Common Antigens Among Varieties of Gossypium hirsutum and Isolates of Fusarium and Verticillium Species

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ABSTRACT

Preparations of antigens from four varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species were compared for common antigens. At least one antigenic substance was common among the varieties of cotton and isolates of *Fusarium oxysporum* f. sp. *vasinfectum*, *F. solani* f. sp. *phaseoli*, *Verticillium albo-atrum*, and *V. nigrescens*. Cotton varieties which were resistant or susceptible to Fusarium wilt as well as pathogenic and nonpathogenic isolates of *F. oxysporum* f. sp. *vasinfectum* shared the common antigen. The common antigen was not shared between *F. moniliforme* (nonpathogenic) and cotton. In gel-diffusion tests, five to eight precipitin bands were observed in the homologous reactions; of these, only one or two bands were common in heterologous reactions between the fungal and cotton preparations. The common antigenic determinant shared by cotton and the fungal isolates does not appear related to the severity of wilt symptoms, but it may affect host-pathogen compatibility during the process of root infection.

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Additional key words: host-pathogen compatibility, serological relationships.

In several instances, it has been found that plant and animal hosts have antigenic substances in common with parasitic microorganisms. These substances have been termed "common antigens".

In animal host-parasite systems, the resistance or susceptibility of an animal to infection and disease development may be dependent on the antigenic relationship of the host and pathogen (4, 6). The greater their antigenic similarity, the greater the possibility of immunological tolerance and the likelihood that the pathogen will become established in the host and cause disease. Damian (4) reviewed this aspect of antigen-sharing by animal hosts and parasitic helminths, and emphasized the significance of immunological cross-reactivity of host and pathogen in regard to disease development. An analogous phenomenon may occur between plant hosts and their pathogens. Common antigens which appear to be involved in disease susceptibility have been shown between host plants and pathogens in flax rust (7, 8, 11), angular leaf spot of cotton (5, 14), crown gall of sunflower and tobacco (13, 15), black rot of sweet potato (5), and common smut of corn (16).

The present study was made to determine the possible presence and significance of common antigens among varieties of *Gossypium hirsutum* L. and isolates of *Fusarium oxysporum* (Schlecht.) f. sp. *vasinfectum* (Atk.) Snyd. & Hans., *F. solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans., *F. moniliforme* Sheld., *Verticillium albo-atrum* Reinke & Berth., and *V. nigrescens* Pethybr. Brief reports of this work have been made (2, 3).

MATERIALS AND METHODS.—A single-spored isolate P10, the most virulent of 14 isolates of *F. oxysporum* f. sp. *vasinfectum* obtained from different cotton-growing regions of the United States, was used. Other fungal isolates used were SS4 and T9 of *V. albo-atrum*, both highly virulent in cotton; 68 of *V. nigrescens*, weakly virulent in cotton; and isolates of *F. solani* f. sp. *phaseoli* and *F. moniliforme*, neither of which was pathogenic in cotton under the environmental conditions used. The fungal isolates were maintained on potato-dextrose agar (PDA). Cotton varieties used were Acala 4-42, Deltapine Smoothleaf, Auburn 56, and Rowden.

Ultraviolet induction of mutants.—Microconidia of isolate P10 of *F. oxysporum* f. sp. *vasinfectum* were obtained from 2-day-old shake cultures grown in 250-ml Erlenmeyer flasks containing 50-ml amounts of the following medium: KNO_3, 10 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $FeCl_3 \cdot 6H_2O$, 0.02 g; and sucrose, 50 g/liter of solution. The cultures were filtered through four layers of cheesecloth under aseptic conditions, centrifuged (2,000 *g* for 15 min), and the conidia resuspended in sterile distilled water at 1×10^4 conidia/ml. Ten-ml amounts of the conidial suspension were pipetted into open 9-cm-diam plastic petri dishes and irradiated from 1 to 5 min at a distance of 30 cm from a Sylvania (G15T8) germicidal lamp. Dilutions of the irradiated conidial suspensions were plated on PDA, and survivors were selected at random on the basis of differences in colony appearance for pathogenicity tests. Plating and germination of conidia were done in total darkness.

