Differences in Sensitivity of Verticillium Species to Ultraviolet Irradiation

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ABSTRACT

Fifteen isolates each of *Verticillium dahliae* and *V. albo-atrum* from several plant hosts and geographical locations were tested for sensitivity to ultraviolet light. Two major groups were found: a very sensitive group, which included 14 of the 15 *V. albo-atrum* isolates, and a relatively resistant group, which included the 15 *V. dahliae* isolates. The one remaining isolate of *V. albo-atrum*, from lucerne in the United Kingdom, was intermediate in sensitivity. Two dark mycelial variants of *V. dahliae*, derived from germinated microsclerotia, were resistant like their progenitors. Ultraviolet sensitivity is apparently a characteristic difference between the two species, and is offered as an additional criterion for separating them.

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Since Klebahn (11) established the species *Verticillium dahliae* in 1913, controversy has been continuous over whether it is a valid species or should be included with the older species *V. albo-atrum* Reinke & Berth. (18). The main distinction between the two species is the type of resting structures formed: *V. dahliae* forms microsclerotia (MS) and *V. albo-atrum* forms dark mycelium (DM). This separation has been challenged by reports that *V. dahliae* can produce variants that form DM (17, 22, 23). Other workers, however, did not observe such variations (7, 10, 12).

The two species have been separated also on biological or physiological bases. *V. albo-atrum* has a lower maximum temperature for growth and pathogenicity than does *V. dahliae* (1, 7, 12, 13, 19). Specific differences between the two have also been reported for effect of pH range on growth (7), growth on various carbon sources (7), protein bands on polyacrylamide gels (3), conidial size (21), and degree of pathogenicity (8). Conversely, other workers have emphasized similarities between the two based on common antigens (20), hyphal anastomosis (2), conidial size (15), and protein bands upon polyacrylamide gels (16).

This paper reports a parameter, sensitivity to ultraviolet light, that clearly distinguishes the two species. Possible bases for this difference in sensitivity and its value in separating the two species are discussed.

MATERIALS AND METHODS.—*Isolates.*—Isolates of *Verticillium* examined in this study along with their origin and type of resting structures are listed in Table 1. Isolates that produced MS, or originally produced them, are listed as *V. dahliae*, and isolates that produced DM are listed as *V. albo-atrum*. The two DM variants of *V. dahliae* (Isolates 16 and 17, Table 1) were isolated from germinated microsclerotia by Tolmsoff (22).

Cultural conditions.—Potato-carrot-dextrose agar

TABLE 1. Isolates of *Verticillium* used in ultraviolet sensitivity tests

Isolate No.	Identification No.	Resting structures formed[a]	Host plant and geographical origin[b]
Verticillium dahliae			
1	T9	MS	Cotton; California
2	V44	MS	Cotton; Texas
3	138	MS	Cotton; Missouri
4	201	MS	Flax; New South Wales
5	277	MS	Sugar beet; Washington
6	383	MS	Peppermint; Washington
7	111	MS	Cotton; Syria
8	104	MS	Sunflower; Canada
9	207	MS	Potato; South Australia
10	p251	MS	Avocado; Florida
11	p252	MS	Okra; Wisconsin
12	219	MS	Pepper; Greece
13	p281	MS	Potato; United Kingdom
14	p294	MS	Japanese red maple; Rhode Island
15	p295	MS	Smoke bush; Rhode Island
16	T9-49-1	DM	Variant of T9
17	222	DM	Variant of V44
Verticillium albo-atrum			
18	211	DM	Potato; South Australia
19	380	DM	Potato; Washington
20	Teccee	DM	Potato; New Hampshire
21	214	DM	Potato; Greece
22	p259	DM	Tomato; Indiana
23	p265	DM	Potato; Maine
24	p260	DM	Potato; Indiana
25	p261	DM[c]	Potato; Maine
26	p274	DM	Hops, United Kingdom
27	p275	DM	Tomato; United Kingdom
28	p285	DM	Hops; Oregon
29	p283	DM	Hops; Oregon
30	p298	DM	Potato; Ontario, Canada
31	p299	DM	Potato; Ontario, Canada
32	p276	DM	Lucerne; United Kingdom

[a] Resting structure formed on PCDA at ca. 24 C. MS = microsclerotia; DM = dark mycelium.

[b] Geographical origin may not necessarily be that of the fungal strain but rather that of the scientist supplying the strain.

[c] This strain formed resting structures only in the presence of 10^{-3} M catechol.

