Genetic Analysis of Isolates of *Botrytis cinerea* Sensitive and Resistant to Benzimidazole and Dicarboximide Fungicides

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ABSTRACT

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A total of 56 isolates of *B. cinerea* collected from ornamental crops from commercial greenhouses were examined by random amplified polymorphic DNA (RAPD) fingerprint analyses. Isolates were examined as two independent sets of 35 and 36 isolates, with 15 isolates common to both sets. The isolates had four phenotypes: 17 were sensitive to two commonly used fungicides, thiophanate-methyl (a benzimidazole) and vinclozolin (a dicarboximide) (S^{TSV}); 18 were resistant to both fungicides (R^{TRV}); 16 were resistant to thiophanate-methyl but sensitive to vinclozolin (R^{TSV}); and 5 were sensitive to thiophanate-methyl but resistant to vinclozolin (S^{TRV}). Relationships among the isolates were determined by cluster analyses of mean character differences using the unweighted pair group method using arithmetic average and cladograms were constructed. Isolates were clustered primarily by fungicide-sensitivity phenotype. In one set of greenhouse isolates, 6 of 10 S^{TSV} isolates clustered together with a bootstrap confidence value of 91%. In the other

fingerprint set of greenhouse isolates, 9 of 11 S^{TSV} isolates clustered together and had a bootstrap confidence value of 98%. Isolates resistant to thiophanate-methyl, vinclozolin, or both fungicides usually were not clustered with other isolates or were clustered with isolates of the same phenotype. To further elucidate these relationships, variant isolates resistant to one or both fungicides were produced on fungicide-amended agar medium from 14 S^{TSV} greenhouse isolates. These 14 S^{TSV} parent isolates, 57 resistant variant isolates, and 11 resistant greenhouse isolates were analyzed as three independent RAPD fingerprint sets. Variants selected from eight S^{TSV} parent isolates were resistant to both thiophanate-methyl and vinclozolin even though parent isolates were exposed to only one of the fungicides. Isolates resistant only to vinclozolin (S^{TRV}) had fingerprint patterns similar to and clustered with those of parent isolates, while fingerprint patterns of isolates resistant to thiophanate-methyl (i.e., R^{TRV} or R^{TSV}), regardless of sensitivity to vinclozolin, clustered differently from both those of S^{TSV} parent isolates and those of S^{TRV} isolates derived from the same parent. R^{TRV} and R^{TSV} variant isolates derived from the same fungicide-sensitive parents only clustered with other variants having the same phenotype.

Botrytis cinerea Pers.:Fr. is a common and economically important pathogen of numerous greenhouse-grown ornamental crops (4,16). Ornamental crops grown in greenhouses and nurseries are some of the most valuable agricultural products in the United States; greenhouse-grown ornamental crops alone had an estimated value of \$3.5 billion in 1993 (8). To reduce the negative impact of *Botrytis* blight, an effective management strategy should include sanitation and other cultural practices as well as fungicide applications to prevent or limit disease spread (4,16). Because host resistance to *B. cinerea* is not available for most plants, fungicides are important for managing leaf, stem, and flower blights in greenhouse crop production. However, resistance to the most commonly used classes of fungicides, the benzimidazoles and dicarboximides, has compromised the effectiveness of these fungicides in many populations of *B. cinerea* (2). Resistance complicates disease management and can lead to serious economic losses. Although benzimidazoles (e.g., thiophanate-methyl) have been used most commonly to control many foliar pathogens in greenhouses (8), dicarboximides (e.g., vinclozolin) may be more effective for managing *B. cinerea* (16).

Benzimidazole-resistant isolates of *B. cinerea* persist long after the use of benzimidazole fungicides is discontinued (9,21,24,37).

Dicarboximide fungicides were introduced in the mid-1970s, and within a decade, dicarboximide-resistant isolates were common on several crops (15,19,23,24,30,31). The incidence of fungicide resistance in populations of *B. cinerea* has become so common on ornamental crops that in some regions these fungicides no longer are recommended for use (29). Reversion of resistant populations to ones with fungicide sensitivity has not been observed even after selection pressure has been removed; resistant populations have persisted even with minimal or no fungicide use (21,31).

