Dispersal of *Verticillium albo-atrum* in the Xylem of Alfalfa

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ABSTRACT

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Alfalfa plants (*Medicago sativa* L. 'Saranac') were inoculated with spores of *Verticillium albo-atrum* and grown in slant-board culture in a growth chamber. Plants were examined histologically at 4-day intervals until permanent leaf wilt and defoliation occurred. Eight days after root-dip inoculation, the fungus had colonized the entire stem. *V. albo-atrum* was found only in the xylem vessel elements, where it sporulated freely. Germinated spores were frequently observed in these vessels. In serial isolations from newly regrown stems from field-grown plants, *V. albo-atrum* was noncontinuous in the host stem during early stages of colonization. Based on histological evidence and isolation data, we concluded that internal sporulation was the mechanism facilitating rapid colonization of the host by *V. albo-atrum*.

Additional key words: lucerne

Wilt of alfalfa (*Medicago sativa* L.) caused by *Verticillium albo-atrum* Reinke & Berth. has been present in Europe since 1918 (4). It was first reported in the United States in the Pacific Northwest in 1976 (4). Since then, Verticillium wilt has been found in Wyoming (6), Montana (6), Wisconsin (5), and as far east as Pennsylvania (11) and Nova Scotia (18). Verticillium wilt is a major factor limiting productivity of alfalfa fields to 3 yr or less (3).

V. albo-atrum causes several distinct foliar symptoms in alfalfa. V-shaped chlorosis of the leaflet tips, narrowing and longitudinal rolling of the apical leaflets, and persistence of an upright, green stem following leaf wilt and defoliation are characteristic of this disease (3,4,8). Stunting is also frequently associated with infected plants in the field (3,12).

Christen and Peaden (3) reported isolation of *V. albo-atrum* from the entire stem of an inoculated alfalfa plant 6 days after inoculation. In view of the slow mycelial growth of the pathogen on media (70 mm in 20 days on prune-lactose yeast agar [2]), we were interested in determining how *V. albo-atrum* spread so rapidly through an alfalfa plant. A portion of this work was reported previously (14).

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MATERIALS AND METHODS

To determine how *V. albo-atrum* moves through the alfalfa plant, we evaluated inoculated plants and naturally infected plants histologically and by serial isolation of the pathogen.

Growth chamber study. Four-week-old seedlings of Verticillium wilt-susceptible alfalfa (cultivar Saranac) were inoculated with an alfalfa isolate of *V. albo-atrum* by soaking the root system in a conidial suspension (about 700,000 spores per milliliter) for 10 min. Roots of control plants were soaked in sterile water. Roots were clipped to 10 cm in length and aerial growth to two stems per plant before inoculation. Plants were then placed on slant-boards according to the technique of Kendall and Leath (10) and maintained in a growth chamber. One-half strength Hoagland's solution (7) was used to water the plants. The growth chamber maintained a 14-hr light/ 10-hr dark photoperiod with a light intensity of 500 mEcm^{-2}s^{-1}. Temperatures were 25 ± 1 and 15 ± 1 C during the light and dark periods, respectively.

