Growth and Pathogenicity of Alfalfa Strain of *Verticillium albo-atrum*

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ABSTRACT

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Thirty-eight isolates of *Verticillium albo-atrum* from alfalfa (*Medicago sativa*) from diverse locations in the northwestern United States and western Canada (British Columbia) appeared the same on prune lactose yeast agar. Eight isolates representative of this area were alike in growth response to temperature on prune lactose yeast agar; ability to induce symptoms in alfalfa cultivars Vertus (moderately resistant to *V. albo-atrum*) and Apalachee (susceptible to *V. albo-atrum*); pathogenicity to certain cultivars in eggplant (*Solanum melongena*), cantaloupe (*Cucumis melo*), and watermelon (*Citrullus lanatus*); and not inducing symptoms in potato (*S. tuberosum*) or tomato (*Lycopersicon esculentum*). The optimum temperature for growth in media was at or near 25 C. Growth of an isolate from alfalfa of *V. albo-atrum* on osmotically adjusted media was greater at 27 and 30 C than it was on the unamended agar medium. These observations indicate that only one alfalfa strain of *V. albo-atrum* exists in the area and that it may have the potential to become more widespread in the United States than strains of *V. albo-atrum* isolated from other crops.

The alfalfa (*Medicago sativa* L.) strain (dark mycelial type) of *Verticillium albo-atrum* Reinke & Berth. was unknown in the United States before 1976 (3). It now occurs in the coastal (cool, high rainfall, low pH) and desert (warm, irrigated, neutral to alkaline) areas of Washington and Oregon, as well as in the desert areas of southwestern Idaho (2). Some 202,000 ha (500,000 acres) of alfalfa are affected. Nearly all alfalfa hay fields more than 1 yr old in the Columbia Basin of Washington are diseased. The prevalence of Verticillium wilt caused by *V. albo-atrum* increases during production, reducing alfalfa stand life from the usual 6–7 yr to 3 yr. Because Verticillium wilt of alfalfa occurs in both cool and warm areas of Washington, and Verticillium wilt caused by strains of *V. albo-atrum* in other crops occurs in cool climates (north central and northeastern United States) but not in Washington deserts, the alfalfa strain appears to have the potential to become more widespread in the United States than strains of *V. albo-atrum* from other crops.

This study was conducted to determine whether geographically diverse isolates of *V. albo-atrum* from alfalfa were the same, to determine their cultural and pathologic characteristics, and to determine whether their temperature-growth requirements were related to the occurrence of the disease in Washington. The growth response of a *V. albo-atrum* isolate to osmotic water potential was determined for comparison with that previously reported for *V. dahliae* Klebahn (8).

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MATERIALS AND METHODS

Thirty-eight isolates of *V. albo-atrum* from alfalfa were obtained from diverse areas of the Pacific Northwest and British Columbia, Canada (2), and stored on silica gel at 5 C (11). All isolates were compared for colony morphology. Eight (or fewer) of the isolates considered to be representative of the areas where they originated were used in all other experiments, as follows: isolate 40-3 from Adams County and 43-2 from Grant County of the Columbia Basin of central Washington; 51-1 from Canyon County of western Idaho; 121-2 from Stevens County of northeastern Washington; 80 A-1 from Pierce County of western Washington; 87-2 from Marion County of western Oregon; and 137-1 and 140 D-1 from the vicinities of Creston and Midway, respectively, in British Columbia, Canada.

Colony morphology on agar media. A mass isolate and two single-conidial isolates from each of the 38 stock cultures were incubated in darkness at 25 C for 20 days on prune lactose yeast agar (PLYA) (15), Difco (Difco Laboratories, Detroit, MI) potato-dextrose agar (Difco PDA), and potato-dextrose agar made from dehydrated potatoes (dehydrated PDA) (7). Colonies were examined macroscopically to compare colony morphology and production of mycelia.

Growth on agar media. A 4-mm-diameter agar plug cut from the edge of a 20-day-old culture of a given isolate growing on PLYA was placed in the center of a plastic petri dish (100 × 15 mm) that contained 20 ml of a given agar medium. Inoculated dishes were enclosed in two polyethylene bags to prevent moisture loss and incubated in constant-temperature chambers. Two measurements of colony diameter, intersecting at right angles, were taken in each of five replicate dishes 20 days after inoculation. Curves for growth in relation to temperature were determined for the eight isolates on PLYA and for one isolate on osmotically adjusted Difco prune agar (PA). The PA was prepared with Milli-Q (Millipore Corporation, Bedford, MA) deionized water and was osmotically adjusted with potassium chloride to 0.1, 0.2, 0.3, 0.6, 0.9, 1.2, and 2 concentrations. The pH of each medium was determined after solidification with a Corning model 12 pH meter and flat surface combination electrode.