Recently, 325 isolates of *B. cinerea* were collected from ornamental crops in 35 commercial greenhouses in 16 counties of South Carolina (42). Isolates resistant to thiophanate-methyl or vinclozolin were found in 32 of 35 greenhouses. Resistance to both fungicides was the most common phenotype found, accounting for 67% of all isolates collected, and only 17% of the isolates were sensitive to both fungicides. Of the remaining isolates, 14% were resistant to thiophanate-methyl and sensitive to vinclozolin, and only 2% (six isolates) were sensitive to thiophanate-methyl and resistant to vinclozolin.

Random amplified polymorphic DNA (RAPD) fingerprint analysis has been a useful technique to study geographic variation within species and to detect clonal diversity (12). It has been used to identify genetic variation in several plant-pathogenic fungi, e.g., *Fusarium oxysporum* (1), *Magnaporthe poae* (14), *Alternaria* spp. (34), and *Ophiostoma piceae* (32). Despite its importance as a plant pathogen, reports of genetic diversity in populations of *B. cinerea* have been limited. In the Netherlands, RAPD-marker

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polymorphisms were found among 29 isolates of *B. cinerea* having different virulence characteristics; however, there was no correlation between genetic polymorphism and virulence, time of sampling, or sampling location (20). Resistance of the isolates to fungicides was not an objective of that study.

A genetic approach to the study of variation among isolates of *B. cinerea* that differ in fungicide sensitivity is reported here. The first objective was to use RAPD technology and cluster analysis to determine the genetic diversity and relatedness among greenhouse isolates of *B. cinerea* that differed in sensitivity to the fungicides thiophanate-methyl and vinclozolin. The second objective was to use fungicide-resistant variant isolates derived from fungicide-sensitive greenhouse isolates to further elucidate genetic relationships among isolates that differed in fungicide sensitivity. A preliminary report has been published (43).

MATERIALS AND METHODS

Fungicides. Commercial flowable formulations of thiophanate-methyl (Cleary's 3336F, 46% active ingredient [a.i.] by weight) and vinclozolin (Curalan, 41.3% a.i. by weight) were used. Fungicides were stored at 15°C in the dark to maintain and preserve fungicide activity.

Isolates of *B. cinerea*. A total of 56 isolates of *B. cinerea* was selected for genetic examination; 55 isolates were selected from among 325 greenhouse isolates recently collected in South Carolina (42). These 55 isolates came from diseased ornamental plants—primarily geranium, poinsettia, impatiens, and begonia—from 30 different greenhouses (Table 1). One additional isolate, DC-5, was recovered from a diseased geranium growing in the landscape in Washington, DC. Previously, phenotype designations were determined by conidium germination and mycelium growth on fungicide-amended minimal nutrient medium, and virulence was determined by a geranium seedling bioassay (42). Four phenotypes were identified: seventeen isolates (including isolate DC-5) were sensitive to both thiophanate-methyl and vinclozolin ($S^T S^V$), eighteen isolates were resistant to both fungicides ($R^T R^V$), sixteen isolates were resistant to thiophanate-methyl and sensitive to vinclozolin ($R^T S^V$), and five isolates were sensitive to thiophanate-methyl and resistant to vinclozolin ($S^T R^V$).

For this study, phenotypes were verified by testing conidia from all isolates for germination on a defined minimal medium, as described previously (42), amended with either thiophanate-methyl or vinclozolin at 5 µg/ml. Conidium suspensions were standardized to a concentration of 2 to 3 × 10⁴ conidia per ml with a hemacytometer; 10-µl drops were pipetted onto the surface of the medium; and approximately 20 drops were added to a dish. Conidium suspensions also were pipetted onto unamended medium as controls. Dishes containing conidia were placed in the dark at 20°C for 14 to 18 h to allow conidia to germinate. Agar surfaces were covered with a solution of 0.025% aniline blue in 50% lactic acid to stop fungus growth and facilitate enumeration of conidia with a microscope (50×). Germination rate was determined by counting the number of conidia out of 100 that had germ tubes with lengths at least three times the diameter of a conidium and that grew into the agar and appeared normal. Conidia of individual isolates were consistent in their responses to fungicide-amended media, i.e., at least 93% of conidia of any isolate responded similarly to fungicide exposure. Isolates with conidia that germinated on fungicide-amended medium were designated resistant to that fungicide.