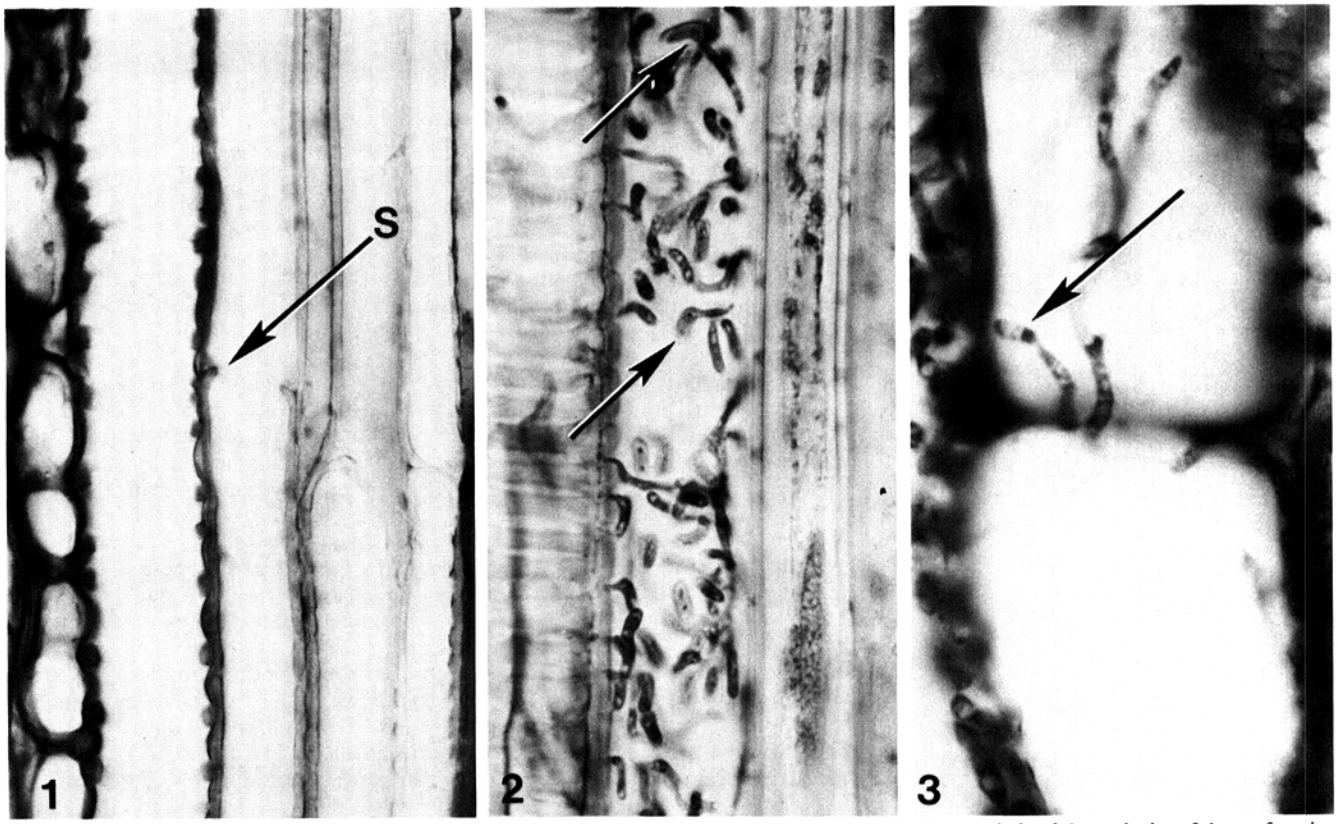
Plants were sampled at 4-day intervals beginning on the fourth day after inoculation and continuing until permanent leaf wilt and defoliation occurred. Three plants, consisting of the taproot and one complete stem per plant, were harvested during each sampling period. A control plant was sampled weekly. Sampled plants were traced on lined paper, surface-sterilized for 5 min in 10% bleach (5.25% sodium hypochlorite), and divided aseptically into numbered, serial 5-mm sections. Odd-numbered sections were fixed in Rawlin's (16) formalin-aceto-alcohol solution no. 1 (FAA). Even-numbered sections were cultured on water agar and examined microscopically after 7 days for the presence of *V. albo-atrum*. The location of sections positive for *V. albo-atrum* was recorded on the plant diagram.

Specimens in FAA were dehydrated using a tertiary butyl alcohol schedule and embedded in paraplast (9). Longitudinal and transverse sections were cut at 10 μm on a rotary microtome, mounted on chemically cleaned slides with Haupt's adhesive, and stained with Johansen's quadruple stain (9).