The osmotic water potential of the basal agar medium was measured by a thermocouple psychrometer to be −1.1 bars, and a published formula (8) was used to calculate water potential values resulting from added potassium chloride from water activities (a_w) (14).

Growth in liquid culture. A few conidia were scraped from the surface of a culture of one isolate grown on PLYA for 20 days, placed in 50 ml of Difco Czapek Dox broth (CDB) in a 125-ml Erlenmeyer flask, and incubated at 25 C in a rotary shaker at 150 rpm for 3 days. Conidia from the culture were filtered through sterile cheesecloth, and the conidial concentration was adjusted with a hemacytometer to 4×10^6 conidia per milliliter. One milliliter of this suspension was added to 49 ml of CDB in 125-ml flasks to produce an initial inoculum concentration of 8×10^4 conidia per milliliter. Incubation was under continuous light (Westinghouse Daylight fluorescent light at approximately 2,400 lux) in a rotary shaker at 150 rpm. At 1- or 2-hr intervals, three replicate flasks were vigorously shaken twice for 4 sec, then a 1-ml sample was withdrawn from each flask and the spore concentration was determined by dilution plating. Samples were taken for 15–30 hr at 20, 22.5, and 25 C to determine the interval before conidia increased in number (lag phase) and the interval for conidia to double in number (doubling time).

The osmotic potential of the basal medium, CDB, was calculated to be −4.7 bars by adding a_w values (12,14) of the ingredients.

Disease reaction. Alfalfa plants of Vertus (moderately resistant to *V. albo-atrum*) and Apalachee (susceptible) were grown in a greenhouse in natural light (24,000–53,000 lux) at 15–21 C. Seeds were planted in flats measuring 35 × 50 × 10 cm and containing a mixture of steamed Ritzville silt-loam soil, sand, and peat (2:2:1, v/v). Four- and twelve-week-old plants were inoculated by soaking roots for 20 min in a suspension (8×10^6 conidia per milliliter) of each of eight isolates singly and of two mixtures of three isolates. The inoculated plants were transplanted into flats containing the same plant-growth medium as the flat used for seeding and were incubated at 20 ± 1 C with a 14-hr day at 8,400 lux and 40–70% relative humidity in a growth chamber. Four replicates of 50–75 plants were inoculated with each isolate. Plants were evaluated for disease incidence and severity 4–6 wk after transplanting, using a rating scale of 1–5 (1). Plants rated 1 and 2 were considered resistant (no more than one or two leaflets chlorotic), and those rated 5 were dead.

Pathogenicity to other crops. Seedlings of eggplant, *Solanum melongena* L. 'Black Beauty'; cantaloupe, *Cucumis melo* L. 'Hales Best'; watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai 'Keckley Sweet'; tomato, *Lycopersicon esculentum* Mill. 'Bonny Best' and 'Roza'; potato, *S. tuberosum* L. 'Norgold Russet' and 'Russet Burbank'; and rooted cuttings of peppermint, *Mentha piperita* L. 'Todd Mitcham' and of spearmint, *M. spicata* L. 'Native' were inoculated with isolate 40-3 or each of the eight isolates in the same way as were the alfalfa plants. Roots of tomato, potato, and mint were sometimes crushed in the inoculum to increase the likelihood of infection. Fifteen to 30 plants inoculated with each isolate were transplanted singly into 10-cm plastic or clay pots containing the soil, sand, and peat mixture and were incubated in cool or warm greenhouses with day-night temperatures of 15–21 and 21–27 C, respectively.

RESULTS

Cultural morphology. A mass isolate and two single-conidial isolates from each of 38 geographic locations were all alike and formed abundant dark mycelia on PLYA (2). Similarly with each isolate, the quantity of resting mycelia varied on replicate plates of Difco PDA or dehydrated PDA.

Growth on PLYA. Colony growth changes at incubation temperatures of 5, 10, 15, 20, 22.5, 25, 27, 30, and 33 C were similar for the eight representative isolates on PLYA. The growth curve of the mean of these isolates is given in Figure 1. Colony growth was good over a range of 20–25 C, was less at 15 and 27 C, and did not occur at 5 and 33 C. Dark mycelia formed in 20 days at temperatures of 20–27 C and several weeks later at 10 or 15 C.

Growth on osmotically adjusted agar. Colony growth on osmotically adjusted PA by isolate 40-3 showed temperature × water potential interactions ($P = 0.01$) (Fig. 2). Growth was greatest at water potentials between 0 and −10 bars at ≤27 C. Growth at temperatures from 20 to 27 C was similar at water potentials of −15 to −90 bars and was less, but substantial, at 30 C and −25 to −60 bars. Growth did not occur at 33 C.

