Classification of Isolates of *Verticillium dahliae* Based on Heterokaryon Incompatibility

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ABSTRACT

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Nineteen isolates of *Verticillium dahliae* from various hosts and geographical areas were tested for ability to form heterokaryons with each other. Two methods of heterokaryon formation were used. In one, doubly auxotrophic mutants were induced with ultraviolet light (UV) and paired on minimal agar medium. Prototrophic growth arising from such pairings was heterokaryotic. In the second method, UV-induced mutants with albino (*alm*) and brown (*brm*) microsclerotia were paired on complete agar medium. In some *alm* × *alm* and *alm* × *brm* pairings, black microsclerotia developed along the line of contact. The isolates involved in such pairings were the same ones that produced prototrophic growth from auxotroph pairings. Thus, the black microsclerotia also indicated heterokaryosis. Among the 19 isolates tested, four subgroups were defined. Isolates within a subgroup formed heterokaryons with each other, but isolates from different subgroups did not. Because heterokaryosis and the subsequent parasexual cycle are the only known means of gene exchange in this asexual fungus, the subgroups may be viewed as genetically isolated populations. The recognition of genetically isolated populations within *V. dahliae* may permit a determination of the origin of new pathotypes or the spread of known pathotypes.

Additional key words: auxotrophic markers, melanin mutants, genetic isolation.

The fungus, *Verticillium dahliae* Kleb., causes wilt disease in a wide variety of plants and is identified and distinguished from other closely related fungi by the production of black microsclerotia. Among isolates of this species, however, there is considerable variation in morphology and pathogenicity (10,16,17). As yet, it is not known whether these natural variants can arise one from another or are discrete biotypes within the species.

Generally, the pathogenicity of isolates of *Verticillium dahliae* is nonspecific, although some isolates, such as those from dahlia and peppermint, have restricted or unique host ranges (4,11). Formae speciales have never been demonstrated in this species. Schnathorst and coworkers (12–15), however, were able to classify isolates of *V. dahliae* into at least two groups based on the type and severity of disease they caused. The isolates in one group produced severe disease and defoliation of cotton plants; those of the second group caused variable, though milder symptoms, and most leaves were nondehiscent until after plant death.

Verticillium dahliae is an imperfect fungus in which hyphal anastomosis, heterokaryosis, and parasexuality have been demonstrated (9). These processes seem to be the sole means by which isolates of the fungus can exchange genetic material; if two isolates cannot form heterokaryons, they are in effect genetically isolated. Such isolates could be viewed as belonging to distinct populations within the species. In the present study, several isolates of *V. dahliae* were assayed for ability to form heterokaryons and four genetically isolated populations within the species were delimited. The significance of these populations in the epidemiology of the fungus and their usefulness in future studies are discussed.

MATERIALS AND METHODS

Fungal strains. The sources and the disease reactions caused in cotton plants by 19 isolates of *Verticillium dahliae* used in this study are listed in Table 1. Eight of the isolates were defoliating types.

Media. Potato-carrot-dextrose agar (PCDA) (6) and minimal agar medium (MM) (9) were used routinely in these studies. For experiments in which restricted colony development was desired, the MM was supplemented with 0.5% L-sorbose and the sucrose content was reduced to 1%.

Inoculation of cotton plants. Each of the 19 isolates was inoculated into plants of the cotton cultivar Acala 4-42 by a stem injection technique (8). The hypocotyl of each plant was injected with approximately 50 μl of a conidial suspension adjusted to 10^6 spores per milliliter. The plants were scored for disease symptoms after 14 days.

Mutant induction. Conidia of each isolate were irradiated with ultraviolet light (UV) at 254 nm as described elsewhere (6,9). Mutants with two biochemical requirements (double auxotrophs) were recovered and characterized by the methods of Puhalla and Mayfield (9).

Mutants of *Verticillium dahliae* with brown microsclerotia also were induced by UV irradiation. Irradiated conidia were spread on plates of MM + sorbose and, after 7–10 days of incubation in the dark at 24 C, the plates were scanned for white and brown variant colonies. The white colonies were transferred to PCDA and incubated for 8 days at 24 C. A few grains of (+)-scytalone (2) were dropped on the surface of those colonies which were still white. Colonies that blackened around the scytalone were found to have colorless microsclerotia. Bell and coworkers (2) described such variants as albino, or *alm*, types. The brown variants also were transferred to PCDA and their color was noted after 8 days at 24 C. These variants had brown microsclerotia and were designated as *brm* by Bell and associates (2).

