Tolerance of *Verticillium dahliae* to Benzimidazoles

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ABSTRACT

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Microsclerotial and album isolates of *Verticillium dahliae* and dark mycelial isolates of *V. albo-atrum* were screened for tolerance to MBC phosphate (MBC-P), thiabendazole, thiophanate-methyl (TM), MBC-HCl, and thiabendazole hydrochloride at 1, 5, 10, 100, and 500 μg/ml. Album isolates grew at all concentrations, whereas microsclerotial and dark mycelial isolates were inhibited by all benzimidazoles except TM at concentrations higher than 1 μg/ml. Sectors from album isolates grew on 1,000 μg/ml of MBC-P. A subculture from a microsclerotial isolate grew at 500 μg/ml MBC-P. Chemical tolerance remained unchanged after 40 wk of storage at 2 C or after 13 serial subcultures onto unamended potato-dextrose agar. Pathogenicity studies with chemically sensitive and tolerant microsclerotial isolates and tolerant album isolates from elm and maple indicated that tolerant and sensitive microsclerotial isolates were more pathogenic than album isolates on both American elm (*Ulmus americana*) and red maple (*Acer rubrum*).

Chemotherapy of vascular diseases has become more feasible with the development of benzimidazole fungicides. These chemicals have shown some potential for control of Verticillium wilt. Verticillium wilt of cotton was controlled in the greenhouse by applications of benomyl and thiabendazole (2,6). Benzimidazoles applied as a soil drench reduced Verticillium wilt in potatoes and strawberry (1,11) and in chrysanthemum when applied to the leaves (3). Rice (17) injected MBC phosphate (MBC-P) into Norway (*Acer platanoides* L.) and sugar (*A. saccharum* Marsh.) maples infected with *Verticillium dahliae* Kleb. Control was more effective in the former species.

Although many plant-pathogenic fungi are controlled by benzimidazoles, tolerant strains have been reported (7,12,13,18,19,23). Talboys and Davies (20) reported benzimidazole tolerance in strawberry isolates of *V. dahliae* up to 12 μg/ml. Emmanouil and Wood (5) found isolates of *V. dahliae* from tomato tolerant to 1,500 μg/ml. E. E. Conaway (*personal communication*) found that microsclerotial or dark mycelial isolates from a culture collection were inhibited, whereas the album isolates grew at 5 μg/ml of MBC-P.

This study reports the frequency, level, and stability of benzimidazole tolerance in *Verticillium* and the pathogenicity of tolerant and sensitive isolates.

MATERIALS AND METHODS

Mass screening of *Verticillium* isolates. We determined the tolerance of 25 isolates of *Verticillium* from our culture collection to Lignasan BLP, a 0.7% solution of methyl 2-benzimidazole-carbamate phosphate (MBC-P); thiabendazole (Arbotect 20-S); and thiophanate-methyl (TM) (Fungo 50WP). The fungitoxicity of the benzimidazoles was tested by incorporating them into potato-dextrose agar (PDA) at 10 μg/ml. MBC-P and thiabendazole were sterilized by vacuum-filtration through a Gelman Metrical filter apparatus. PDA was amended by adding the appropriate volumes of the sterilized chemical to molten PDA. PDA was amended with TM by adding the appropriate weights to molten agar to achieve the desired concentrations.

The following procedure for determining benzimidazole tolerance was used throughout the study unless otherwise noted. Isolates were transferred to PDA

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plates and grown for 14 days at 22 ± 2 C in the dark. Mycelial plugs 5 mm in diameter were transferred to each of three replicate plates of amended and unamended PDA and incubated for 14 days at 22 ± 2 C either on a laboratory bench or in the dark. Colony growth was measured as the average of two diameters per replicate and was expressed as a percentage of the diameter of the controls.

Four isolates were selected for further study of their tolerance to benzimidazoles. V-Ea and V-Ma, album isolates, and V-Ems and V-Mms, microsclerotial isolates from American elm (*Ulmus americana* L.) and sugar maple (*Acer saccharum* Marsh.), respectively, were grown on PDA amended with MBC-P, thiabendazole, TM, MBC-HCl, and thiabendazole-HCl at 0, 1, 5, and 100 μg/ml.

Rapidly growing fan-shaped sectors developed from album isolates on PDA amended with 50, 75, 100, and 500 μg/ml of MBC-P. Twelve supertolerant (ST) album sectors (V-Ea 50, 75, 101, 102, 501, 502; V-Ma 75, 100, 101, 102, 103, and 501) were compared for their levels of benzimidazole tolerance with parent isolates.