All isolates were stored in an ultra-low-temperature freezer (−80°C) in 15% glycerol in 2-ml cryogenic vials (Nalgene Corporation, Rochester, NY) and were retrieved from storage as described previously (42). Retrieved conidia were used as propagules to start mycelium cultures for DNA extraction (described below) and to verify phenotype designation with the conidium germination assay (described above).

Selection of fungicide-resistant variants. To further study genetic variation and relationships identified among the 56 greenhouse isolates of *B. cinerea*, isolates resistant to one or both of the fungicides with known pedigrees were needed. Therefore, resistant variants (i.e., asexual clonal derivatives) were selected from parent isolates sensitive to both fungicides. In 1997, conidia of 15 $S^T S^V$ isolates of *B. cinerea* (each from a distinct collection site) were harvested from dishes of potato dextrose agar (PDA) and placed in suspension in 0.01% Tween 80. Conidium suspensions (1 to 5 × 10⁶ conidia per ml) were spread on petri dishes containing the minimal medium described above amended with a single fungicide—either thiophanate-methyl at 10 µg/ml or vinclozolin at 5 µg/ml. Conidia from each of the 15 isolates were spread on medium amended with thiophanate-methyl and on medium amended with vinclozolin, with five dishes per isolate for each fungicide; dishes were placed at 20°C in the dark for 5 days. All 15 isolates produced some normal colonies after 5 days on fungicide-amended medium; colonies were subcultured by placing hyphal tips on the same fungicide-amended medium from which they had been selected. These variants were placed at 20°C in the dark for 3 days, and 12 of the 15 isolates continued to produce apparently normal colonies. Tips of hyphae from these colonies were transferred to unamended PDA for further growth and conidium production. Parent isolates yielded at least one normal-appearing variant isolate that was resistant to at least one fungicide. A total of 70 variant isolates were selected and assayed by conidium germination for phenotype designation; conidia then were stored in a freezer at −80°C.

A similar selection procedure was conducted in 1998, with 26 $S^T S^V$ isolates used as parents. After 5 days on fungicide-amended medium, 20 of the 26 isolates yielded at least one normal-appearing colony. After hyphal tips were transferred to fungicide-amended media and incubated for 3 days, 12 of the 20 sensitive parent isolates yielded viable and normal-appearing colonies. A total of 78 variant isolates were selected from these 12 parents, and hyphae of these isolates were transferred to PDA for conidium production. Conidia were tested with the germination assay to verify phenotypes, and conidium suspensions were stored at −80°C. Six parent isolates were common to both selection procedures that were conducted in 1997 and 1998. The rate of occurrence of variant isolates in the two selection procedures ranged from approximately 10^{−4} to 10^{−8}. Of the 148 resistant variants selected in both years, 57 isolates were used in this study. EC₅₀ values were calculated (42) for variant isolates based on a mycelium growth assay on nutrient agar medium amended with thiophanate-methyl at 0, 0.05, 0.1, 0.5, 1, 2, 5, 50, or 500 µg/ml and vinclozolin at 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 10, or 100 µg/ml. Isolates were considered resistant when EC₅₀ values were equal to or greater than 1 µg per ml of thiophanate-methyl or vinclozolin (Table 2).

Isolate nomenclature. Greenhouse isolates were given identification numbers that consisted of uppercase letters to indicate the geographic site of collection (Table 1), a number indicating the individual isolate from that site, and a lowercase letter, *a* or *b*, if two isolates were collected from the same piece of diseased plant tissue. Variant isolates were designated by the number of the parent isolate followed by /T or /V to indicate selection on thiophanate-methyl- or vinclozolin-amended medium, respectively, and a number or letter to identify individual variants (Table 2).

