadic and it was not detected in all racemes. Within each cultivar or hybrid, the percentage of




**Figs. 1–4.** *Verticillium albo-atrum* developing from peduncle, pedicel, pod, and a seed produced on diseased alfalfa stems after incubation on the selective medium for 3 days. **1,** An infected peduncle with mycelia growing from two of the pedicel bases (arrows) (×13). **2,** An infected pedicel with mycelia growing from both ends (×34.6). **3,** The basal portion of an infected pod with mycelia growing from both ends of the main rib on the ventral side (×30.4). **4,** An infected seed with mycelium growing from a brown patch on seed coat near the hilum (×28.3).

infected tissue was invariably highest in peduncles and lowest in seeds (Table 1). No infected pod tissue or contaminated seed was found in the three stems of Beaver × Lutèce that were examined, and no contaminated seeds were found in three stems of Vela.

In 80 racemes harvested from 17 stems of five plants of Anchor, the frequency of *V. albo-atrum* in peduncles, pedicels, pods, and seeds was 84, 36, 18, and 5%, respectively. The scattered distribution of the fungus in the pedicels, pods, and seeds borne on two infected peduncles of Anchor is shown in Fig. 5. In one raceme (R1), only the peduncle was infected. In the second raceme (R2), *V. albo-atrum* was detected not only in the peduncle but also in 11 of 19 pedicels, in two pods, and in six seeds. The 11 infected pedicels were randomly distributed on the peduncle and were not arranged in an acropetal or basipetal order. Three seeds contaminated by *V. albo-atrum* were found within each of two pods, and in each case, there were other uncontaminated seeds within the same pods. In some pods borne on other racemes of Anchor, all seeds within the pod were contaminated, but in others only one seed was contaminated. The seeds contaminated by *V. albo-atrum* often germinated and developed into young seedlings without visible lesions on cotyledons and hypocotyls during the 1 wk of incubation on the selective medium.

Mycelia of *V. albo-atrum* were derived from infected peduncles, pedicels, pods, and seeds within 2–4 days after placement on the selective medium. They further developed into colonies with numerous whorl-branched conidiophores bearing small spore droplets. The fungus in the infected tissue appeared to be confined to the vascular tissue as mycelia were always first derived from the pod attachment point on the peduncle (Fig. 1), the ends of the pedicel (Fig. 2), and the ends of the main rib on the ventral side of the pod (Fig. 3). When the seeds contaminated by *V. albo-atrum* were incubated on the medium, the fungus was derived from the discolored patch of the seed coat near the hilum (Fig. 4). Localized distribution of *V. albo-atrum* was obvious, particularly in racemes harvested before the infected tissue turned brown and dried. Many seeds produced from the diseased alfalfa plants grown in the growth room were small but remained free of seedborne *V. albo-atrum*.

**Seedborne *V. albo-atrum* from flower-inoculated plants.** When spores of *V. albo-atrum* were placed on the stigmas of individual florets of the cultivar Vernal, browning of the stigma and the distal portion of the style occurred within about 4 days and remained evident as the pod matured (Fig. 6). The rest of the style also showed a slight discoloration. In contrast, no discoloration of stigma and style was observed on the flowers tripped with toothpicks carrying only pollen (Fig. 6). *V. albo-atrum* was isolated from the discolored stigmas and styles but not from the white ones from control plants.