A sector from V-Mms growing on TM-amended PDA at 100 μg/ml was designated as a benzimidazole-adapted microsclerotial (BAM-5) isolate. The tolerance levels of the BAM-5 and its parent isolate were compared by transferring mycelial plugs from 10-day-old cultures to PDA amended with 0, 1, 5, 100, 500, and 1,000 μg/ml MBC-P and measuring diameter growth as described previously.

Stability of benzimidazole tolerance. We determined the stability of tolerance of seven album isolates of *V. dahliae* stored at 2 C on unamended PDA and PDA amended with 1 μg/ml MBC-P. Tolerance was tested at 5- to 15-wk intervals for about 40 wk on PDA plates amended with MBC-P ranging from 1 to 1,000 μg/ml.

As a second measure of stability of tolerance, the seven album and four ST isolates used before and two microsclerotial isolates were serially subcultured onto PDA after an initial test of their tolerance levels. Isolates were transferred from stock cultures and grown 10 days at 22 ± 2 C in the dark. Agar plugs were transferred to the centers of plates of PDA or PDA amended with MBC-P at 5 μg/ml for album isolates, 500 μg/ml for ST album isolates, and 1 μg/ml for microsclerotial isolates. Tolerance was determined as described for stored isolates, then one unamended PDA plate of each isolate was selected at random for the next serial transfer to amended and unamended PDA plates. This serial procedure was repeated 14 times with album isolates, 7 with ST isolates, and 8 with microsclerotial isolates.

Pathogenicity studies. We examined the pathogenicity of six *V. dahliae* isolates from elms and maples. These included microsclerotial isolates V-Ems, V-Mms, and BAM-5 and album isolates V-Ea, V-Ea 50, and V-Ma. American elm and red maples (*A. rubrum* L.) were used as host plants. For experiment 1, a soil-peat-perlite (1:1:1) mix was steamed in flats for two 1-hr periods several days apart. Seeds were planted 1 wk after steaming.

Inoculum was prepared by transferring mycelial plugs of each isolate into 60 ml of potato-dextrose broth (PDB) in 125-ml flasks and agitating cultures on a Model 75 Burrell wrist-action shaker for 3 days at 22 ± 2 C. The resulting spore suspensions were diluted with distilled water to 10^7 cells per milliliter. Roots of 6-wk-old seedlings were immersed in the conidial suspensions of isolates V-Ems, V-Ea, and V-Ea 50 for 18 hr at 22 ± 2 C. Roots of control seedlings were immersed on diluted PDB. Plants were enclosed in plastic bags during inoculation to prevent wilting.

Immediately after inoculation, stems of 10 seedlings with each isolate and 10 controls were surface-sterilized with 2.7% sodium hypochlorite solution for 5 min. A basal stem section of each plant was plated onto PDA and another section was placed in a moist chamber to determine initial fungus intake. The remaining seedlings were planted in steamed soil mix in flats.

For experiment 2, spore suspensions of isolates BAM-5, V-Mms, and V-Ma were prepared as described previously. One-year-old potted American elms and red maples were inoculated with a drop of spore suspension through a scalpel wound in the base of the stem.

Foliar symptoms were rated at 2, 4, 6, 8, and 10 wk using a five-point scale, where 1 = no symptoms, 2 = mottling and/or necrosis of the lower leaf pair, 3 = mottling and/or necrosis of less than 50% of the plant, 4 = mottling and necrosis of more than 50% of the plant, and 5 = dead or completely defoliated plant.

After 10 wk, vascular discoloration in the stem was noted and fungus isolations were made from 2.5-cm sections cut from each third of the stem and 9-mm-diameter disks from leaves from the same stem section. All tissue sections were sterilized for 5 min in 2.7% sodium hypochlorite plus 2 drops of Tween 80 per 100 ml of solution. The tissue was rinsed twice with sterile distilled water and placed in a moist chamber or onto Czapek-Dox agar. A plant was rated as infected if *Verticillium* grew from any tissue section.

RESULTS

Mass screening of *Verticillium* isolates. Seven album isolates of *V. dahliae* had an average diameter of 19.7, 44.4, and 59.7% of control on PDA amended with 10 μg/ml of MBC-P, thiabendazole, and TM, respectively. Nine *V. dahliae* microsclerotial isolates had 0, 0, and 7.9% diameter growth, and two *V. albo-atrum* isolates 0, 0, and 6% diameter growth for the respective treatments.