Preparation of mycelia. Gamborg's B-5 basal medium (broth) with vitamins and minimal organic compounds (Sigma BioSciences, St. Louis, MO) was prepared according to the label (3.2 g/liter). Sterile 30% glucose (final concentration 0.3%; 10 ml) and sterile 10% K₂HPO₄ (final concentration, 0.25 g/liter; 2.5 ml) were added to 1 liter of sterile medium that was cooled to ambient temperature (final pH of 6.6). Broth (20 ml) was dispensed into sterile disposable petri dishes (15 × 100 mm), and a conidium

suspension (0.5 ml), containing approximately $1 \text{ to } 2 \times 10^7$ conidia per ml, was added to each dish. Dishes were placed at 20°C in the dark, and, after 3 days, the mycelium cultures were harvested. Mycelium mats were washed with sterile, distilled water and blotted to remove excess liquid. For each isolate, a volume of 300 to 500 µl of mycelium was placed in a sterile microcentrifuge tube, and the tubes were stored at -80°C.

DNA extraction. The procedure for extracting DNA was modified from the method of Möller et al. (27). Frozen mycelia were thawed, and the mats were ground with a mortar and pestle in liquid nitrogen. The powdered mycelium was added to sterile 1.5-ml microcentrifuge tubes containing 500 µl of TES extraction buffer (100 mM Tris, pH 8; 10 mM EDTA, pH 8; 2% sodium dodecyl sulfate [SDS]). RNase A (stock = 10 mg/ml; Sigma BioSciences) was

TABLE 1. EC₅₀ values^a for 56 isolates of *Botrytis cinerea* collected from ornamental plants from greenhouses in South Carolina that were used in genetic analyses