Field study. Twenty-five alfalfa plants showing apparent symptoms of Verticillium wilt were identified in a 4-yr-old, unirrigated stand of alfalfa at the Rock Springs Research Center near State College, PA. One stem was collected from each plant and the plant was tagged for future reference. The remaining stems were cut to a 10-cm stubble approximating standard hay-crop practice. Harvested stems were surface-sterilized as described previously and cultured on water agar for the presence of *Verticillium* sp.

Stem regrowth from plants that were positive for *Verticillium* sp. was reexamined at weekly intervals for 3 wk during August 1982. The first week, sampling involved all the plants and consisted of collecting two stems from each plant. The stems were traced, surface-sterilized, and serially cultured on water agar as described earlier. The location of sections positive for *Verticillium* sp. was recorded on the appropriate diagram, thus producing a map of the colonized stem. Sections were also selected and fixed in FAA for histological examination. The same procedure was followed during the remaining two sampling periods, with one exception. The plants were divided into two groups, one sampled after 2 wk of stem regrowth and the other after 3 wk of regrowth.

Pathogenicity test. V. dahliae and *V. albo-atrum* are the only species of *Verticillium* that are pathogenic on alfalfa (8). To determine the species of *Verticillium* isolated from the naturally infected plants in the field study, we



Figs. 1–3. (1) Longitudinal section through a healthy alfalfa stem. The simple-perforation plate (S) is characteristic of the majority of the perforation plates in the xylem vessel elements of alfalfa (×588). **(2 and 3)** Longitudinal sections through alfalfa stems infected with *V. albo-atrum*. **(2)** Numerous conidia in a xylem vessel element. Note that many of the conidia are germinating (arrows) (×806). **(3)** Sporulation of *V. albo-atrum* within the xylem vessel. Note the conidiophore and attached conidium (arrow) (×930).

conducted a pathogencity test on a representative isolate from each plant. Pathogenicity on alfalfa and the absence of microsclerotia in culture were considered evidence that the isolate was *V. albo-atrum*.

Seedlings of alfalfa (cultivar Saranac-AR) were grown in Terra-Lite Reddi-Earth Peat-Lite mix (W. R. Grace & Co., Cambridge, MA 02140) in trays (34.5 × 12 × 6 cm) in a greenhouse under natural light supplemented with metal-halide lamps.

Five-week-old plants were removed from the flats and the roots were cleaned and clipped to provide ready access to the vascular system. The stems were also trimmed to maintain a reasonable root:shoot ratio. The *Verticillium* isolates tested were grown on potato-dextrose agar slants from single-spore transfers. Inoculum was prepared by placing the spores from one slant into a flask containing 40 ml of sterile water. Trimmed root systems of three plants were soaked in this spore suspension for 1 hr. Roots of control plants were soaked in sterile water for 1 hr. After inoculation, three plants per isolate were potted in a 10-cm-diameter (400-cc) plastic pot containing the potting mix described before and placed on a greenhouse bench. Pots were elevated individually on upended plastic pots to prevent cross-contamination. After 3 wk, plants were evaluated for disease symptoms.

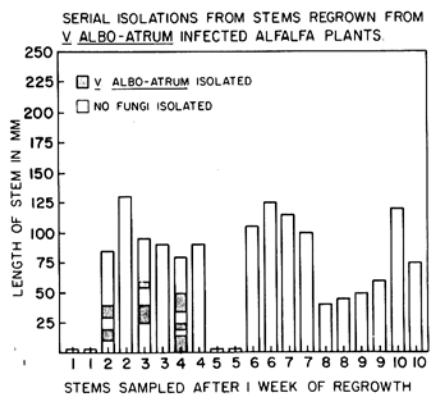


Fig. 4. Histogram of results of serial isolations for *V. albo-atrum* performed on 1-wk-old stems from 10 naturally infected alfalfa plants. Two stems were cultured from each plant. Numbers on the horizontal axis correspond to the plant from which the stems were harvested.