When examined microscopically, the surface of the stigma in each floret appeared to be covered with a layer of amorphous mucilaginous substances. Spores of *V. albo-atrum* placed on the flower by hand tripping were often submerged in this layer. Most of the spores had germinated within 1–4 days and produced mycelia either on the surface (Fig. 7) or within the layer (Fig. 8). Some germ tubes from spores penetrated the mucilaginous layer (Fig. 9) and subsequently attacked the tissue beneath this layer; this resulted in the destruction and disintegration of parenchymal cells in the stigma (Figs. 10 and 11). The hyphae in the stigmatic tissue further spread down to the discolored tissue of the style. They grew in the parenchymal tissue of the style both intracellularly (Figs. 12 and 13) and intercellularly. The fungus ramified in the host cell by hyphal branching (Fig. 12). It penetrated the cell wall directly by a small hyphal peg and appeared constricted at the point of penetration (Fig. 13). Destruction of stigmatic tissue (Figs. 10 and 11) and cell walls of style (Fig. 14), presumably by enzymatic activity, was also evident. In addition to the tissue of stigma and style, *V. albo-atrum* invaded pollen grains as hyphae of the pathogen were frequently found in the pollen grain and/or the pollen tube.

The remnant stigma and style remained attached to the pod even after the pod and seeds had matured, about 5–7 wk after

TABLE 1. Frequency of *Verticillium albo-atrum* in peduncles, pedicels, pods, and seeds from root-inoculated alfalfa plants

| Cultivar or cross | Infected stems (no.) | Infection (%) in: | | | |
|---|---|---|---|---|---|
| | | Peduncles | Pedicels | Pods | Seeds |
| Beaver × Lutèce | 3 | 56(9)[a] | 33(49) | 0(49) | 0(293) |
| Vela | 3 | 44(10) | 13(55) | 4(55) | 0(384) |
| Anchor | 17 | 84(80) | 36(488) | 18(450) | 5(1526) |
| Vernal | 8 | 85(26) | 38(186) | 13(186) | 0.8(666) |

[a]The figures in parentheses are the actual numbers of plant parts examined.

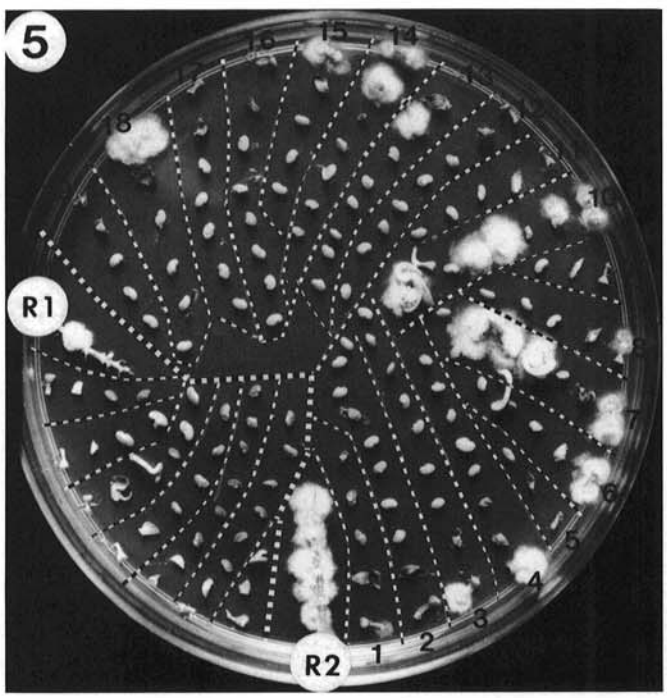



**Fig. 5.** Alfalfa seeds of cultivar Anchor produced on two peduncles, R1 and R2, infected by *Verticillium albo-atrum*. Each dotted line compartment contains a pedicel (near the edge of the petri dish), a piece of pod tissue (next to the pedicel), and seeds from one pod. Note that raceme R2 had 19 pods and the fungus was detected in 11 pedicels, two pods, and six seeds. Three of the infected seeds in pods 7 and 10 germinated during 1 wk of incubation on the selective medium.