Isolate	Source			Thiophanate-methyl		Vinclozolin	
	Host plant and organ	County	Phenotype ^b	EC ₅₀ -germ	EC ₅₀ -growth	EC ₅₀ -germ	EC ₅₀ -growth
A-2-b	Petunia flower	Georgetown	S ^T S ^V	0.48	0.10	2.52	0.26
A-7-b	Geranium flower	Georgetown	R ^T S ^V	>500	>500	1.01	0.25
A-8-a	Impatiens leaf	Georgetown	S ^T R ^V	0.40	0.13	500	1.85
B-14-b	Pansy flower	Saluda	S ^T S ^V	1.06	0.08	2.21	0.25
B-5-b	Poinsettia bract	Saluda	R ^T R ^V	>500	>500	>500	21.08
B-9-b	Poinsettia leaf	Saluda	R ^T S ^V	>500	>500	0.51	0.22
BT-3-b	Geranium flower	Richland	S ^T R ^V	2.35	0.33	>500	1.68
BT-4-a	Geranium leaf	Richland	R ^T S ^V	>500	>500	0.89	0.16
BU-3-b	Begonia leaf	Charleston	R ^T R ^V	>500	>500	>500	1.90
BU-5-a	Impatiens flower	Charleston	R ^T S ^V	>500	>500	0.81	0.21
BW-1-a	Geranium leaf	Charleston	R ^T S ^V	>500	>500	1.28	0.27
BW-1-b	Geranium leaf	Charleston	R ^T R ^V	>500	>500	>500	3.14
BW-3-b	Geranium flower	Charleston	S ^T S ^V	0.63	0.07	0.87	0.03
C-3	Camellia flower	Berkeley	R ^T S ^V	>500	>500	1.87	0.27
CC-4-a	Begonia leaf	Charleston	S ^T R ^V	0.74	0.05	>500	1.11
CC-5-a	Geranium leaf	Charleston	R ^T S ^V	>500	>500	0.87	0.20
CC-6-a	Geranium flower	Charleston	R ^T R ^V	>500	>500	>500	1.98
CC-6-b	Geranium flower	Charleston	S ^T S ^V	0.52	0.10	1.40	0.20
CG-1-b	Geranium leaf	Colleton	R ^T R ^V	>500	>500	>500	1.03
CG-2-a	Ivy-geranium leaf	Colleton	S ^T S ^V	1.87	0.09	0.93	0.21
CH-5-a	Geranium leaf	Charleston	S ^T S ^V	0.22	0.15	1.40	0.28
CH-5-b	Geranium leaf	Charleston	R ^T S ^V	>500	>500	0.72	0.36
CH-6-b	Geranium leaf	Charleston	S ^T R ^V	0.88	0.08	>500	2.04
CL-1-b	Azalea flower	Oconee	S ^T S ^V	0.95	0.10	2.70	0.35
CN-1-a	Gerbera daisy leaf	Horry	S ^T S ^V	0.53	0.16	1.65	0.46
CN-2-b	Gerbera daisy flower	Horry	R ^T R ^V	>500	>500	>500	1.11
DC-5	Geranium flower	Washington, DC ^c	S ^T S ^V	0.23	0.14	1.62	0.07
DD-10-a	Calendula flower	Newberry	S ^T S ^V	1.05	0.05	1.34	0.28
DD-4-b	Geranium leaf	Newberry	R ^T R ^V	>500	>500	>500	2.17
DD-9-b	Cosmos flower	Newberry	R ^T S ^V	>500	>500	1.55	0.26
FL-4-b	Geranium leaf	Florence	S ^T S ^V	2.07	0.36	0.85	0.12
GA-1	Chrysanthemum leaf	Dorchester	S ^T S ^V	0.89	0.30	2.93	0.26
GG-4-a	Pansy flower	Georgetown	R ^T R ^V	>500	>500	>500	1.81
GG-4-b	Pansy flower	Georgetown	R ^T S ^V	>500	>500	2.57	0.34
GO-1-b	Geranium leaf	Marion	R ^T R ^V	>500	>500	>500	1.52
GO-2-a	Begonia flower	Marion	R ^T S ^V	>500	>500	2.11	0.29
H-3-a	Geranium flower	Dorchester	S ^T R ^V	0.43	0.06	>500	1.65
H-3-b	Geranium flower	Dorchester	R ^T S ^V	>500	>500	0.75	0.24
H-4-b	Begonia flower	Dorchester	S ^T S ^V	2.22	0.07	1.17	0.19
HC-1-b	Artemesia leaf	Calhoun	R ^T R ^V	>500	>500	>500	1.25
HC-2-a	Impatiens flower	Calhoun	S ^T S ^V	0.81	0.09	1.17	0.24
HH-5-a	Petunia flower	Richland	S ^T S ^V	0.10	0.12	2.64	0.16
HH-9-b	Begonia flower	Richland	R ^T S ^V	>500	>500	1.16	0.18
HK-1-b	Geranium leaf	Lexington	R ^T S ^V	>500	>500	1.07	0.25
HK-3-a	Geranium leaf	Lexington	R ^T S ^V	>500	>500	1.30	0.19
HL-7-1	Poinsettia leaf	Oconee	S ^T S ^V	0.54	0.08	3.51	0.31
MD-3-a	Impatiens flower	Georgetown	R ^T R ^V	>500	>500	>500	1.76
MD-6-a	Geranium flower	Georgetown	S ^T S ^V	0.48	0.16	2.95	0.20
PB-1-a	Geranium leaf	Richland	R ^T R ^V	>500	>500	>500	1.31
PL-4-b	Snapdragon flower	Richland	S ^T S ^V	1.12	0.10	2.21	0.27
PP-4-b	Oregano leaf	Charleston	S ^T S ^V	0.43	0.10	2.66	0.21
PS-4-b	Geranium flower	Colleton	R ^T R ^V	>500	>500	>500	1.97
PW-6-b	New Guinea impatiens leaf	Richland	R ^T R ^V	>500	>500	>500	1.41
S-1-b	Poinsettia leaf	York	R ^T R ^V	>500	>500	>500	11.90
S-8-a	Cattlea petal	York	R ^T S ^V	>500	>500	1.72	0.29
SI-4-b	Begonia leaf	Charleston	R ^T R ^V	>500	>500	>500	1.37
UG-2	Geranium leaf	Greenville	S ^T S ^V	1.83	0.07	0.98	0.34
UG-9	Geranium flower	Greenville	R ^T R ^V	>500	>500	>500	1.30
WD-1	Cherry tomato fruit	Oconee	R ^T R ^V	>500	>500	>500	1.60

^a EC₅₀ values were the effective concentrations of fungicides that inhibited conidium germination (EC₅₀-germ) or mycelium growth (EC₅₀-growth) by 50%.