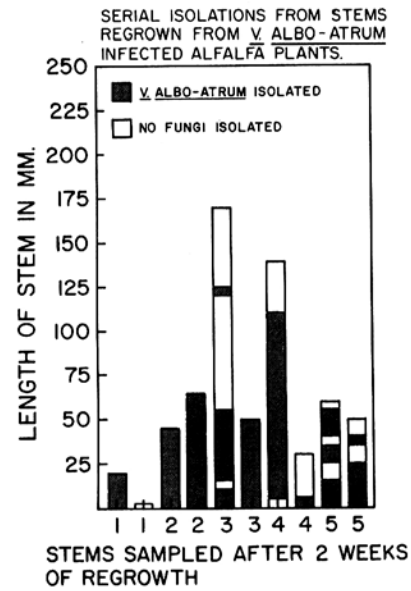


Fig. 5. Histogram of results of serial isolations for *V. albo-atrum* performed on 2-wk-old stems from five naturally infected alfalfa plants. Two stems were cultured from each plant. Numbers on the horizontal axis refer to the plant from which the stems were harvested and correspond to the numbers in Figure 4.

RESULTS

Growth chamber study. *Isolation data.* Although symptoms of wilt did not appear until 16 days after inoculation, *V. albo-atrum* was first isolated 8 days after inoculation. At this time, it was isolated from the entire aerial portion of two plants and was discontinuous in the third plant. The pattern of discontinuous isolation of the pathogen persisted in one plant during each sampling period. No fungi were isolated from the control plants.

V. albo-atrum was dispersed in the alfalfa stems to varying distances, depending on the stem length. Maximum stem length colonized was 354 mm in 16 days.

Histology. Xylem vessel elements of the root, stem, and petiole of healthy

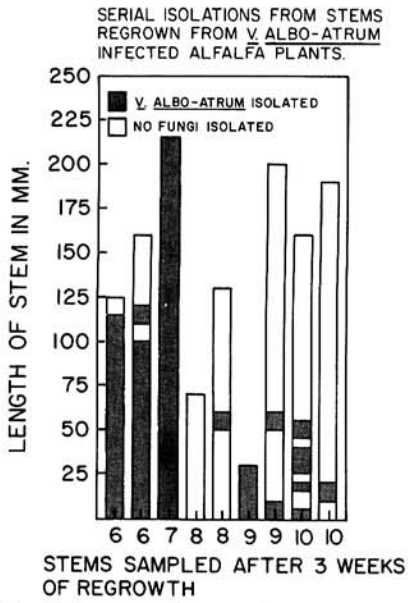


Fig. 6. Histogram of results of serial isolations for *V. albo-atrum* performed on 3-wk-old stems from five naturally infected alfalfa plants. Two stems were cultured from each plant, with the exception of plant 7. Numbers on the horizontal axis refer to the plant from which the stems were collected and correspond to the numbers in Figure 4.

alfalfa plants were quite straight and had simple-perforation plates (Fig. 1). Multiple-perforation plates were noted occasionally in metaxylem vessel elements in the stem. No potential impediments to spore translocation were noted in the xylem vessels.

Eight days after inoculation, numerous conidia and germinating conidia were observed in the xylem vessel elements of the stem (Fig. 2). Hyphae were frequently observed in conjunction with conidia, possibly indicating colonization of the xylem vessel by hyphae from the germinating conidia. Hyphae were restricted to the xylem vessels at this point in pathogenesis.

Conidiophores with conidia were found in the xylem vessels of the stem (Fig. 3). Internal sporulation, coupled with the rapidity with which *V. albo-atrum* spores germinated in culture, led us to conclude that the conidia found within the vascular system 8 days after inoculation were probably produced within the host rather than a portion of our initial inoculum.