Pathogenicity tests.—Isolates of *Fusarium* and *Verticillium* were tested on the four cotton varieties by stem inoculations (1) using 25-50 µliter droplets containing 1×10^6 conidia/ml. Conidia for this purpose were obtained from liquid shake cultures as described above. In each of two tests, 20 plants were inoculated for each isolate. Mycelial fragments of *F. solani* were used for inoculum due to sparse conidial formation by this isolate. Cotton plants were grown in sterile soil in a controlled environment chamber at

33 C for pathogenicity with *Fusarium* isolates, or at 27 C for tests with *Verticillium*. Plants were 14 or 21 days old when inoculated with *Fusarium* or *Verticillium*, respectively. The virulence of the isolates was rated from the type and severity of the symptoms and the number of plants that died over a period of 4-5 weeks after inoculation.

Pathogenicity of *Fusarium* isolates which survived the ultraviolet irradiation was tested on the variety Rowden, by the following method: A glass tube (1.8 X 10 cm), filled with soil and plugged at the lower end with cotton, was introduced into a 125-ml flask containing 25 ml of sterile water. The tube and soil were also sterilized. One surface-sterilized seed was planted in each tube, and germinated in a growth chamber at 33 C. When the seedling was 3-4 days old, it was inoculated by pouring 5 ml of an unwashed suspension of conidia and mycelium from 3- to 5-day-old liquid shake cultures into the tube, and 20 ml of the suspension into the flask. After 3 days, the seedling was transferred to sterile water culture. Symptoms appeared about the 7th day after inoculation, and tests were completed by the 21st day. Isolates of *Fusarium* that produced visible disease reactions were eliminated. Those that appeared to be avirulent were then retested by stem inoculations on older plants grown in sterilized soil, using standardized inoculum (1 X 10^6 conidial or mycelial fragments/ml) as previously described.

Preparation of fungal and plant-host antigens.—The fungi were grown in liquid shake cultures in the synthetic medium for 3 to 5 days as described for the pathogenicity tests. Conidial suspensions were prepared by passing the liquid cultures through four layers of cheesecloth and centrifuging the resulting suspension of conidia at 7,000 *g* for 15 min. Four cc of packed cells were then resuspended in 50 ml of buffer (KH_2PO_4-K_2HPO_4, 0.05 M; $MgSO_4 \cdot 7H_2O$, 0.001 M; and NaCl, 0.14 N) at pH 7.2. The conidia were sonicated for 8 min at a frequency of 20 khz (Biosonik III, Bronwill Scientific Co.) at 5-10 C. The suspension of sonicated conidia was then ground for 8 min with 20 g of glass beads (0.17- to 0.18-mm-diam) in a Braun cell homogenizer (Bronwill Scientific Co.) at 5-10 C. The cell homogenate was centrifuged at 20,000 g for 40 min, and the clear supernatant layer was decanted and used for immunization of rabbits.

Host antigens were prepared from both roots and shoots of healthy cotton plants. Plants grown in sterilized soil in controlled environment chambers at 33 C for 2 weeks were washed and used for preparation of antigens. The antigens were extracted by grinding the plant parts in a mortar (about 50 g fresh wt) under liquid nitrogen, followed by further grinding in 50 ml of buffer (KH_2PO_4-K_2HPO_4, 0.05 M; $MgSO_4 \cdot 7H_2O$, 0.001 M; NaCl, 0.14 N; and sodium ascorbate, 0.05 M) at pH 7.2 with 20 g glass beads (0.17- to 0.18-mm-diam) and 5 g of insoluble polyvinyl pyrrolidone (PVP). The homogenate was stirred at 4 C for 2 hr, then centrifuged at 20,000 *g* for 40 min. The clear supernatant layer was decanted and used as the preparation of plant antigens for immunization of rabbits.

The amount of protein in the preparation of antigens (PA) was estimated by the method of Lowry et al. (10). Before analysis, samples of the PA from plant materials were dialyzed for 24 hr in two changes of 10 volumes of the buffer (no ascorbate added) used for preparation of fungal antigens.

The preparations of fungal and plant antigens were stored in 5- to 8-ml quantities in serum bottles at −20 C and were used within 2 weeks after preparation. Antigens for serological tests were prepared by the same methods.

Preparation of antisera.—Antisera for the fungal and plant antigens were produced in New Zealand white does (5- to 9-lb. size). Two rabbits were immunized for each PA. Normal sera were obtained before immunization. For immunization, PA contained ca. 800 μg protein/ml (fungal preparation) or 5 mg protein/ml (plant preparation). The immunization procedure was as follows: starting with a 0.25-ml intravenous injection, daily intravenous injections were given, increasing the dosage by 0.25 ml/day up to 2.0 ml, an amount which was given on the 8th, 9th, 10th, and 14th days. Rabbits were rested on 11th, 12th, and 13th days. For intramuscular injections, 0.5 ml of PA was homogenized with an equal volume of Freund's complete adjuvant (Difco) and administered on the 7th and 14th days. On the 5th through the 10th day, and on the 14th day, 1-ml amounts of the PA were administered intraperitoneally. The animals were starved for 24 hr prior to bleeding by cardiac puncture on the 18th day. The blood was allowed to clot, the clot loosened from the tube, and the blood stored at 4 C overnight. Sera were collected the following day and stored at −20 C in 5- to 8-ml quantities.

