Immunological Comparison of Isolates of Verticillium albo-atrum and V. nigrescens Pathogenic to Cotton

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ABSTRACT

Serological and electrophoretic comparisons were made of cotton defoliating and nondefoliating pathogenic isolates of *Verticillium albo-atrum* from California and Missouri. A similar comparison was made with *V. nigrescens* isolates mildly virulent on cotton. The *Verticillium* spp. were distinctly differentiated from one another on an antigenic basis. The nondefoliating strain of *V. albo-atrum* SS4 was shown to differ antigenically from the defoliating strain T9; it appeared to be more closely related serologically to the mildly virulent *V. nigrescens* isolates than was the defoliating T9 isolate. Phytopathology 60:1682-1686.

Additional key words: Root pathogen, wilt.

Verticillium albo-atrum and *V. nigrescens* are pathogens of cotton in California and Missouri. In both localities, severe forms of verticillium wilt are characterized by darkening of vascular tissues, epinasty, stunting, and interveinal chlorosis of leaves followed by necrosis and defoliation. Some isolates, considered intermediate in virulence, cause all these symptoms except defoliation. *Verticillium nigrescens* which is weakly virulent may cause slight stunting and a mild chlorosis of the lower leaves, with no defoliation.

In California prior to 1960, cotton fields in the San Joaquin Valley were infested with the intermediate strains of *V. albo-atrum,* similar to strain SS4. Since 1960, however, these same fields have become infested with more virulent strains of *V. albo-atrum,* such as T9, that cause defoliation (3). Isolates causing severe wilt (involving defoliation) are also present in southeast Missouri.

The severe and intermediate strains of *V. albo-atrum* in California are apparently district pathotypes. Their relationship to the strains causing severe wilt in Missouri has never been established. Relationships among strains of *V. nigrescens* from both California and Missouri also are unknown. This paper compares the antigenic structure among strains of *V. albo-atrum* and *V. nigrescens* from California and Missouri which differ in virulence.

MATERIALS AND METHODS.—Table 1 lists the isolates of *Verticillium* used. Antigen preparations of *Verticillium* spp. were made by growing the isolates in a liquid medium consisting of $NaNO_3$, 5 g; $MgSO_4 \cdot 7H_2O$, 3 g; KH_2PO_4, 3 g; $FeCl_3 \cdot 6H_2O$, 0.24 mg; H_3BO_3, 0.06 mg; $ZnCl_2$, 0.15 mg; $CuCl_2 \cdot 2H_2O$, 0.05 mg; $MnCl_2 \cdot 4H_2O$, 0.04 mg; $NaMoO_4 \cdot 2H_2O$, 0.03 mg; D-glucose, 30 g; malt extract, 25 g; and distilled water to 1,000 ml. Fifty ml of the above medium were dispensed into 250-ml Erlenmeyer flasks and autoclaved for 20 min at 121 C. The flasks were seeded with mycelial discs, then incubated on a rotary shaker for 1 week at 24 C. The cultures (primarily conidia) were harvested by centrifuging for 15 min at 8,000 *g*. The supernatant layers were decanted and the cellular material was resuspended in sterile distilled water and recentrifuged. The cellular material, approximately 4 cc, was resuspended in 70 ml of buffer at pH 7.2 (NaCl, 0.14 N; $MgSO_4 \cdot 7H_2O$, 0.01 M; and KH_2PO_4-K_2HPO_4, 0.05 M). The cell suspensions were dispensed into serum bottles, and chloroform (0.2 ml/100 ml antigen) was added as a preservative. The antigen preparations were stored at 4 C and used within 2 weeks.

Antigens for serological tests were prepared as above, except that the cell suspensions were agitated in an OmniMixer in an ice bath for 3 min, then ground for 1 min with 20 g of 0.5-mm-diam glass beads in a Bronwill mechanical cell homogenizer. The mixture was then centrifuged at 4 C for 30 min at 20,000 *g*. The supernatant (antigen) layer was collected and stored as above.