Forced heterokaryon synthesis. Doubly auxotrophic strains carrying different biochemical requirements were paired on MM at 24 C. The procedures for pairing auxotrophs and the detection and isolation of heterokaryons were those of Puhalla and Mayfield (9).

Pairing of color variants. Pairings were made either between two *alm* mutants or between an *alm* and a *brm* mutant. Small blocks of agar medium with mycelia of each mutant were placed 8–10 mm apart on a plate of PCDA. Four pairings were made on each plate

(Fig. 1). Plates were incubated at 24 C in the dark. In some pairings, cellophane (#124-PD Cellophane, E.I. duPont de Nemours & Co., Wilmington, DE 19899) was interposed between the two paired blocks. During the period of observation the cellophane was not penetrated by the fungus, but it allowed the passage of small molecules, including the intermediates in melanin biosynthesis.

RESULTS

Cotton plant inoculations. The 19 isolates listed in Table 1 were classified as defoliating and nondefoliating types based on trial inoculations made earlier in this and other laboratories. The results of inoculations of Acala 4-42 cotton plants in the present study confirmed these groupings. The defoliating isolates caused severe disease, as well as premature defoliation. The disease symptoms caused by the nondefoliating isolates varied from almost none (isolates 207 and 277) to quite severe (isolates TS-1), but none caused defoliation of the cotton plants.

Recovery of *alm* and *brm* mutants. Albino mutants were recovered from all 19 isolates; brown mutants suitable for pairing (see below) were recovered from only 14 isolates which are listed in Fig. 2. The color of microsclerotia of brown mutants ranged from very deep brown to a pale reddish tan and some of them secreted pigment into the growth medium. They were similar to those reported by Bell and colleagues (2). The frequencies of *alm* variants and *brm* variants induced by UV irradiation were similar, and they ranged 0.01–0.05% of the surviving conidia.

Pairings of *alm* × *alm*. Most pairings of *alm* mutants showed no sign of interaction. One exception was an *alm* mutant from isolate T9 that formed black microsclerotia along the line of contact with certain other *alm* types (Fig. 1A). This mutant was described by Bell and coworkers (2) who labelled it *alm-3*. When *alm-3* was paired with *alm* mutants of each of the 19 isolates, lines of black microsclerotia formed with mutants of all eight defoliating isolates. No reaction was observed with any of the albino nondefoliating isolates. The line of black microsclerotia did not form when cellophane was interposed between the paired *alm* mutants.

Pairings of *alm* × *brm*. Pairings of some *alm* mutants with *brm* mutants produced black microsclerotia well in advance of the line of contact (Fig. 1B). The blackening is due to the secretion of intermediates in the melanin biosynthetic pathway (2). Such mutants were not suitable for pairings and were discarded.

The *alm* and *brm* mutants of 14 isolates were paired in all possible combinations (Fig. 2). When an *alm* mutant was paired with a *brm* mutant of the same isolate, black microsclerotia formed along the line of contact (Figs. 1C, 2). That reaction did not occur if cellophane was interposed between the two mutants.

The line of black microsclerotia also formed between *brm* and *alm* mutants from different defoliating isolates (Fig. 2). However, when defoliating and nondefoliating isolates were paired in this way, no reaction was seen (Fig. 1D, 2). Pairings between nondefoliating isolates revealed three reacting groups. Isolates within each group, when paired, formed a line of black microsclerotia. Pairings between isolates from different groups showed no such reaction. One group included isolates 106, PH, WM, GR, TS-1, and TS-2; a second group consisted of isolates 115 and 207; and isolates 227, TA, and BB fell into a third group. The interposition of cellophane eliminated the line of black microsclerotia in the several pairings that were tested.

Forced heterokaryons. Doubly auxotrophic mutants were obtained from 12 of the 19 isolates, and these 12 are shown in Fig. 3. The auxotrophs were paired; however, not all combinations were possible because some isolates had the same biochemical requirements. Heterokaryons formed very readily between defoliating types, but never between paired defoliating and nondefoliating isolates (Fig. 3). Among pairings between auxotrophs of nondefoliating types, heterokaryons formed only in those combinations that also produced the black line of microsclerotia in *alm* × *brm* pairings (Fig. 2). The forced heterokaryons between defoliating isolates grew faster and were more robust than those between nondefoliating types.