Serological techniques.—Determination of titers of immune sera.—Titers of antisera were determined by agglutination tests using either washed conidia of the fungi or soluble plant antigens adsorbed on polystyrene latex particles (Colab) (12). Antiserum dilutions from 2 to 25,600 were prepared with normal saline. The antigens used for all tests contained ca. 500 μg protein/ml.

Agar gel double diffusion technique.—Agar gel plates were prepared by dispensing 8 ml of the following agar into plastic petri plates (9-cm-diam): 0.5% Ionagar No. 2 (Colab) in KH_2PO_4-K_2HPO_4, 0.01 M; and NaCl, 0.14 N. Two-tenths ml of 80% phenol was added/500 ml of this agar as preservative. Plates were incubated either at 4 C for a week or 25 C overnight with similar results, except that the precipitin bands appeared sooner in plates incubated at 25 C. Soluble preparations of antigens (ca. 500 μg protein/ml) and undiluted sera were used.

RESULTS.—*Pathogenicity of fungal isolates.*—The four cotton varieties tested varied in their susceptibility to the isolates of *Fusarium* and *Verticillium*. Among the *Fusarium* isolates, only *F. oxysporum* f. sp. *vasinfectum* was pathogenic on the host varieties under the conditions of the tests.

TABLE 1. Presence of common antigens among cotton varieties and isolates of *Fusarium* species and *Verticillium albo-atrum* based on agar-gel double diffusion tests

Antigens	Antisera												
	F. oxysporum f. sp. *vasinfectum*–P10	*F. oxysporum* f. sp. *vasinfectum*–11	*F. oxysporum* f. sp. *vasinfectum*–13	*V. albo-atrum* SS4	*V. albo-atrum* T9	Acala 4-42 root	Acala 4-42 shoot	Auburn 56 root	Auburn 56 shoot	Rowden root	Rowden shoot	Deltapine Smoothleaf root	Deltapine Smoothleaf shoot
Acala 4-42 root	+[a]	–[b]	+	–	–	+	+	+	+	+	+	+	+
Acala 4-42 shoot	+	–	+	–	–	+	+	+	+	+	+	+	+
Auburn 56 root	+	(–)[c]	+	(–)	(–)	+	+	+	+	+	+	+	+
Auburn 56 shoot	+	–	+	–	–	+	+	+	+	+	+	+	+
Rowden root	+	+	+	(–)	–	+	+	+	+	+	+	+	+
Rowden shoot	+	+	+	–	–	+	+	+	+	+	+	+	+
Deltapine Smooth leaf root	+	+	+	(–)	–	+	+	+	+	+	+	+	+
Deltapine Smooth leaf shoot	+	+	+	(–)	–	+	+	+	+	+	+	+	+
F. oxysporum f. sp. *vasinfectum* P10	+	+	+	+	+	(–)	(–)	+	–	+	(–)	(–)	(–)
F. oxysporum f. sp. *vasinfectum* 11	+	+	+	+	+	–	–	+	–	+	(–)	(–)	(–)
F. oxysporum f. sp. *vasinfectum* 13	+	+	+	+	+	(–)	+	+	+	+	(–)	(–)	(–)
V. albo-atrum SS4	+	+	+	+	+	–	–	+	–	–	–	–	+
V. albo-atrum T9	+	+	+	+	+	–	–	+	–	–	–	–	–
F. solani f. sp. *phaseoli*	+	+	+	+	+	–	–	+	–	+	–	–	–
F. moniliforme	+	+	+	+	+	–	–	–	–	–	–	–	–

[a] Common precipitin band(s) detected.
[b] Common precipitin band was not detected.
[c] Reactions represented within parentheses failed to develop the precipitin band(s), but reactions were observed when preparations of antigens and antisera for the fungal and plant materials were reversed.