Antisera were prepared from New Zealand white rabbits (2-4 kg) that were immunized by intramuscular, intraperitoneal, and intravenous injections of antigens. Intramuscular injections were made using antigen which was emulsified with 1 volume of Freund's incomplete adjuvant. Two rabbits were used for each antigen, and the antisera were pooled. Normal sera were withdrawn before immunization of the rabbits. The immunization schedule used was initiated by the intravenous injection of 0.25 ml of antigen. Each successive day through day 8 the dosage was increased 0.25 ml. Two ml of antigen was then administered on day 9, 10, and 14. Concurrently, beginning on day 5 and continuing thereafter on each scheduled immunization day (intravenous), 1 ml of antigen was administered intraperitoneally. Intramuscular immunizations were made at weekly intervals using a 0.50 ml antigen dosage each time. In all cases, blood was collected on day 18.

The rabbits were bled by either cardiac puncture or by making a small incision in a dorsal ear vein. A Bellco partial-vacuum apparatus was used to facilitate collection of blood from ears. The blood samples were collected in 50-ml centrifuge tubes, and were kept for 1 hr at 24 C. The clots were then loosened and kept overnight at 4 C. The antisera were then decanted and

TABLE 1. Characteristics of cultures of *Verticillium albo-atrum* and *V. nigrescens* used in immunological studies

Isolate	Source	Culture type Potato-dextrose agar	Virulence on cotton
V. albo-atrum Rke. & Berth.			
VTAA-1	*Gossypium hirsutum* L. DPL-45A; Mo.	Black-sclerotial	Severe
VTAA-2	*Gossypium hirsutum* L. Auburn M; Mo.	Black-sclerotial	Severe
VTAA-3	*Gossypium hirsutum* L. SJ-1; Ark.	Black-sclerotial	Severe
T9	Soil in Calif.	Black-sclerotial	Severe
SS4	*Gossypium hirsutum* L. Acala 4-42; Calif.	Black-sclerotial	Intermediate
V. nigrescens Pethybr.			
VTNG-2	*Gossypium hirsutum* L. Stardel; Mo.	Gray-mycelial	Mild
VTNG-3	*Gossypium hirsutum* L. Stardel; Mo.	Gray-mycelial	Mild
68	*Amaranthus retroflexus* L. Calif.	Gray-mycelial	Mild

clarified by low-speed centrifugation. The straw-colored antisera were dispensed into 2-5 ml serum bottles and frozen.

Agglutination tests were made with antiserum dilutions ranging from 2 to 25,600. One-half-ml amounts of antiserum and antigen (approx 500 μg protein/ml) were mixed in Kahn tubes, incubated 1 hr at 37 C, then placed overnight at 4 C. Degree of agglutination was measured on a scale from (−) for no agglutination to (+++) for max agglutination.

Reciprocally absorbed antisera were prepared by absorbing the antisera twice with amounts of antigen equivalent to that needed for the max homologous reactions. All cross-reactions were made using antisera adjusted to a reciprocal homologous titer of 400.

Agar-gel double-diffusion tests were made in plastic 90-mm petri dishes containing 8 ml of 0.5% Ionagar No. 2 (Colab) (0.01 M) at pH 7.2. Phenol was added as a preservative at the rate of 0.2 ml of 80% phenol/500 ml of agar. Following placement of the antigens and antisera in the agar wells, precipitin reactions were developed for 5-7 days at 4 C.

Electrophoretic comparisons of the proteins in antigens of the isolates were made on an ED 470 vertical gel electrophoresis apparatus. Separations were made in polyacrylamide gel of Cyanogum-41 consisting of 95% acrylamide and 5% bis acrylamide in a 5% solution in a buffer containing Tris[tris (hydroxymethyl) amino methane], 43.1 g; Na_2EDTA (disodium salt of ethylene diamine tetraacetic acid), 3.7 g; boric acid, 22.0 g; and water to 4 liters, at pH 8.4. To prepare the gel, 10 g of Cyanogum-41 were placed in 200 ml of Tris-Na_2EDTA-boric acid buffer and 0.2 ml of *N, N, N′, N′*-tetramethylethylene diamine was added. This was catalyzed chemically with 0.3 g of ammonium persulfate. The gel was poured and allowed to set for 15-30 min. A prerun was made for 15-30 min at 300 v.