DISCUSSION

Puhalla and Mayfield (9) showed that the prototrophic growth arising from a pairing of complementary auxotrophs of *Verticillium dahliae* is heterokaryotic. They also found that

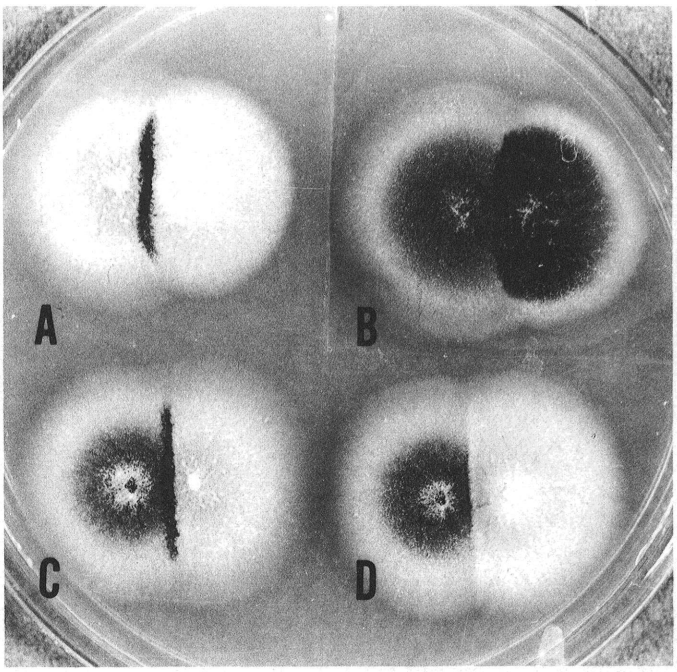

Fig. 1. Pairings of microsclerotial color variants of *Verticillium dahliae* on potato-carrot-dextrose agar medium. **A,** Two *alm* mutants forming black microsclerotia along their line of contact. **B,** A *brm* and an *alm* mutant in which the *brm* colony excretes diffusible substances that blacken most of the *alm* colony. **C,** A *brm* and an *alm* mutant forming black microsclerotia only at their line of contact. **D,** A *brm* and an *alm* mutant showing no apparent black interaction.

brm mutants of isolate	alm mutants of isolate: T9	PT-1	BA	106	PH	WM	GR	TS-1	TS-2	115	207	277	TA	BB
T9	+	+	+	−	−	−	−	−	−	−	−	−	−	−
PT-1	+	+	+	−	−	−	−	−	−	−	−	−	−	−
BA	+	+	+	−	−	−	−	−	−	−	−	−	−	−
106	−	−	−	+	+	+	+	+	+	−	−	−	−	−
PH	−	−	−	+	+	+	+	+	+	−	−	−	−	−
WM	−	−	−	+	+	+	+	+	+	−	−	−	−	−
GR	−	−	−	+	+	+	+	+	+	−	−	−	−	−
TS-1	−	−	−	+	+	+	+	+	+	−	−	−	−	−
TS-2	−	−	−	+	+	+	+	+	+	−	−	−	−	−
115	−	−	−	−	−	−	−	−	−	+	+	−	−	−
207	−	−	−	−	−	−	−	−	−	+	+	−	−	−
277	−	−	−	−	−	−	−	−	−	−	−	+	+	+
TA	−	−	−	−	−	−	−	−	−	−	−	+	+	+
BB	−	−	−	−	−	−	−	−	−	−	−	+	+	+

Fig. 2. Interactions in pairings between albino-microsclerotia (*alm*) and brown-microsclerotia (*brm*) variants of isolates of *Verticillium dahliae*. Inocula of the members of each pairing were placed 8–10 mm apart on potato-carrot-dextrose agar medium and incubated in the dark at 24 C for 7–10 days. The "+" symbol indicates that black microsclerotia formed along the line of contact of the two mutants, "−" indicates no black microsclerotia were formed.

heterozygous diploids derived from these "forced" heterokaryons could undergo the parasexual cycle. In the present study heterokaryons were made between auxotrophs of defoliating isolates of *V. dahliae* or between nondefoliating isolates. The studies of Puhalla and Mayfield (9) involved only defoliating isolates.

The line of black microsclerotia formed in certain pairings of microsclerotial color variants resulted from genetic complementation similar to that between different auxotrophs. These color variants arise from different lesions in the melanin biosynthetic pathway (2). Furthermore, such complementation between color variants probably requires true heterokaryon formation between the two mutants and not just cross feeding. Unlike the pairings of auxotrophs, there is no selection for the heterokaryon in pairings of color variants. Proof of such "nonforced" heterokaryosis is indirect but strong: there was a strict correlation between the formation of forced heterokaryons and the formation of the line of black microsclerotia in pairings, and interposition of cellophane between paired isolates eliminated this blackening reaction. Cellophane would not prevent simple cross feeding because melanin precursors can pass through it.