(PCDA) contained the following: $MgSO_4 \cdot 7\ H_2O$, 0.3 g; $CaCO_3$, 0.2 g; Difco yeast extract, 0.5 g; Difco peptone, 2.5 g; Difco potato-dextrose agar, 40 g; carrot juice, 15 ml; Difco agar, 10 g; distilled water to make one liter. Complete medium (CM) contained: KH_2PO_4, 1.36 g; K_2HPO_4, 1.68 g; $Na_2SO_4 \cdot 10\ H_2O$, 7.32 g; $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.4 g; KNO_3, 4.6 g; $MgSO_4 \cdot 7\ H_2O$, 0.7g; NaCl, 0.5 g; glucose, 20 g; Difco yeast extract, 5 g; Difco malt extract, 5 g; Difco peptone, 5 g; Difco agar, 20 g; minor element solution, 1.0 ml; distilled water to make one liter. The minor element solution was prepared by adding the following to one liter of distilled water: $ZnSO_4 \cdot 7\ H_2O$, 1.0 g; $MnCl_2 \cdot 4\ H_2O$, 1.0 g; H_3BO_3, 1.0 g; $FeCl_3 \cdot 6\ H_2O$, 0.5 g; $CuSO_4 \cdot 5\ H_2O$, 0.1 g; KI, 0.1 g; $Na_2MoO_4 \cdot 2\ H_2O$, 0.1 g. Unless otherwise specified, cultures were grown at room temperature (ca. 24 C).

Ultraviolet light treatment.—The source of ultraviolet light was a low-pressure mercury arc lamp, model R51 Mineralight (Ultra-violet Products, Inc., San Gabriel, Calif.). The lamp was used without the nickel cobalt filter, and was allowed to warm up for at least 20 min before use. Dosage was measured with a meter sensitive to radiation only at 254 nm. Conidia were scraped from the outer edges (in advance of resting structures) of 6-day-old colonies on PCDA or CM and suspended in water. The suspensions were filtered through sterile glass wool or Whatman No. 1 filter paper. Some suspensions were subsequently centrifuged and resuspended in water. However, all treatments yielded comparable results. The conidial suspensions were then adjusted to approximately 10^6 spores/ml before irradiation.

Ten ml of the conidial suspension was poured into a sterile glass petri dish (100 X 15 mm). The dish was uncovered during irradiation, and the suspension was agitated continuously with a magnetic stirring bar. The surface of the suspension was 25 cm from the light source, and received a dosage of ca. 35 ergs/mm^2 per s.

The irradiated conidial suspension was diluted, and 0.1 to 0.2 ml spread on each of two or more plates of complete medium with a sterile glass rod. The plates were wrapped in aluminum foil to exclude light during subsequent incubation. The number of colonies (ranging from 20 to 300) on each plate were counted after 2, 3, and 4 days. The percentage of viability was determined by comparing numbers of colonies from irradiated and control suspensions.

Photoreactivation.—To test the effect of visible light on killing of conidia by ultraviolet light, some plates were incubated after irradiation under continuous light from a 30-W fluorescent lamp ~1,076 lx (~100 ft-c) instead of in the dark.

Conidial size.—Conidia were scraped from the periphery of colonies on CM and mounted in water, photographed, and the dimensions of at least 50 conidia of an isolate were measured directly from the photographs.

Nuclear staining.—Conidia scraped from the periphery of colonies on CM were streaked on microscope slides. The slides were frozen and then immersed in Carnoy's fixative (absolute alcohol: chloroform:glacial acetic acid, 6:3:1) for 15 min. The slides were washed with distilled water, immersed in a solution of acridine orange (5 mg acridine orange in 100 ml of 0.1 M potassium phosphate buffer, pH 7.0) for 2 min, washed again, and mounted in water. They were examined under a Leitz Orthoplan microscope equipped for fluorescence microscopy. Under fluorescent illumination, the cytoplasm was orange and the nuclei yellow-green.

RESULTS.—*Sensitivity to ultraviolet.*—Fig. 1 shows the log of the average survival and limits of survival after various doses of ultraviolet light in 15 isolates of *V. dahliae* (isolates 1-15 in Table 1) and 14 isolates of *V. albo-atrum* (isolates 18-31). All isolates of *V. albo-atrum* were much more sensitive than those of *V. dahliae*. There was no overlapping in the

range of survival values between *V. dahliae* and *V. albo-atrum* at any dose. For example, after 30 s of exposure to ultraviolet light, the survival range in the 15 isolates of *V. dahliae* was 8.0 to 46%; among the 14 isolates of *V. albo-atrum* it was 0.02 to 0.24%.