Each antigen, prepared as previously described, was adjusted to 130 μg of protein/ml as determined by the method of Lowry et al. (1). Sucrose and enough bromphenol blue were added to color the sample and permit easy placement of the sample (40 μliters) in the gel. After placement, the samples were equilibrated for 10 min, and a potential of 300 v was then applied for 2 hr. The gel was cooled to less than 20 C by continuous circulation of iced distilled water. Following development, the gel was removed and immersed for 2 hr in 0.25% amido black 10B stain in a 5:5:1 solution of methanol, water, and glacial acetic acid. The gel was destained with successive washes of 5:5:1 methanol, water, and glacial acetic acid until the bands were apparent. After destaining, the gel was cleared with 5% aqueous glycerin.

RESULTS.—Isolates T9 and SS4 were compared by using reciprocally absorbed antisera and tube agglutination tests (Table 2). Titers of homologous reactions and heterologous cross-reactions were 400. T9 antiserum when absorbed by T9 antigen was incomplete, having a titer of 25 when reacted with T9 antigen; however, when reacted with SS4 antigen, the titer was zero, indicating complete absorption of antibodies reactive with antigens of SS4. This antigenic difference between T9 and SS4 was also observed when T9 antiserum was absorbed with SS4 antigen. In this case, the T9-absorbed serum gave a titer of 0 when reacted with

TABLE 2. Agglutinin titer determinations of reciprocally absorbed antisera of isolates SS4 and T9 of *Verticillium albo-atrum*

Antiserum	Antigen	Titer[a]
T9	T9	400
T9	SS4	400
SS4	SS4	400
SS4	T9	400
T9 absorbed with T9	T9	25
T9 absorbed with T9	SS4	0
T9 absorbed with SS4	T9	50
T9 absorbed with SS4	SS4	0
SS4 absorbed with SS4	T9	0
SS4 absorbed with SS4	SS4	0
SS4 absorbed with T9	T9	10
SS4 absorbed with T9	SS4	5

[a] Reciprocal of final antiserum dilution with a definite reaction.

SS4 antigen, and 50 when reacted with T9 antigen. Antiserum of SS4 when absorbed by either T9 or SS4 antigens was completely absorbed. It appeared that the antigenic structure of T9 was broader but inclusive of that of SS4.

The results of the tube agglutination tests were supported by those obtained in agar-gel double-diffusion tests. The agar-gel tests also included reactions with other isolates of *V. albo-atrum* and *V. nigrescens*. These results are shown in Fig. 1-5. When antiserum of T9 was reacted with homologous antigen and antigens of isolates VTAA-1, VTAA-2, VTAA-3, SS4, VTNG-2, VTNG-3, and 68 (Fig. 1), strong common precipitin reactions occurred among all the *V. albo-atrum* isolates, but were absent or hardly apparent with the *V. nigrescens* isolates. An additional band of less