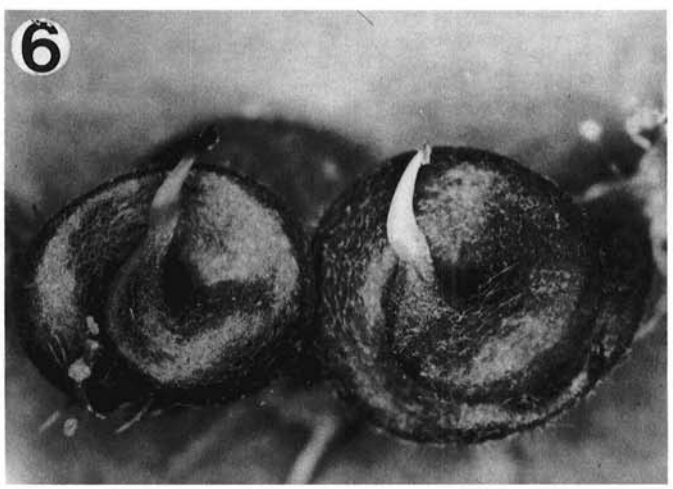
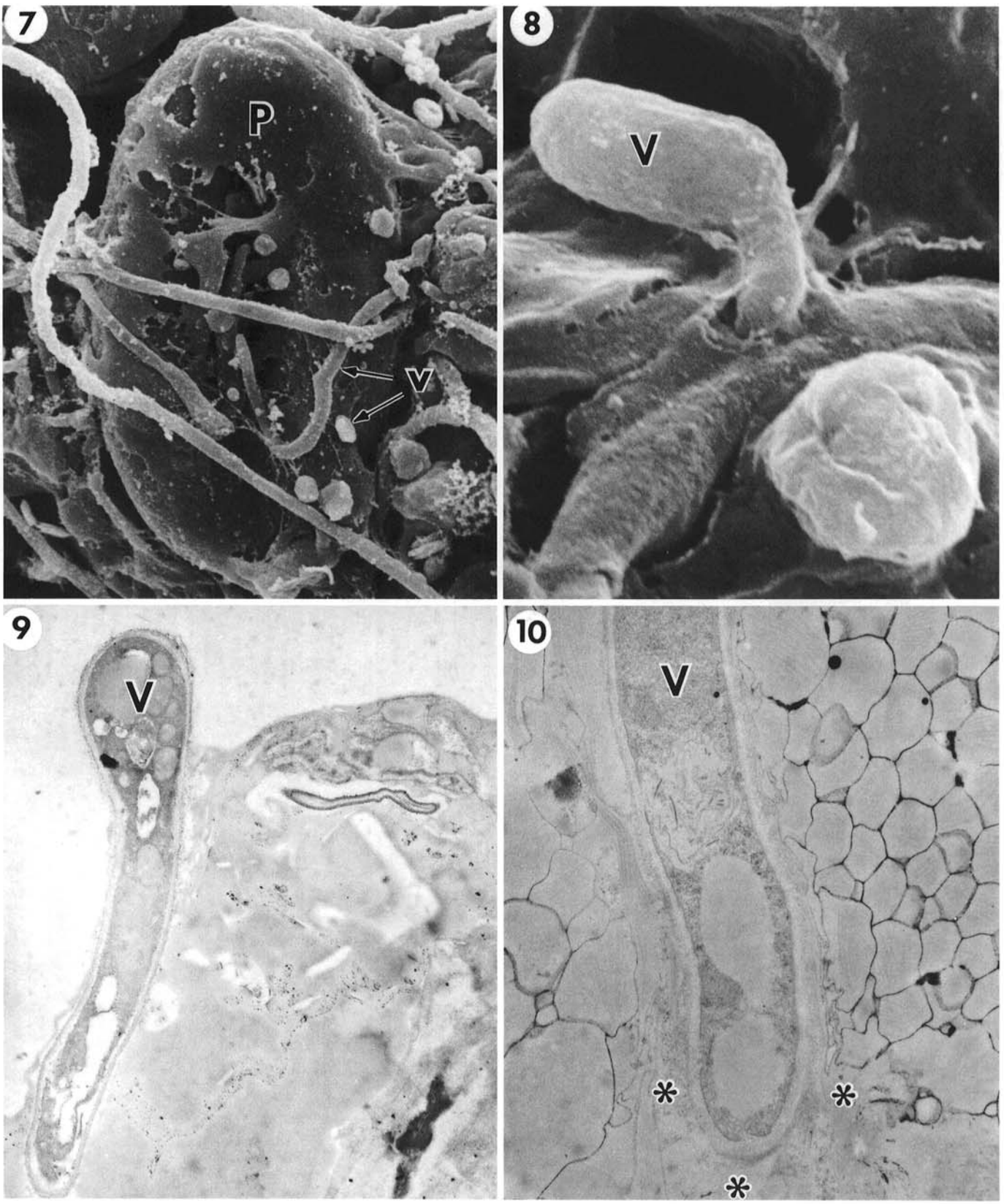