Through the mechanisms of heterokaryosis and parasexuality two isolates of *Verticillium dahliae* can exchange genetic information. Because the fungus has no known sexual stage, these mechanisms are probably its only means of genetic exchange. Isolates of *V. dahliae* that can form heterokaryons could therefore be viewed as belonging to one distinct population. Two isolates that cannot form heterokaryons with each other would be genetically isolated.

Among the 19 isolates of *Verticillium dahliae* that were studied, only certain pairings yielded heterokaryons. Four groups, or populations, were defined; paired isolates within a group formed heterokaryons with each other but not with isolates from another group (Fig. 4). One population contained all eight defoliating isolates. Even isolates as geographically separated as T9 (California, USA) and PT-1 (Peru) readily formed heterokaryons. This finding supports the contention of Schnathorst and Mathre (15) that all defoliating isolates are similar. The designation P1 also will be used here for the population containing defoliating isolates (Fig. 4).

A second population that corresponded to the pathogenicity type designated SS-4 by Schnathorst and Mathre (15) included all nondefoliating isolates from California as well as one from Texas. This population will be designated P2. Whereas most of the isolates of population P1 were isolated from cotton, isolates of population P2 came from several different hosts.

A third population included isolates 115 and 207 from cotton in

TABLE 1. Isolates of *Verticillium dahliae* used in heterokaryon incompatibility analysis

Isolate	Host	Geographical origin	Pathotype[a]	Disease Score in cotton[b]
T9	Cotton	Calif., USA	D	5.0
V44	Cotton	Texas, USA	D	5.0
V76	Cotton	Mexico	D	5.0
PT-1	Cotton	Peru	D	5.0
AR	Cotton	Ark., USA	D	5.0
BA	Cotton	Calif., USA	D	5.0
OL	Olive	Calif., USA	D	4.8
138	Cotton	Mo., USA	D	4.3
TS-1	Tomato	Calif., USA	N	4.5
PH	Pistachio	Calif., USA	N	3.0
GR	Grape	Calif., USA	N	3.0
106	Cotton	Calif., USA	N	2.8
WM	Cotton	Texas, USA	N	2.8
115	Cotton	Syria	N	2.6
BB	Potato	Idaho, USA	N	2.5
TS-2	Tomato	Calif., USA	N	2.4
TA	Potato	Idaho, USA	N	2.2
207	Potato	S. Australia	N	1.5
277	Sugarbeet	Wash., USA	N	1.3

[a] Disease type in cotton plants as determined previously in this and other laboratories. D = defoliation. N = no defoliation.

[b] Disease intensity in plants of cotton cultivar Acala 4-42 inoculated by hypocotyl puncture. 5 = complete defoliation and death and 1 = no disease symptoms.

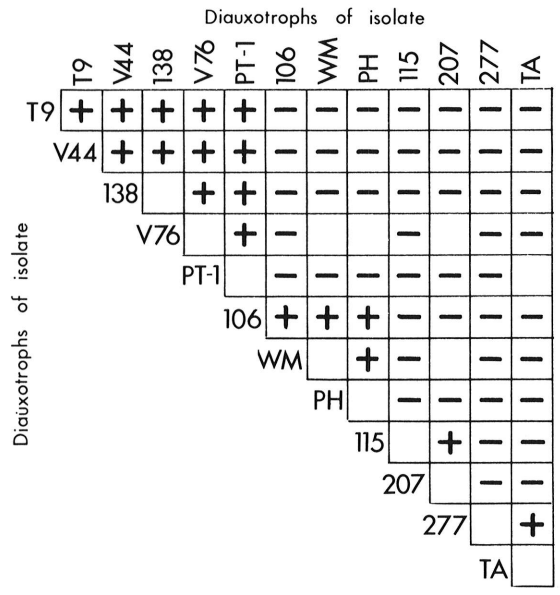

Fig. 3. Forced heterokaryons between doubly auxotrophic mutants of isolates of *Verticillium dahliae*. Small blocks of mycelial growth of complementary auxotrophs were paired on minimal medium at 24 C. The pairings were scored for prototrophic or heterokaryotic growth after 2–4 wk of incubation. The "+" symbol indicates heterokaryon formation, "–" indicates no heterokaryosis and blank squares refer to those pairings which could not be made because complementary auxotrophs were not available.