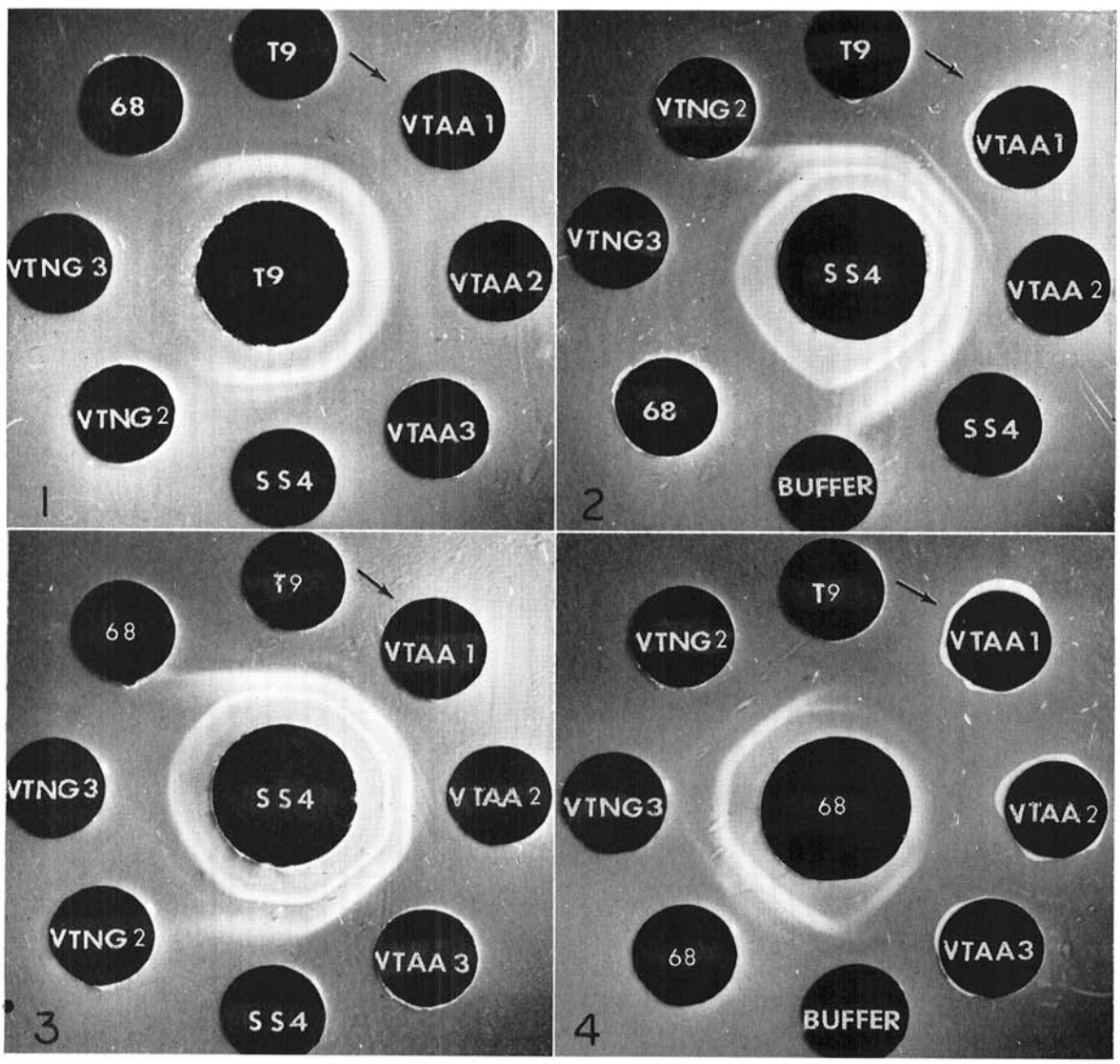
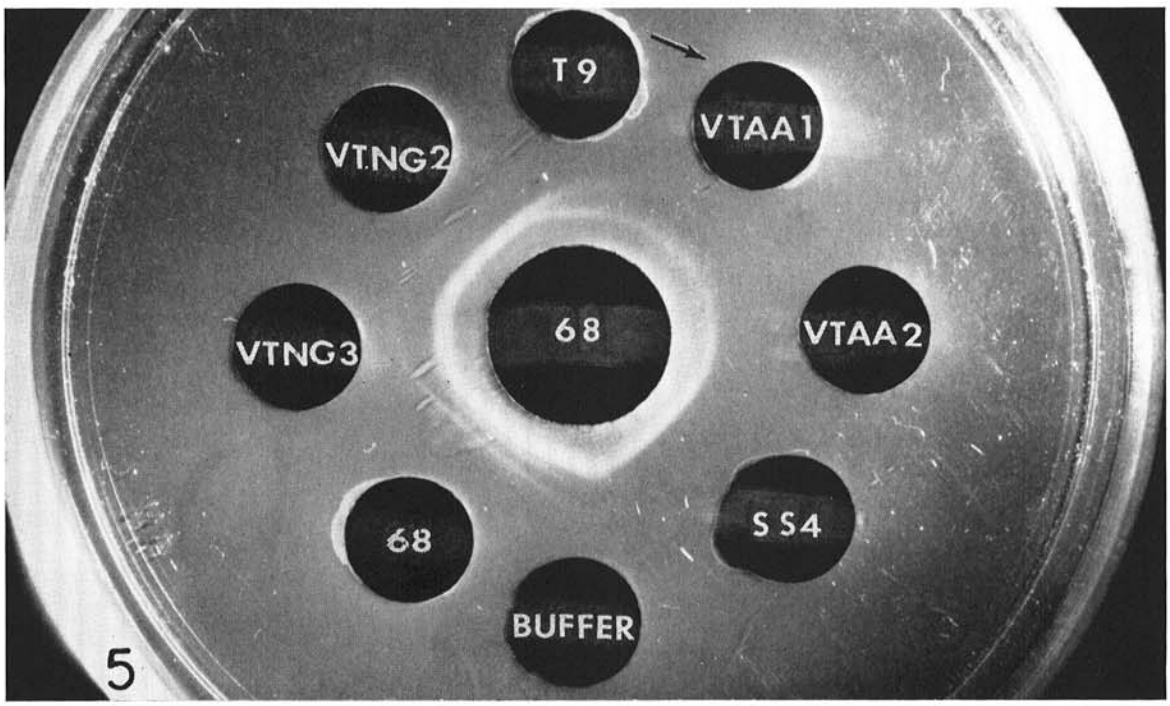