^b Phenotypes were sensitive (S) or resistant (R) to thiophanate-methyl (^T) or vinclozolin (^V); isolates were resistant to fungicides if EC₅₀ values for conidium germination or mycelium growth were ≥5 µg/ml or ≥1 µg/ml, respectively.

^c Isolate collected in Washington, DC.

dispensed into each suspension for a final concentration of 50 µg/ml, and tubes were held at 37°C for 30 min. Proteinase K (stock = 10 mg/ml; Sigma BioSciences) was added to each suspension to give a final concentration of 100 µg/ml, and suspensions were held at 60°C for 60 min. Two hundred microliters of 5 M NaCl and 70 µl of warm 10% CTAB (hexadecyltrimethyl-ammonium bromide) (Sigma BioSciences) were added to each solution, and solutions were held at 65°C for 10 min. An aliquot of 700 µl of chloroform:isoamyl alcohol (24:1) was added to each tube, and tubes were placed on ice for 30 min. Tubes with solutions were centrifuged at 13,000 rpm for 10 min. The upper phase was transferred to a new tube, and 225 µl of 5 M NH₄OAc (1.2 M final concentration) was added. Solutions were mixed gently and placed on ice for at least 30 min. To precipitate DNA, 0.55× (510 µl) of cold isopropanol was added to each tube, and tubes were centrifuged at 13,000 rpm for 5 min. DNA pellets were washed once with 70% ethanol containing 10 mM NH₄OAc and once with 70% ethanol and were suspended in 200 to 300 µl of nuclease-free water (Promega Corp., Madison, WI). For each isolate, DNA was extracted independently from two cultures and used as template DNA for separate RAPD fingerprint replicate sets.

Primers. An initial screen of 130 primers against 12 isolates of *B. cinerea* (3 isolates of each phenotype) was conducted with Operon 10-mer oligonucleotide kits OP-A, -B, -C, -D, -K, and -T (Operon Technologies Inc., Alameda, CA) and 10 laboratory primers (courtesy of Y. Wang and R. A. Dean, Genomics Institute, Clemson University, Clemson, SC) to search for polymorphisms. Based on this screen, 12 primers were selected for use in this study: OPB-9, -11, -19, and -20, OPC-3, -9, and -14, OPD-4, -6, -9, and -10, and laboratory primer no. 162 (5' AACTTACCGC 3').

RAPD-Polymerase chain reaction procedures. DNA solutions were quantified by fluorometry (TKO 100, Hoefer Scientific Instruments, San Francisco, CA) and diluted in nuclease-free water to working concentrations of 3.33 ng/µl (i.e., 500 ng/150 µl of water). Two microliters of template DNA (or water as a negative control) was dispensed into 0.2-ml polymerase chain reaction (PCR) tubes (Corning, Corning, NY). A PCR reaction mixture was made for each individual primer (all constituents of the mixtures except primers were purchased from Promega Corp.) and contained 11.8 µl of nuclease-free water, 2.0 µl of 10× *Tfl* reaction buffer, 1.2 µl of MgSO₄ (25 mM), 0.8 µl of dNTPs (2.5 mM each), 2.0 µl of 10-base primer (6.25 µM), and 0.2 µl of *Tfl* polymerase (approximately 1.0 unit). Eighteen microliters was pipetted into each PCR tube containing 2 µl of template DNA. The tubes containing 20 µl were placed in a AmpliTron II thermocycler (Barnstead/Thermolyne Corp., Dubuque, IA) with an initial denaturing temperature of 94°C for 4 min. Each cycle was programmed as follows: (i) fast ramp to 94°C; (ii) 94°C (15 s hold); (iii) fast ramp to 44°C; (iv) 44°C (30 s hold); (v) 60 s ramp to 72°C; and (vi) 72°C (60 s hold). After 45 cycles, a final extension temperature of 72°C for 5 min was followed by a dwell temperature of 4°C.