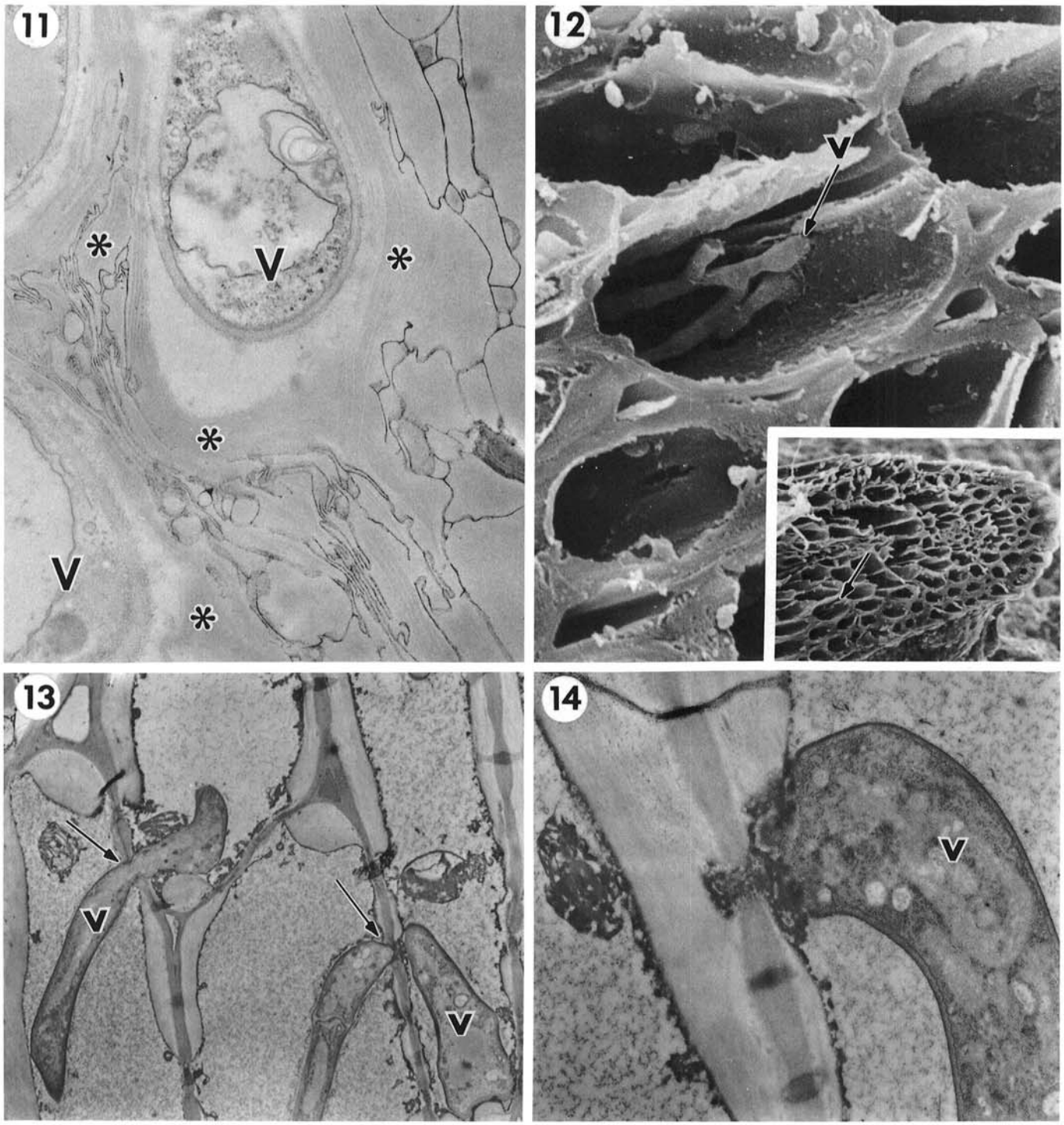