Fig. 1-4. **1)** Precipitin band reactions in agar gel diffusion tests of California isolates (T9 and SS4) and three Missouri isolates (VTAA-1, 2, and 3) of *Verticillium albo-atrum* and California isolate (68) and Missouri isolates (VTNG-2, 3) of *V. nigrescens* when T9 antiserum was used. Clockwise from the top: T9; VTAA-1; VTAA-2; VTAA-3; SS4; VTNG-2; VTNG-3; 68; antiserum of T9 in center well. **2)** Precipitin band reactions in agar gel diffusion tests of California isolates T9 and SS4, Missouri isolates VTAA-1 and VTAA-2 of *Verticillium albo-atrum* and California isolate 68, Missouri isolates VTNG-2, and VTNG-3 of *V. nigrescens* when SS4 antiserum was used. Clockwise from the top: T9; VTAA-1; VTAA-2; SS4; Buffer; 68; VTNG-3; VTNG-2; antiserum of SS4 in center well. **3)** Reactions of the same isolates as shown in Fig. 2 except that the antigen sequence is clockwise from the top: T9; VTAA-1; VTAA-2; VTAA-3; SS4; VTNG-2; VTNG-3; 68; antiserum of SS4 in center well. **4)** Precipitin band reactions in agar gel diffusion tests of California isolates T9 and Missouri isolates VTAA-1, VTAA-2, and VTAA-3 of *Verticillium albo-atrum* and California isolate 68 and Missouri isolates VTNG-3 and VTNG-2 of *V. nigrescens* when 68 antiserum was used. Clockwise from the top: T9; VTAA-1; VTAA-2; VTAA-3; Buffer; 68; VTNG-3; VTNG-2; antiserum of 68 in center well.

density developed in the heterologous reaction with SS4 that was not present in the homologous pairing or with the other defoliating strains, indicating a different and additional antigen-antibody system in the SS4 isolate. This difference in antigenic structure was not apparent in the tube agglutination tests. A very weak common band developed between the defoliating strains, T9 (homologous), VTAA-1, and VTAA-2, but not with VTAA-3 or SS4, indicating that this defoliating strain and SS4 differed antigenically from the other defoliating strains tested.

When antiserum of SS4 was reacted with homologous and heterologous antigens (Fig. 2, 3), three distinct precipitin bands in the homologous reaction were shared by the four other strains of *V. albo-atrum*. But the density of the third (from the center well) precipitin