Fig. 2 shows the survival curves for a representative isolate of *V. dahliae*, and one for *V. albo-atrum*. The ultraviolet dosage was extended to 70 s at 10-s intervals to show the limiting slopes in the curves of both species.

Isolate 32 is a strain of *V. albo-atrum* from lucerne (alfalfa) in the United Kingdom. This strain forms normal dark hyphae sparingly on PCDA medium. The survival of this isolate is intermediate between the two main groups shown in Fig. 1.

The two DM variants (isolates 16 and 17, Table 1) of *V. dahliae* were also subjected to ultraviolet light treatment. Both variants exhibited survival curves similar to the average for MS types of *V. dahliae* (Fig. 3). The resting structures of one of these variants, along with typical MS of *V. dahliae* and DM of *V. albo-atrum*, are shown in Fig. 4.

Cytology.—The difference in sensitivity to ultraviolet light between the two species is not due to any obvious differences in conidial morphology or nuclear condition. The average size of conidia of five isolates of *V. albo-atrum* (5.8 × 3.4 μ) is comparable to that of three isolates of *V. dahliae* (6.0 × 3.4 μ). Staining experiments with acridine orange showed that both species produced predominantly uninucleate conidia. Binucleate or multinucleate conidia were not observed among 200-400 conidia from each of four isolates of *V. dahliae* and two isolates of *V. albo-atrum*.

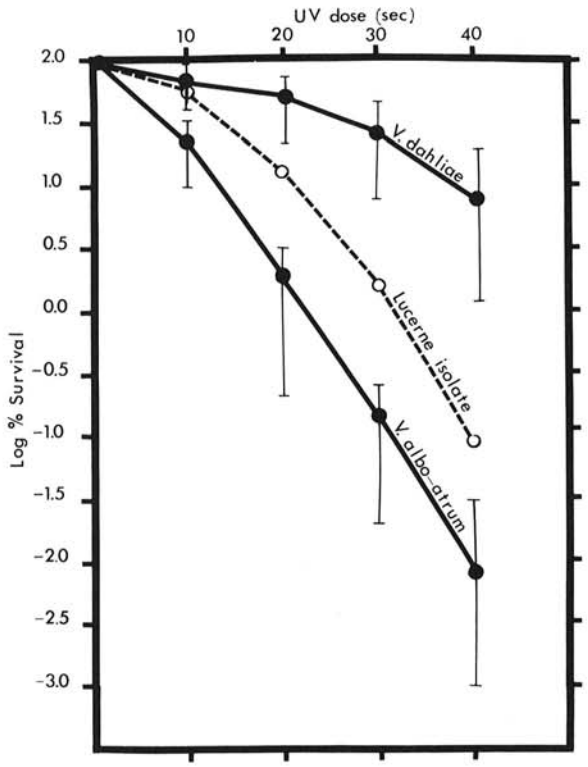

Fig. 1. Survival curves of isolates of *Verticillium* irradiated with ultraviolet light at a dose of ca. 35 ergs/mm² per sec. The top curve is the average survival of 15 isolates of *V. dahliae*. The bottom curve is the average survival of 14 isolates of *V. albo-atrum*. The vertical bars show the range of survival at each dose for each group. The middle curve is the survival of an isolate of *V. albo-atrum* from lucerne (Isolate 32).

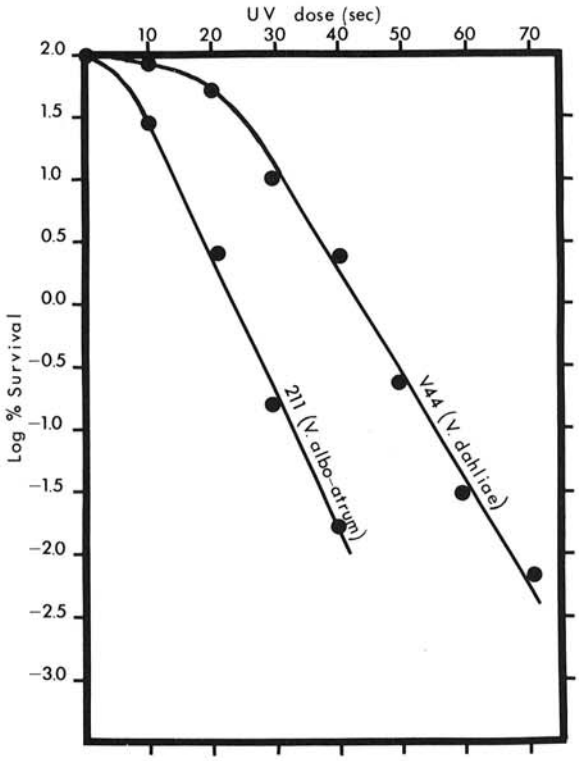

Fig. 2. Survival curves of representative isolates of *Verticillium dahliae* and *V. albo-atrum* irradiated with ultraviolet light at a dose of ca. 35 ergs/mm² per sec.