Among the 14 isolates of *F. oxysporum*, P10 was highly virulent on Rowden, whereas the other isolates showed lesser degrees of virulence or were nonpathogenic. In pathogenicity tests of *V. albo-atrum*, isolate T9 was highly virulent, whereas isolate SS4 was less virulent on all the cotton varieties. Isolate 68 of *V. nigrescens* was mildly virulent on Rowden and Auburn 56, and nonpathogenic or weakly virulent on the other varieties. Among the four cotton varieties, Rowden was most susceptible to *F. oxysporum* and the *Verticillium* isolates.

Selection of avirulent ultraviolet mutants of F. oxysporum f. sp. vasinfectum.—One hundred and seventy-five isolates of P10 were selected at random from germinating conidia which prior to germination had been exposed to ultraviolet irradiation for varying lengths of time. Based on pathogenicity tests with Rowden, two were selected (isolates 11 and 13) that were avirulent in both seedling and stem inoculations of test plants.

Serological tests.—In homologous reactions, agglutinin titer values of the fungal antisera were in the range of 1,600 to 12,800, while those of the plant antisera were between 400 and 25,000. Agar gel diffusion tests with fungal and cotton antisera revealed the presence of at least one common antigen among the *Fusarium* and *Verticillium* isolates (except *F. moniliforme*) and cotton varieties in reactions between various combinations of antigens and antisera (Table 1). As expected, antigens were common among the roots and shoots of the host varieties (Fig. 1). The PA from shoots gave 5-8 precipitin bands when reacted with various host antisera, whereas PA from roots gave four or five bands. Four to eight bands were formed in homologous reactions with fungal antigens. When PA of the fungal isolates were reacted with antisera to cotton roots and shoots, precipitin bands were observed in certain combinations. Antisera to Auburn 56 root and Rowden root (homologous titers 400-800) were the best in this respect, reacting with most of the fungal PA tested except those of *F. moniliforme* and isolate 68 of *V. nigrescens*. PA of isolate SS4 of *V. albo-atrum* reacted strongly with antisera to roots of Auburn 56, Rowden, and Deltapine Smoothleaf, whereas antiserum to Acala 4-42 roots was nonreactive with PA of SS4. PA of isolate T9 reacted only with antiserum to Auburn 56 root tissues. The antisera for T9 or SS4 were not reactive with antigen preparations of the cotton varieties. Of the many bands observed in homologous and heterologous combinations of host and fungal antigens and antisera, only one or two appeared to be common in the agar gel diffusion tests. The common antigens among the fungal preparations and between the cotton varieties appeared to be serologically identical, as the bands joined completely in agar gel tests (Fig. 1). It appeared that in most cross reactions of PA of cotton plants and fungal isolates, there was only one precipitin band. At present it is not known if the common antigen band observed was due to a single antigenic substance. However, an antigenic

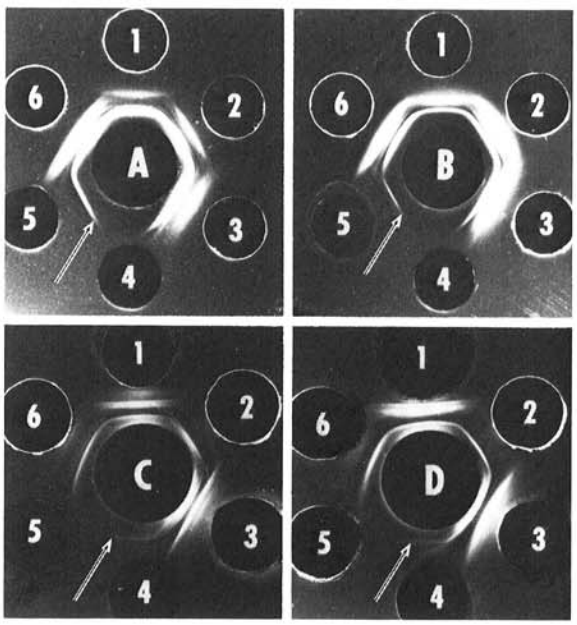

Fig. 1. Agar gel precipitin tests showing common antigen relationships shared by *Fusarium* species and several cotton varieties. Arrows indicate common antigen precipitin bands. **A)** Antiserum for Auburn 56 root tissues (center well) reacted with antigen preparations of Rowden shoot tissues (1), Auburn 56 root tissues (2), Auburn 56 shoot tissues (3), buffer (4), *F. oxysporum* f. sp. *vasinfectum*, isolate P10 (5), and Rowden root tissues (6). **B)** Same as (A) except that antiserum for Rowden root tissues is in the center well. **C)** Antiserum for Rowden root tissues (center well) reacted with antigen preparations for Rowden shoot tissues (1), Auburn 56 root tissues (2), Auburn 56 shoot tissues (3), *F. solani* f. sp. *phaseoli* (4), *F. moniliforme* (5), and Rowden root tissues (6). **D)** Antiserum for Auburn 56 root tissues (center well) reacted with antigen preparations for Deltapine Smoothleaf shoot tissues (1), Acala 4-42 root tissues (2), Acala 4-42 shoot tissues (3), *F. solani* f. sp. *phaseoli* (4), *F. moniliforme* (5), and Deltapine Smoothleaf root tissues (6).