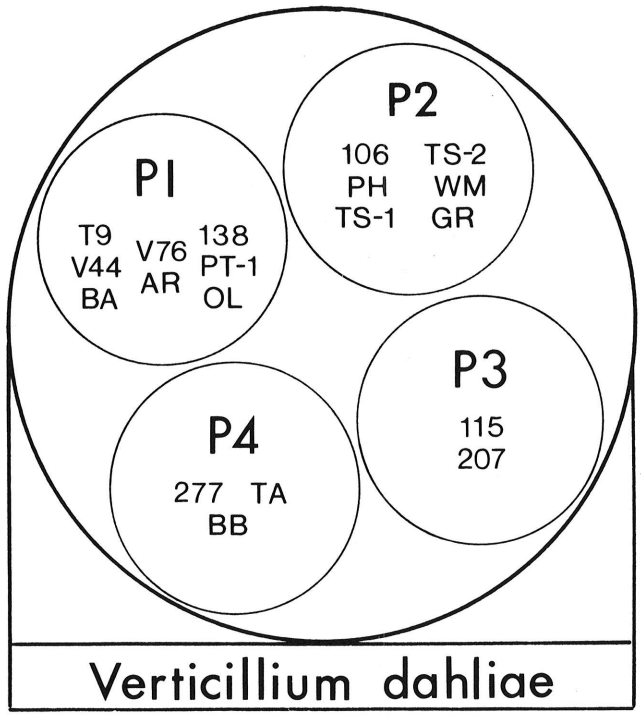

Fig. 4. Diagrammatic representation of the species *Verticillium dahliae*. The large circle represents the whole species and the smaller circles represent the four populations, P1, P2, P3, and P4, which have so far been found within the species. The isolates within each population are listed in its circle.

Syria and from potato in South Australia, respectively. The designation P3 will be applied to this population (Fig. 4). Schnathorst and Fogle (14) found that isolate 115 (their isolate V107) could be distinguished from SS-4 types because it caused much more severe disease and partial defoliation in the cotton cultivar Acala 4-42. They referred to this type as Int-1 (intermediate). In the inoculation tests reported here, however, isolate 115 was not more virulent than the SS-4 types.

All isolates of the remaining population came from the northwestern USA. This population is labeled P4 in Fig. 4.

The inability to form heterokaryons between populations may indicate that the populations have diverged genetically to the extent that prevents further interaction. Lack of heterokaryosis also could be due to the presence of vegetative incompatibility genes. In the latter case, two genetically similar isolates will not form heterokaryons if they carry different alleles at one or more so-called incompatibility loci. Such loci have been found in several fungi, including the plant pathogen, *Endothia parasitica* (1). Incompatibility genes do not prevent sexual union of two isolates, nor do they always prevent hyphal anastomoses. The present studies do not preclude the possibility of anastomosis by isolates from different populations. Ability to anastomose has been used to classify the species *Thanatephorus cucumeris (Rhizoctonia solani)* into subgroups (5).

The presence of incompatibility genes can be assessed only through sexual analysis. Because *Verticillium dahliae* has no known sexual cycle, the basis for the lack of heterokaryosis between breeding populations cannot be determined directly. If two populations are very divergent, however, this divergence may be reflected in significant differences in morphology, biochemistry, and pathogenicity. Isolates within a population, on the other hand, may share some characteristics in common. Such common traits can now be cited for isolates of population P1; all P1 isolates defoliated cotton, caused severe disease in safflower but no disease in tomato (15), detoxified sanguinarine (3), and produced a distinctive type of microsclerotia on water agar (13). The fact that Schnathorst and Fogle (14) could distinguish three of the four populations by means of pathogenicity tests in cotton suggests that these populations may have very specific and characteristic host ranges and virulence patterns. Recently Puhalla reported the presence of an unstable gene locus in *Verticillium dahliae* (7). Isolates from populations P1, P2, and P3 have this locus, whereas the three isolates of population P4 do not (J. E. Puhalla, *unpublished*). Clearly, many more such comparisons of isolates within and between populations need to be made.

The designation of genetically isolated populations within *Verticillium dahliae* should permit a determination of the origin of new pathotypes or the spread of known pathotypes. For example, when the defoliating type was first found in California 15 yr ago, some scientists believed it had arisen as a variant of the then prevalent SS-4 type. That the defoliating type and the SS-4 type are in different populations does not support this belief. On the other hand, isolates TS-1 and TS-2 have different virulence reactions on tomatoes with *Verticillium* resistance, and yet both are in the same population. Isolate TS-1 will not cause disease in tomatoes carrying the *Ve* gene for Verticillium wilt resistance; isolate TS-2 will cause disease in tomatoes having the *Ve* gene. Isolate TS-2 could have arisen as a variant of TS-1. Moreover, because the two isolates can form heterokaryons with each other, a parasexual genetic analysis of differences in virulence might be possible.

The 19 isolates of *Verticillium dahliae* used here do not represent a random sampling of the species. Therefore, an estimate of the total number of populations within the species *V. dahliae* throughout the world cannot be made at present.

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