Bacteria and some fungi have enzymes that can repair DNA damage from ultraviolet light, and thereby reverse some of its lethal effects. Certain of these enzymes require visible light; their activity is called "photoreactivation" (9). All 11 isolates of *V. dahliae* and *V. albo-atrum* tested had photoreactivating enzyme systems (Table 2).

Effect of temperature.—Table 3 shows the growth rates at 21 C and 30 C of four isolates of *V. dahliae*, six isolates of *V. albo-atrum*, and the two DM variants of *V. dahliae*. The data confirm the findings of others that the isolates of *V. albo-atrum* show little or no growth at 30 C, whereas the isolates of *V. dahliae* still grow well. The two DM variants of *V. dahliae* also grew well at 30 C and thus retain this physiological characteristic of the species.

DISCUSSION.—The different sensitivity to ultraviolet light seems to be characteristic of *Verticillium dahliae* and *V. albo-atrum*, since the

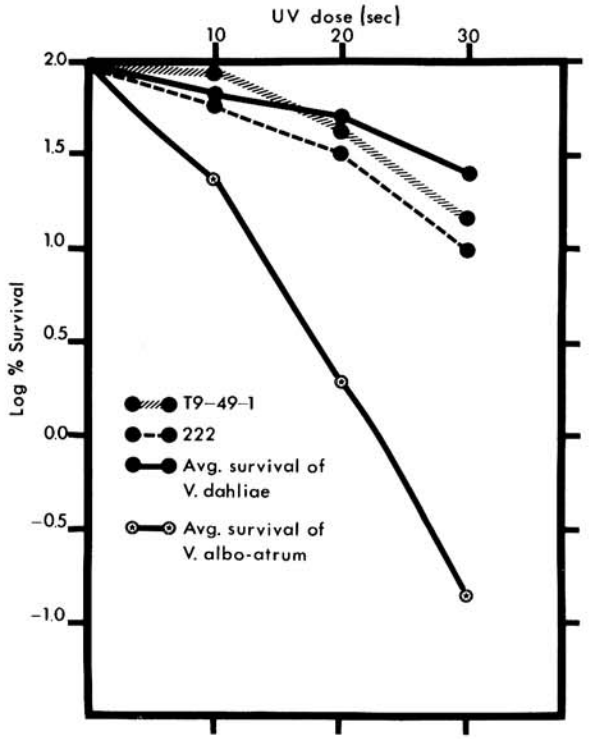

Fig. 3. Survival curves of dark mycelial variants of *Verticillium dahliae* irradiated with ultraviolet light at a dose of ca. 35 ergs/mm² per s. The average survival curves of *V. dahliae* and *V. albo-atrum* are given for comparison.

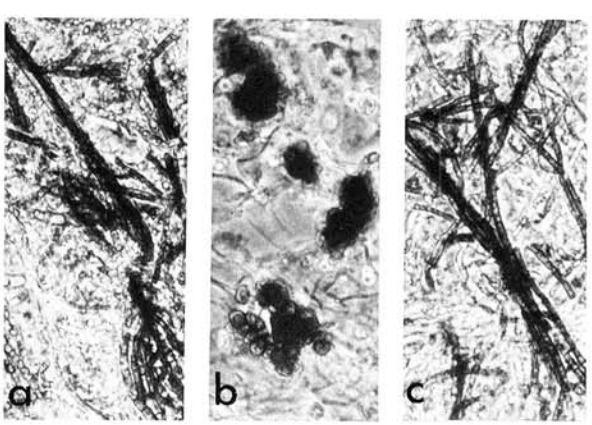

Fig. 4. Photomicrographs of the resting structures formed by *Verticillium* sp. on PCDA medium. a) Strain 222, a dark-mycelium (DM) variant of an isolate of *V. dahliae*. b) Strain T9, a *V. dahliae* isolate with typical microsclerotia. c) Strain 214, a *V. albo-atrum* isolate with typical dark mycelium. (× 280).

isolates tested represent a broad range of host plants and geographical locations. The difference is very clear-cut; and, except for the isolate from lucerne, there is no overlap between the ranges of the two species. The intermediate sensitivity of the lucerne isolate may actually reflect characteristics which set it apart from either *V. dahliae* or *V. albo-atrum*. Heale & Isaac (5) and Heale (4) found that isolates of *Verticillium* from other hosts do not attack lucerne, and lucerne isolates do not form heterokaryons with other *V. albo-atrum* strains. Nevertheless, the lucerne isolate does produce dark mycelium typical of *V. albo-atrum*, its growth is retarded at 30 C, and it is still much more sensitive to ultraviolet than any of the isolates of *V. dahliae*. For the present, it must be considered to be a strain of *V. albo-atrum*.