substance common to both plant and fungal preparations has been isolated and purified; it is a polysaccharide-protein complex which will be described in a subsequent paper. In control plates, antiserum to Freund's complete adjuvant did not react with the antigen preparations.

Common antigen relationship of avirulent isolates with cotton.—To see if loss of virulence by a fungal pathogen affected the common antigen relationship between host and pathogen, the avirulent mutants 11 and 13 of P10, induced by ultraviolet irradiation, were tested for their serological relationship with the cotton hosts. Agar gel tests involving fungal antigens and host antisera (homologous titers 400-800) showed the presence of at least one precipitin band between the avirulent isolates and antisera for Rowden and Auburn 56 root tissues. There were also one or two precipitin bands formed between PA of roots and shoots of Rowden and Deltapine Smoothleaf and antiserum of isolate 11; however, PA of Auburn 56 and Acala 4-42 gave negative reactions with antiserum of isolate 11. In contrast, antisera of isolates P10 and 13 gave positive reactions when reacted with the PA of all the cotton varieties (Table 1).

Antigenic relationships among the fungal isolates.—In serological tests with either homologous or heterologous fungal antisera, PA of P10, 11, and 13 of *F. oxysporum* f. sp. *vasinfectum* gave four or five precipitin bands. PA of other *F. oxysporum* isolates in serological cross reactions with P10 antiserum gave three to six bands, of which at least two showed complete identity with P10 antigens. Serological comparisons of the isolates of *Verticillium* species, which confirmed the results of Wyllie & DeVay (17), showed that the nondefoliating isolate SS4 of *V. albo-atrum* could be distinguished from defoliating isolates, and that isolates of *V. albo-atrum* and *V. nigrescens* were serologically distinct. Moreover, serological comparisons of these isolates with isolate 68 of *V. nigrescens* indicated that at least three common bands between antisera and antigen preparations of *V. albo-atrum* were absent. Among the virulent isolates T9 and SS4 of *V. albo-atrum* and P10 of *F. oxysporum* f. sp. *vasinfectum*, there were one or more common precipitin bands.

DISCUSSION.—Earlier views (5, 8) on the occurrence of common antigens between host and pathogen emphasized a quantitative relationship in regard to their effect on disease susceptibility. Thus, the antigenic relationship between Rowden and a virulent isolate like P10 should be reflected in a greater number and intensity of common precipitin bands than there would be between P10 and Auburn 56. Likewise, a highly virulent isolate such as P10 would be more closely related serologically to Rowden than avirulent isolates such as 11 and 13. However, there were no apparent differences in the common antigen relationship between cotton varieties and virulent and avirulent isolates of *F. oxysporum* f. sp. *vasinfectum*, *F. solani*, and *Verticillium*. There was one major antigenic constituent common among the above hosts and fungi. Furthermore, the common antigen band of virulent and avirulent isolates against host root antisera appeared serologically identical in gel diffusion plates. Possibly then, this antigen was not related to the severity of wilt symptoms in cotton plants. Since resistance in cotton to Fusarium and Verticillium wilts is expressed after infection of roots and invasion of the vascular system (1, 9), the concept of antigenic compatibility proposed earlier (5, 8) should also encompass the primary process of infection. The common antigen relationship was probably the same among the four varieties of cotton and the isolates of *F. oxysporum* f. sp. *vasinfectum*, *F. solani* f. sp. *phaseoli* and *V. albo-atrum* and *V. nigrescens* because the precipitin bands produced by the common antigens were identical in agar gel tests. However, in several agar gel tests (Table 1), the formation of the common precipitin bands between the preparations of fungal antigens and plant antisera was not duplicated when fungal antisera were reacted with preparations of plant antigens. Possible reasons

could involve blocking substances in the antigen preparations or limiting amounts of antigens or antibodies. However, it is apparent that isolates of both *F. oxysporum* f. sp. *vasinfectum* and *V. albo-atrum* share a common antigenic determinant with the cotton varieties, although the significance of this relationship is unknown in regard to host-pathogen compatibility.

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