**Fig. 6.** Three-week-old pods developing from florets tripped with wooden toothpicks smeared with either a mixture of pollen and spores of *Verticillium albo-atrum* (left) or pollen alone (right). Note the discoloration of stigma and tip of the style of the pod at left (×7.4).




**Figs. 7–10.** Electron microscopy of infection of alfalfa flower stigmas by *Verticillium albo-atrum* (V). **7,** Scanning electron micrograph (SEM) showing alfalfa pollen (P) and ungerminated spores and hyphae from germinated spores of *V. albo-atrum* adhering to the sticky surface of the stigma (×4,000). **8,** SEM of a germinated spore of V showing the germ tube penetrating into the gelatinous substance on the stigma surface (×13,700). **9,** Transmission electron micrograph (TEM) of a sectioned stigma showing the germ tube of a spore of V extending through the mucilaginous surface layer (×16,500). **10,** TEM of a sectioned stigma showing a hypha of V penetrating the thin-walled cells of the parenchymal tissue of the stigma. Note signs of lysis (*) and the destruction of cells (×19,800).

pollination. Although *V. albo-atrum* was able to colonize the stigma and the tip of the style on a young pod, it was unable to spread further to the pod and seeds during seed development and maturation. Of the 804 seeds from 247 harvested pods of cultivar Vernal, *V. albo-atrum* was isolated from 86% of the stigmas and styles of florets tripped with a mixture of spores and pollen, but it was not isolated from any seeds and pods attached to the infected stigmas and styles. Furthermore, the pathogen was latent in the stigma and style, and it was still viable long after the seeds had matured and the pod had dried (Figs. 15–18). All 173 pods harvested from the control plants were healthy and contained no *V. albo-atrum* in the stigmas, styles, pods, or seeds. When a maturing or matured pod was incubated in a moist chamber, *V. albo-atrum* in the infected stigma and style became active and produced spores on the infected tissues (Figs. 15–17). It soon ramified throughout the entire style and spread further onto the pod which resulted in heavy sporulation on the pod and even the seed coat (Fig. 18). The hypocotyls of young seedlings appeared to be quite resistant to infection by *V. albo-atrum* (Fig. 18). In contrast to the matured pods, *V. albo-atrum* grew slowly in the stigma and style of young,