Conidia of the pathogen were found throughout the xylem of the stem and were present in the xylem vessels of both the petioles and leaf midveins. The xylem vessels of the petioles and especially those of the leaf midveins were narrower than those of the stem and were occasionally plugged with conidia and mycelium.

Field study. *Isolation data. Verticillium* sp. was isolated from 15 of the 25 field-grown alfalfa plants showing apparent symptoms of Verticillium wilt. Ten of the positive plants were used during the remainder of the field study. Use of newly regrown stems rather than older stems allowed us to trace the pattern of colonization in these stems. Results of the serial isolations for *Verticillium* sp. conducted on 1-, 2-, and 3-wk-old stems are shown in Figures 4–6. No symptoms other than stunting were present in the stem regrowth sampled during this study.

After 1 wk of growth, only three of the 20 stems serially cultured were positive for *Verticillium* sp. (Fig. 4). In these stems, isolation of the fungus was discontinuous. The same discontinuous pattern was present in some stems during the second and third weeks of sampling (Figs. 5 and 6). Spore translocation within the host would lead to a pattern of discontinuous fungal isolations.

Plants that were positive for *Verticillium* sp. during the first sampling, along with plants that showed insufficient regrowth (less than 5 mm) at that time, were sampled after the second week of regrowth and the stems were serially cultured (Fig. 5). The remaining five plants, which were negative for *Verticillium* sp. during the first sampling, were sampled after 3 wk of regrowth (Fig. 6). Possibly as a consequence of the earlier fungal penetration, plants sampled after 2 wk of regrowth had a higher percentage (40%) of totally colonized stems than plants sampled after 3 wk of growth (22%) (Fig. 6).

In addition to the discontinuous pattern of colonization, *Verticillium* sp. penetrated naturally infected stem tissue a distance of 125 mm after 14 days (Fig. 5) and 225 mm after 21 days (Fig. 6).

Pathogencity test. All isolates of *Verticillium* sp. tested for pathogenicity on alfalfa caused symptoms typical of Verticillium wilt in the inoculated plants. None of the isolates produced microsclerotia in culture; therefore, we concluded that the isolates were *V. albo-atrum* and that the 10 plants used in the field study were infected with this species of *Verticillium*.

Histology. Conidia were found within the xylem vessel elements of field-grown, naturally infected stems from which *Verticillium* sp. was isolated in a discontinuous pattern.

DISCUSSION

Spore translocation in xylem vessels occurs in wilts of tobacco (19), cotton (15), and chrysanthemum (1) caused by *V. dahliae*; however, it has been implicated in only one wilt disease caused by *V. albo-atrum*. Sewell and Wilson (17) found that conidial movement facilitated the spread of *V. albo-atrum* in the vascular system of hops.

Panton (13), working with Verticillium wilt of alfalfa, attributed host colonization to hyphal elongation and discounted any role for conidia in the internal spread of this pathogen. Isaac (8) did not note any internal sporulation in alfalfa infected with *V. albo-atrum*, although he reported extensive external sporulation late in pathogenesis.

The concept of colonization via hyphal elongation does not explain the rapid colonization of alfalfa reported by Christen and Peaden (3) and verified by our growth chamber and field isolation data. Host colonization by means of conidial movement through the xylem of the alfalfa plant is strongly implicated by: 1) our histological evidence of internal sporulation and germination of *V. albo-atrum* in inoculated plants, 2) observations of conidia in the xylem vessel elements of naturally infected alfalfa plants, and 3) the discontinuous pattern of *V. albo-atrum* isolation from field-grown, naturally infected plants.

Spore movement within the xylem appears to be an important mechanism in pathogen spread through the host during early stages of pathogenesis. Once spores spread through the vascular system of the host and germinate, a continuous fungal presence is established in the stem. Continuous colonization may occur as early as 6 days in inoculated plants (3) and between 8 and 14 days in naturally infected plants. Isolations conducted after this period would not be a valid assay for spore translocation in alfalfa infected by *V. albo-atrum*.

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