Growth in liquid culture. Conidial production by isolate 40-3 in CDB proceeded more rapidly at 25 C than at lower temperatures. The temperature-growth response was affected mainly by the duration of the lag phase, which was 9, 13, and 18 hr at 25, 22.5, and 20 C in the first experiment and 10, 14, and 21 hr at these temperatures in the second experiment, respectively. The doubling time was similar at 25 and 22.5 C (1.8 and 2.0 hr; 1.5 and 2.4 hr) and at 20 C was 2.8 and 3.0 hr.

Disease reactions. Susceptible cultivar Apalachee was affected equally by the eight isolates singly and by the two mixtures, with the mean percentage of plants resistant to *V. albo-atrum* ranging from 0 to 3% when plants inoculated at 4 and 12 wk of age were assessed at 31 and 42 days, respectively. The percentage of resistance to *V. albo-atrum* in the moderately resistant cultivar Vertus varied widely in plants inoculated at 4 wk of age and read at 31 days, but the differences became insignificant when read later at 39 days (Table 1). Less variation in percentage of resistance to *V. albo-atrum* in Vertus was obtained with plants inoculated at 12 wk of age (Table 1).

Pathogenicity to other plant species. The eight representative isolates caused leaf symptoms in eggplant, cantaloupe, and watermelon but not in potato or tomato. In tests with one isolate (isolate 40-3), symptoms were not produced in mint. *V. albo-atrum* was reisolated from

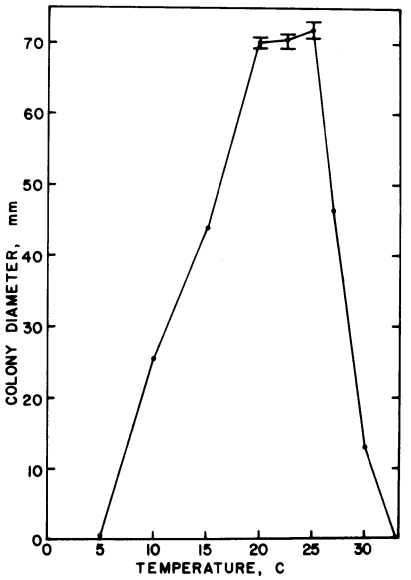

Fig. 1. Effect of temperature on growth of alfalfa *Verticillium albo-atrum* isolates grown on prune lactose yeast agar with a pH of 5.8. Colony diameter measured 20 days after incubation. Points represent the mean of eight isolates, and vertical bars indicate the confidence interval ($P = 0.95$).

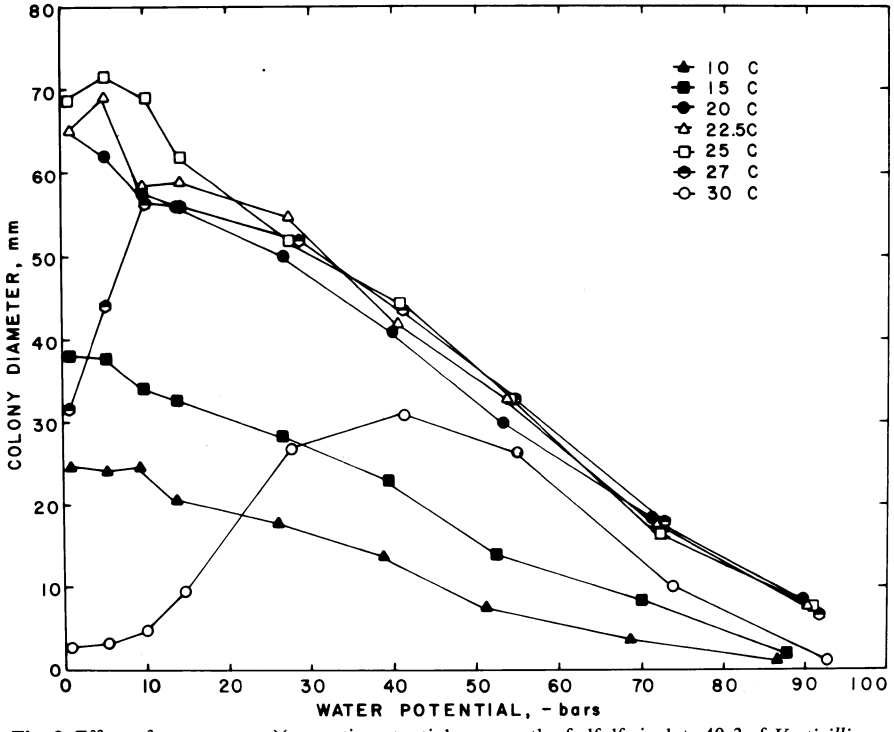

Fig. 2. Effect of temperature × osmotic potential on growth of alfalfa isolate 40-3 of *Verticillium albo-atrum* grown on osmotically adjusted Difco prune agar with a pH of 5.4. Colony diameter was measured 20 days after incubation.