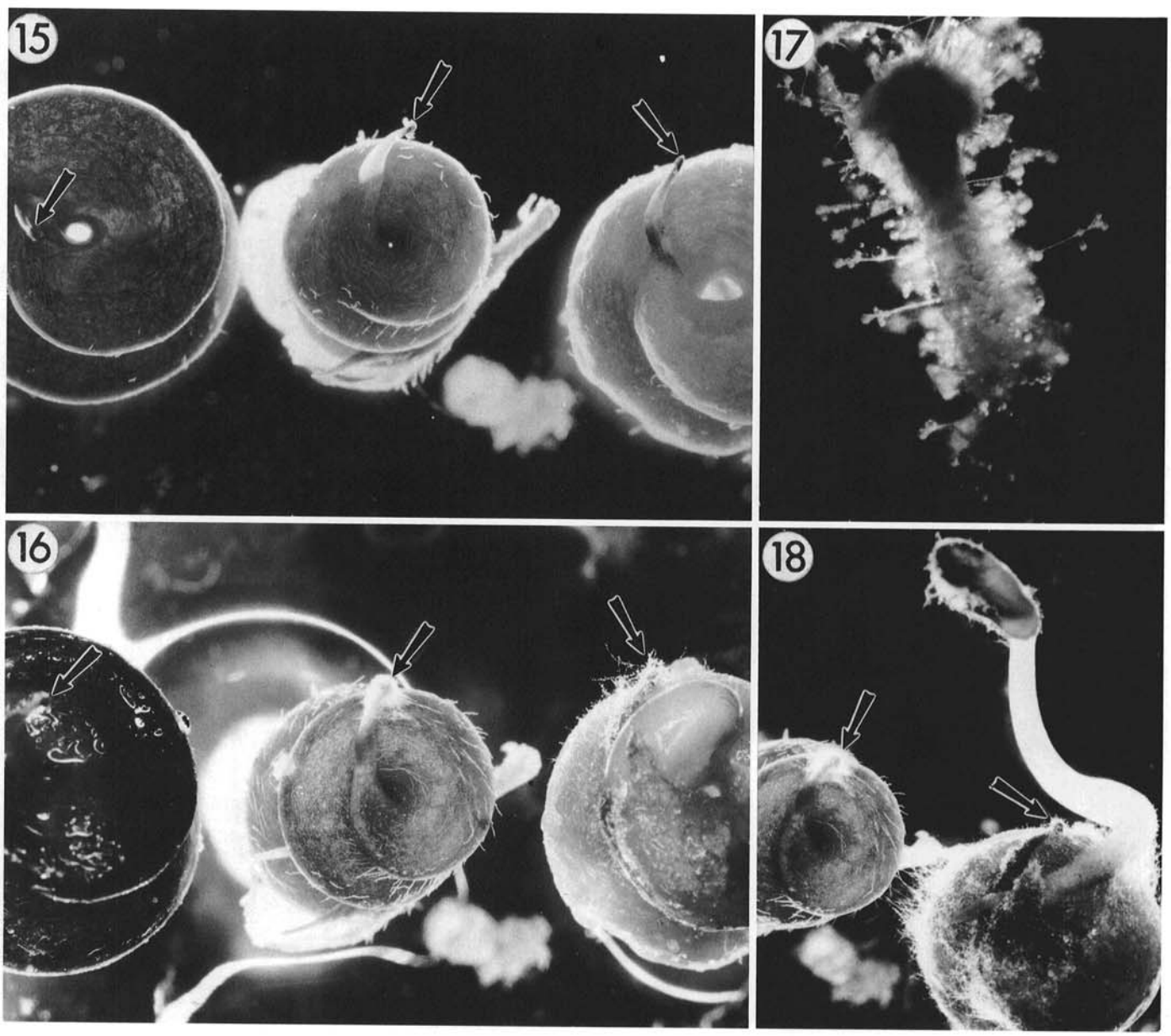

**Figs. 11–14.** Transmission electron micrograph (TEM) showing the advanced stage of infection of stigma by *Verticillium albo-atrum* (V). Note signs of lysis (*) and the disorganization of parenchymal tissue (×21,500). **12,** Scanning electron micrograph, a sectional view through the mid-region of the style (insert) showing hyphae of V (arrows) in a parenchyma cell of the style (×2,830) (inset, ×265). **13 and 14,** TEM micrographs showing hyphae of V penetrating (arrows) thick-walled parenchymatous cells of the style. Note the constricted hyphae at the point of penetration. (**13,** ×5,230; **14,** ×14,750)

green pods and sporulated sparsely when they were incubated under the same moist conditions (Figs. 15, 16, and 18).