With the exception of limited growth at 1 μg/ml, microsclerotial isolates V-Ems and V-Mms were completely inhibited by all fungitoxicant treatments except TM. Album isolates V-Ea and V-Ma grew up to 44% of controls at 100 μg/ml. MBC chemicals were most toxic, followed by thiabendazole and TM (Table 1).

ST sectors appeared the same macroscopically on amended and unamended PDA; however, the undersides of the parent colonies were convoluted and the mycelium was sometimes appressed to the amended medium. ED_{50}s for parent isolates V-Ea and V-Ma were 30 and 35 μg/ml, respectively, whereas those of the ST sectors were all higher than 1,000 μg/ml. There were no correlations between tolerance of ST sectors and concentration of MBC-P in the medium from which they were isolated. For example, ST sectors V-Ea 50, 75, 101, and 501, selected from isolate V-Ea on PDA amended with MBC-P at 50, 75, 100, and 500 μg/ml, respectively, grew at 91, 99, 92, and 94% of controls on PDA amended with MBC-P at 100 μg/ml.

BAM-5 grew appressed and produced microsclerotia. BAM-5 grew at 98, 91, 93, and 42% of controls on PDA amended with 1, 5, 100, and 500 μg/ml MBC-P but was completely inhibited at 1,000 μg/ml.

Stability of benzimidazole tolerance.

Table 1. Diameter growth of colonies of *Verticillium dahliae* isolates on potato-dextrose agar amended with MBC phosphate (MBC-P), thiabendazole, and thiophanate-methyl (TM)

	Benzimidazole (μg/ml)														
	MBC-P			MBC-HCl			Thiabendazole			Thiabendazole HCl			TM		
Isolate[a]	1	5	100	1	5	100	1	5	100	1	5	100	1	5	100
V-Ems	7[b]	0	0	0	0	0	6	0	0	0	0	0	76	1	0
V-Mms	0	0	0	0	0	0	0	0	0	0	0	0	90	14	5
V-Ea	57	20	19	55	27	16	99	63	15	90	54	20	95	70	37
V-Ma	64	34	26	65	38	27	95	71	18	90	68	22	93	73	44

[a] V-Ems and V-Mms are microsclerotial isolates and V-Ea and V-Ma are album isolates of *V. dahliae* from American elm and sugar maple, respectively.

[b] Two colony diameters were averaged after 14 days at ambient temperature in the dark. Growth measurements represent three replicates per treatment and are expressed as a percentage of controls.

Table 2. Pathogenicity of microsclerotial (ms) and album (a) isolates of *Verticillium dahliae* (V) from American elm (E) and sugar maple (M) to American elm and red maple

Isolate	American elm				Red maple			
	No. plants treated	Disease rating[u]	Plants with vascular discoloration (%)	Infected plants[v] (%)	No. plants treated	Disease rating	Plants with vascular discoloration (%)	Infected plants (%)
				Experiment 1[w]				
V-Ems	64	3	30 a[x]	88 a	34	2	64 a	89 a
V-Ea	64	1	2 b	25 b	35	1	0 b	50 a
V-Ea 50	61	1	2 b	24 b	29	1	0 b	41 a
Control	62	1	0 b	2 c	38	1	0 b	3 b
				Experiment 2[y]				
V-Mms	27	...[z]	37 a	35 a	22	3	75 a	56 a
V-Ma	30	...	0 b	0 b	26	1	42 b	13 b
BAM-5	30	...	31 a	16 b	25	3	75 a	13 b
Control	29	...	0 b	0 b	25	1	0 c	0 b

[u] Foliar symptoms were rated after 10 wk: 1 = no symptoms, 2 = necrosis of lower leaf pair, 3 = necrosis of less than 50% of plant, 4 = necrosis of more than 50% of plant, 5 = dead or defoliated plant.
[v] A plant was considered infected if *V. dahliae* was isolated from any leaf or stem tissue.
[w] Six-week-old seedlings were inoculated by dipping roots in a conidial suspension of test isolate for 18 hr. After inoculation, seedlings were planted in steamed soil mix (1 part soil:1 part peat:1 part perlite).
[x] Values followed by the same letter within a column for each species are not significantly different at the 0.05 level as determined by a χ^2 test.
[y] One-year-old American elms and red maples were inoculated with a drop of conidial suspension through a scalpel wound in the lower stem.
[z] Mite damage precluded reliable determination of foliar symptoms.