Table 1. Percentage of Vertus alfalfa plants rated resistant[x] to *Verticillium albo-atrum* after root-soak inoculation of 4- or 12-wk-old plants with eight alfalfa-strain isolates, alone or in combination, and 31 or more days incubation in a cool (20 ± 1 C) growth chamber

	4-wk-old plants rated after		12-wk-old plants rated after
Isolate	31 days (%)	39 days (%)	42 days (%)
137-1	61 a[y]	48[z]	40[z]
121-2	58 ab	41	42
80 A-1	50 abc	40	40
51-1, 40-3, 80 A-1	50 abc	38	39
43-2	47 bc	42	40
40-3	46 bc	38	40
140 D-1	46 bc	40	36
51-1	45 bc	42	37
87-2	42 c	36	39
43-2, 87-2, 140 D-1	42 c	33	40

[x] Rating of 1 or 2 on a five-point disease severity scale. For 12-wk-old plants, 1 = no symptoms, 2 = one or two leaflets chlorotic, 3 = trifoliolates on one shoot chlorotic, 4 = trifoliolates on more than one shoot chlorotic, 5 = plant dead. For 4-wk-old plants, 3 = two to several trifoliolates chlorotic, 4 = most trifoliolates chlorotic.

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

[z] Differences between means within a column are not significant at the 5% level.

the lower part of the stem of potato and tomato but not from the mint plants.

DISCUSSION

Isolates of *V. albo-atrum* from alfalfa representing 38 locations in the Pacific Northwest and British Columbia, Canada, had identical colony characteristics on PLYA. Eight of these isolates selected to represent diverse areas were similar in their temperature-growth relations on PLYA, their pathogenicity to several plant species, and their ability to induce disease symptoms on cultivars of alfalfa that were susceptible and moderately resistant to *V. albo-atrum*. These observations indicate that only one strain of *V. albo-atrum* occurs on alfalfa in the area. This may be the result of its recent occurrence and narrow host range.

Temperature requirements for growth of the eight isolates from alfalfa on PLYA were similar to those reported for other isolates of *V. albo-atrum* on laboratory media (9,10). The optimum growth range was 20–25 C, with growth numerically but not statistically greater ($P = 0.05$) at 25 C, which was consistent with the range found for other *V. albo-atrum* isolates (10). Duration of the lag phase for conidial production in liquid culture at 20, 22.5, and 25 C for a representative isolate supported the conclusion that optimum growth occurred at 25 C. *V. albo-atrum* isolates from other crops have been reported to have a growth optimum of 22.5 C (5,13) or between 20 and 25 C (A. Morehart, *personal communication*). Osmotic water potentials between −15 to −90 bars on agar increased the optimum temperature range of 20–25 C to 20–27 C. Growth at 30 C was nearly tenfold greater at −25 to −60 bars than at 0 to −10 bars. A similar effect of water potential on *V. dahliae* occurred at 20–30 and 35 C (8).

Symptoms of Verticillium wilt occurred in eggplant, cantaloupe, and watermelon, but not in potato, tomato, or mint in greenhouse inoculations with the alfalfa isolate of *V. albo-atrum*. Because the fungus was readily reisolated from greenhouse-inoculated potatoes, we collected 100 potato plants in July 1979 from each of three fields in which alfalfa infected with *V. albo-atrum* had been grown in 1978, but we were unable to isolate *V. albo-atrum* from the stems or roots. This does not rule out the possibility that potato or other crops act as symptomless carriers of *V. albo-atrum*. In Europe, *V. albo-atrum* from alfalfa has been reported to be mildly pathogenic to potato and tomato, as well as to additional crops and weeds (4,6).

In Washington, Verticillium wilt caused by *V. dahliae* occurs in crops such as potato, mint, sweet cherry (*Prunus avium* L.), cantaloupe, and grape (*Vitis vinifera* L.), whereas the wilt caused by *V. albo-atrum* is limited to alfalfa. The alfalfa strain is apparently more adapted to the Pacific Northwest than the strains of *V. albo-atrum* isolated from other crops. This occurrence of the alfalfa strain may not be explained solely on the basis of temperature response of the fungus.

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