## DISCUSSION

Results of this study confirm previous findings that *V. albo-atrum* can be carried with alfalfa seeds produced from infected plants (4), that the frequency of seed contamination by *V. albo-atrum* is low (4,20), and that the fungus is mainly confined to the seed coat (4,15). Pennypacker and Leath (18) reported that the fungus is confined to the xylem vessels of stems, where it sporulates readily. They observed a discontinuous distribution pattern of *V. albo-atrum* in recently infected plants and concluded that the colonization of new host tissue is due to conidial movement in the xylem vessels. The findings of this study, that the pathogen is systemically distributed in the stem but sporadically distributed in the peduncles, pedicels, and seeds suggest that the fungus is confined to only certain xylem vessels of the stems prior to reaching the advanced stage of infection. Bishop and Cooper (2) studied the infection of tomato by *V. albo-atrum* and found that vessel-to-vessel colonization was uncommon but that it occurred occasionally by penetration of intertracheary pit membranes. Thus, it is possible for *V. albo-atrum* to colonize the alfalfa seed coat only when the vessel elements connecting the hilum of the seed and the main rib of the pod are infected with the pathogen. Similarly, the presence of *V. albo-atrum* in the main rib of a seed pod (Fig. 3) does not necessarily guarantee infestation of all the seeds in that pod unless the xylem vessels in the main rib connecting to the seeds are all colonized by the pathogen.

Silow (21) investigated the dissemination of spores of *Botrytis anthophila* Bond. on blossoms of red clover by bees and found that the spores on stigmas of healthy plants germinated and that the hyphae rapidly traversed the stylar canal causing a systemic infection of the plant. In contrast, we found that *V. albo-atrum* can colonize the stigma and style readily, but it did not attack the pod and ovules during seed development. The period of latent infection




**Figs. 15–18.** Three seed pods developed from flower-inoculated plants, showing growth and sporulation of the fungus after incubation for **15,** 1 hr; **16,** 3 days; and **17–18,** 7 days in a moist chamber. Left to right: a 5-wk-old brown mature pod; a 2-wk-old green young pod; and a 5-wk-old yellow maturing pod (magnifications: Figs. 15 and 16, ×7.1; Fig. 17, ×57.5; and Fig. 18, ×5.7). Note the profuse growth of the pathogen from the stigma and style of the matured and maturing pods (arrows in Figs. 16 and 18) but only limited growth of the pathogen from the stigma and style of the young green pod after incubation for 7 days (arrow in Fig. 18, left).

appears to be long, and the fungus will colonize the pod only under moist conditions and only after the pod changes color with maturity (Figs. 15, 16, and 18). This suggests that the immature, green pods are resistant to invasion, and that the fungus remains latent in the stigma and the tip of the style. Mechanisms involved in the latent infection and the failure of the fungus to invade ovules and immature, green pods are unknown and warrant further investigation.

Recent studies have shown that numerous spores of *V. albo-atrum* are produced on old infected tissue of alfalfa plants grown under irrigation (13). Insects, including pests, predators (10,13), and pollinators (12), are effective dispersal agents for spores produced in the field. Pests such as the pea aphid (13) and alfalfa weevil (10) are effective vectors capable of transmitting the spores to healthy alfalfa and inducing Verticillium wilt. Leafcutter bees released into a diseased field may carry spores of *V. albo-atrum* on their bodies (H. C. Huang and K. W. Richards, *unpublished*) and thus serve as an effective vector for the seedborne *V. albo-atrum* because of the potential for transporting spores of the pathogen to alfalfa blossoms via flower tripping.

Although both internal (4) and external (5) seedborne *V. albo-atrum* occurred in naturally infected alfalfa plants, the externally seedborne pathogen appeared to be more common in commercial seed lots (5,20). Since the infected plant debris such as pieces of pods, pedicels (14), and styles may be an important source of inoculum for infection of alfalfa seeds, efforts should be made to protect the seeds from contamination by such debris under field and storage conditions. Recommended practices include: avoidance of irrigation after the seeds have matured to prevent the pathogen from producing conidia on infected pedicels, pods, and styles; removal of infected crop debris by seed cleaning; and application of a fungicide to seeds harvested from diseased plants (15,16).

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