Benzimidazole tolerance remained unchanged in album isolates V-Ea and V-Ma stored at 2 C on either PDA or PDA amended with 1 μg/ml MBC-P after 40 wk or after 14 biweekly serial transfers on PDA. Microsclerotial isolate V-Ems showed no significant change in tolerance after eight serial transfers on PDA, whereas V-Mms increased significantly in colony diameter from 12 to 23% of controls. There were no significant changes in benzimidazole tolerance of any other ST album sectors after 37 wk storage at 2 C on either PDA or amended PDA nor after 14 biweekly serial transfers onto unamended PDA.

Pathogenicity studies. After 10 wk, foliar symptoms were present only in American elms and red maples inoculated with microsclerotial isolates V-Ems, V-Mms, and BAM-5 (Table 2). Significantly more elms and maples inoculated with microsclerotial isolates developed vascular discoloration than those inoculated with album isolates V-Ea, V-Ea 50, and V-Ma. V-Ems and V-Mms caused higher percentages of infection in both elms and maples and BAM-5 caused a higher percentage of infection in elms than V-Ea 50 or V-Ma. V-Ea and V-Ea 50 were reisolated from stem and leaf segments in the lower portion of the plant only, whereas V-Ems was recovered from stem and leaf tissue from all plant sections. *Verticillium* was reisolated from one uninoculated elm and maple.

DISCUSSION

Many pathogens, including *Verticillium*, have developed tolerance to benzimidazoles after several years of successful use (8,20,23). The impact of tolerant isolates on control of *Verticillium* with benzimidazoles is influenced by the occurrence of tolerant strains in nature, the levels and stability of their tolerance, and their pathogenicity.

There is little information on the prevalence of tolerant album isolates in nature. Several workers reported that *Verticillium* loses its ability to form microsclerotia after prolonged culturing. However, nonmicrosclerotial isolates have been cultured from diseased plants (14,15,21).

Verticillium tolerance to benzimidazoles, at concentrations of 10–12 μg/ml, has been reported in nature prior to chemical exposure (20,23). Tolerant album isolates used in this study, too, were in culture before the use of benzimidazoles or were from hosts where such chemical treatments were unlikely. Thus, benzimidazole-tolerant isolates apparently occur in nature in the absence of chemical selection.

It is difficult to predict the levels of fungal tolerance necessary to overcome chemical concentrations in treated plants. It is likely, however, that tolerance levels of our album, ST album, and BAM-5 isolates would exceed chemical concentrations found in treated plants under control conditions. The stability of tolerance in these isolates increases the difficulty of controlling them with benzimidazoles.

Pathogenicity of tolerant isolates will influence their role as disease-causing agents. Others (16,23) reported that album isolates were less pathogenic than microsclerotial strains. Although album isolates did not produce foliar symptoms, they were reisolated from both elms and maples. Isaac (10) and Tompkins and Ark (22), however, reported that hyaline and microsclerotial isolates were equally pathogenic.

Our sensitive, microsclerotial isolates from elm and maple were more pathogenic on both elms and maple than were tolerant album isolates. Tolerant strains, however, can survive in such symptomless hosts and may be returned to the soil in plant debris and serve as a source of inoculum. Others (4,9) have reported that album isolates do not survive on soil as long as microsclerotial types. This, combined with reduced pathogenicity, may lessen their impact as disease agents.

Isolates such as BAM-5 that combine high levels of pathogenicity, chemical tolerance, and microsclerotial production may significantly reduce disease control. Microsclerotial production and benzimidazole sensitivity appear to be independent but closely linked genetic characteristics. Tolerant, microsclerotial, pathogenic isolates such as BAM-5 and that reported by Emmanouil and Wood (5) may result from genetic recombination.

The close association we found between chemical tolerance of album isolates and sensitivity of microsclerotial cultures was unexpected. Because pigmented, thick-walled microsclerotia are the resting stage that enables *Verticillium* to survive biotic and abiotic stresses of the soil environment, they might be expected to be more resistant to chemical activity than thin-walled, unpigmented album cells. Because the reverse was true, chemical tolerance or sensitivity appears related to factors other than cell-wall structure.

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