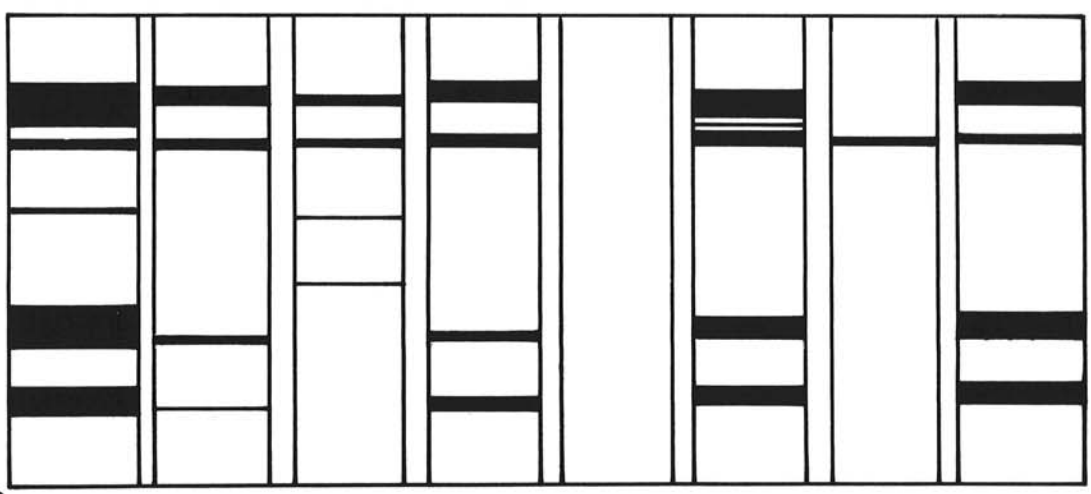


Fig. 5-6. 5) Precipitin band reactions in agar gel diffusion tests of California isolates T9 and Missouri isolates VTAA-1, VTAA-2, and VTAA-3 of *Verticillium albo-atrum* and California isolate 68 and Missouri isolates VTNG-3 and VTNG-2 of *V. nigrescens* when 68 antiserum was used. Clockwise from the top: T9, VTAA-1; VTAA-2; SS4; Buffer; 68; VTNG-3; VTNG-2; antiserum of 68 in center well. **6)** Electrophoretic patterns comparing antigen proteins of California and Missouri isolates of *Verticillium*. From left: T9; SS4; 68; VTAA-1; VTNG-2; VTAA-2; VTNG-3; VTAA-3. Thickness of band denotes the intensity of the electrophoretic band.

band in the homologous reaction was less than corresponding bands in the heterologous reactions (T9, VTAA-1, VTAA-2, and VTAA-3). This may indicate that the third precipitin band in the heterologous reactions with the defoliating strains was composed of two separate antigen-antibody systems that have precipitated in close proximity to one another. Splitting of the dense bands in front of wells VTNG-2 and V68 (Fig. 1) supports this interpretation. A precipitin band (Fig. 2, 3) which appeared only between the SS4 antiserum and VTAA-2 antigen wells and a band which appeared only between SS4 antiserum and T9 and VTAA-1 antigens (band closest to antigen wells) distinguished SS4 (nondefoliating) from the other (defoliating) isolates of *V. albo-atrum*. Two precipitin bands were common among all antigens, including the *V. nigrescens* isolates. A third weak band present in the reaction with VTNG-2 was not shared by VTNG-3 or 68.

When antiserum of 68 was reacted with homologous antigen and heterologous antigens (Fig. 4, 5), at least three precipitin bands developed in the homologous reaction but only one band was shared with VTNG-3; whereas at least two bands were shared with VTNG-2. Only one band was shared with the *V. albo-atrum* isolates, indicating distinct immunological differences between *V. albo-atrum* and *V. nigrescens*. The increased density of the band in VTAA-3 as compared to VTAA-1 and VTAA-2 demonstrates mild strain differences between this defoliating Missouri isolate and the other defoliating isolates from Missouri, VTAA-1, and VTAA-2. This was also demonstrated in Fig. 1, where T9 antiserum was used.

A great similarity between the virulent T9 isolate from California and the virulent isolates from Missouri was also evident in a comparison of electrophoretic patterns (Fig. 6). Four bands can be seen that are the same for these isolates. Protein separation, however, is different for SS4 and the *V. nigrescens* isolates from California and Missouri.

DISCUSSION.—Serological data and corroborative electrophoretic patterns on polyacrylamide gel that compare the antigenic structure of the defoliating California and Missouri isolates of *V. albo-atrum* indicate that the isolates from each area are very similar, but different from the nondefoliating strain. Antigenic comparisons between *V. nigrescens* isolates from cotton in Missouri and V68, originally from pigweed, *Amaranthus retroflexus,* in California indicated they were alike.

Within each species, strain differences were detected serologically. Major antigenic differences were apparent between the nondefoliating and defoliating strains. The nondefoliating strain (SS4) appears to have a closer antigenic affinity to the *V. nigrescens* isolates than does the defoliating isolate T9 (Fig. 1, 2, 3). The serological differences inherent in *V. albo-atrum* and *V. nigrescens* were clearly demonstrated (Fig. 1-5). Serological differences between these species have also been reported by Schnathorst (2).

It is significant that the nondefoliating *V. albo-atrum* isolate, SS4, from California differed markedly from the defoliating strains from California and Missouri and from the mildly pathogenic *V. nigrescens* isolates. Thus, SS4 differs basically from the defoliating strains in more characters than virulence (3, 4). It is possible that differences in virulence may be expressed in specific serological reactions or by electrophoretic patterns. It is not assumed, however, that the differences observed in this study parallel or are related to virulence in cotton.

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