The literature contains scant data on the comparative killing of *Verticillium* species by ultraviolet light. While searching for variability within an isolate of *V. dahliae* and an isolate of *V. albo-atrum*, Robinson et al. noticed that the *V. albo-atrum* isolate was much more sensitive to ultraviolet light (19). Their methods were similar to those used here. These investigators, however, did not test other isolates of the two species and were unaware that greater sensitivity to ultraviolet light was characteristic of *V. albo-atrum*.

The greater sensitivity of *V. albo-atrum* to ultraviolet light might have several possible causes. A high proportion of binucleate conidia would increase resistance to ultraviolet light, but these studies show that both species produce uninucleate conidia. No differences in spore size or nuclear size were found. Furthermore, it seems unlikely that resistance to ultraviolet in *V. dahliae* can be ascribed to diploidy. The high frequency of auxotrophic mutants (1.5% at 95% kill) recovered from irradiated conidia of *V. dahliae* (author's *unpublished*) indicates haploidy.

Survival after ultraviolet irradiation is scored by the percentage of conidia that can form visible colonies after irradiation. This percentage reflects both the severity of the initial damage to the cell and the efficiency of the enzyme systems in repairing this damage. Figure 2 shows that the major difference

TABLE 2. Photoreactivation in conidia of isolates of *Verticillium dahliae* and *Verticillium albo-atrum*

Isolate No.	Ultraviolet dose (s)[a]	Survival (%)	
		In dark	In light[b]
V. dahliae:			
1	40	0.7	12
2	40	1.7	32
5	40	4.4	37
8	30	11.0	49
9	40	1.3	13
12	30	29.0	43
V. albo-atrum:			
18	20	2.4	65
19	20	4.3	41
21	20	0.4	58
22	20	3.0	73
24	20	1.7	45

[a] Conidial suspensions irradiated at a distance of 25.4 cm (10 inches), which delivers a dose of 35 ergs/mm² per sec.

[b] Incubation under continuous light from a 30-watt fluorescent light [1,076 lx (100 foot-candles)].

TABLE 3. Comparative growth rates of *Verticillium dahliae* and *Verticillium albo-atrum* on complete medium at 21 and 30 C

Isolate No.	Growth (mm)[a] 21 C	30 C
V. dahliae:		
1	42	45
2	45	43
13	51	33
15	56	33
V. albo-atrum:		
18	50	2
21	50	5
23	53	0
24	51	0
28	39	3
32	50	7
DM variants of *V. dahliae*[b]:		
16	53	47
17	50	49

[a] Growth measured in diam (mm) of a colony on complete agar medium after 14 days of incubation. Each measurement is the average of three replicates.

[b] DM variants are those that produce a dark mycelium.

between the two survival curves is the more pronounced shoulder in the curve for *V. dahliae*. In bacteria (6) and yeast (14), such an enhanced shoulder has been ascribed to a more efficient enzymatic repair of damage caused by ultraviolet, and this may be the case in *V. dahliae*. Enzymatic repair can be divided into light-dependent repair and dark repair. Light-dependent repair, or photoreactivation, was found in all strains of *Verticillium* tested in this study.

Of all the physiological differences between the two forms of *Verticillium*, only two – maximum temperature for growth and ultraviolet light sensitivity – are clear-cut. These findings establish that the two species are definitely distinct. Since the two DM variants of *V. dahliae* still retained the temperature response and ultraviolet sensitivity of the MS parent, any other variants of *V. dahliae* which resemble *V. albo-atrum* will probably not be true conversions.

Whether the differences in temperature response and ultraviolet sensitivity warrant separating the two morphological forms into separate species can still be argued. Any decision must reflect a certain amount of subjectivity; the final argument must rest on convenience and clarity. I believe that the two species should be kept separate. Anything less than this masks the fact that *V. dahliae* and *V. albo-atrum* are two clearly distinct groups of organisms.

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