RAPD fingerprints. Gels (100 × 250 mm) were prepared with 1.4% agarose (Mallinckrodt Baker Inc., Paris, KY) in 100 ml of 0.5× Tris-boric acid-EDTA (TBE) buffer (33), and wells were formed with a 40-well comb (3 mm per lane). Gels were placed in an electrophoresis apparatus containing 0.5× TBE. Each PCR reaction was provided with 2 µl of 10× gel-loading buffer, and 20 µl were loaded into each well. Each of three lanes was loaded with approximately 1 ng of a 123-bp DNA ladder (Gibco BRL, Life Technologies Inc., Gaithersburg, MD). Electrophoresis was carried out for 3 h at 70 V and gels were stained for 25 min in 200 ml of 0.5× TBE with 4 µl of ethidium bromide (10 mg/ml). Gels were washed in water for 45 min before photographing with type 667 (positive) film and type 665 (positive/negative) film with a No. 16A Wratten filter (Polaroid Corp., Cambridge, MA) and UV light from a transilluminator.

Fingerprint analyses. In all, five RAPD fingerprint sets were generated for this study. Each set contained 12 gels—1 for each of

the selected primers. The 56 greenhouse isolates were separated into 2 sets of 20 and 21 isolates, with the remaining 15 isolates used in each set. In this way, genetic relationships identified for one set of isolates could be confirmed and validated by independent analysis of the second set of isolates. Therefore, Greenhouse (GH) set 1 contained 35 greenhouse isolates: 10 S^{TSV} isolates, 10 R^{TRV}, 10 R^{TSV}, and 5 S^{TRV}; and GH set 2 contained 36 isolates: 11 S^{TSV}, 11 R^{TRV}, 9 R^{TSV}, and 5 S^{TRV}. The 15 isolates that were common to both sets included 4 that were sensitive to both fungicides, 3 that were resistant to both fungicides, 3 that were resistant to thiophanate-methyl and sensitive to vinclozolin, and 5 that were sensitive to thiophanate-methyl and resistant to vinclozolin.

To further study genetic variation and the relationships identified among the greenhouse isolates, 82 isolates (57 variant isolates, 14 sensitive greenhouse isolates that were parents of the variant isolates, and 11 resistant greenhouse isolates) were analyzed as 3 RAPD fingerprint sets, with 36 isolates used in each set. Variant set 1 contained 32 variant and 4 S^{TSV} parent isolates; Variant set 2 contained 24 variant, 6 S^{TSV} parent isolates, 4 R^{TRV}, and 2 R^{TSV} greenhouse isolates; and Variant set 3 contained 23 variant isolates, 8 S^{TSV} parent, and 5 S^{TRV} greenhouse isolates. Some isolates were used in more than one set. A single trial consisted of running the 12 gels for one fingerprint set. Each trial was conducted twice, and the data initially were analyzed separately. Data for the two trials of each fingerprint set were nearly identical so they were combined and analyzed together. Binary matrices were constructed according to the presence or absence of bands. Cluster analyses were performed by the unweighted pair group method using arithmetic average (UPGMA) (7,38) with the software program PAUP* (Phylogenetic Analysis Using Parsimony and other methods; version 4.0b2, Sinauer Associates, Sunderland, MA). Distances were measured by mean character differences. Bootstrap analyses of 500 replicates by full heuristic general searches were conducted to evaluate the confidence level of cluster patterns. Consensus cladograms were constructed based on the 50% majority rule. Cladograms were labeled above the branches with mean character difference values and bootstrap confidence values, listed in parentheses.

RESULTS

Greenhouse isolates. In all, 56 isolates of *B. cinerea* collected from ornamental plants were subjected to RAPD fingerprint analyses to examine the genetic variation among isolates with different sensitivities to thiophanate-methyl and vinclozolin. To verify relationships and establish reproducibility, isolates were analyzed as 2 independent sets with 15 isolates common to both sets. Both sets produced similar results. Relationships among isolates with the same phenotypes were consistent in both fingerprint sets of greenhouse isolates, and the 15 isolates that were common to both fingerprint sets clustered similarly in the 2 sets. When clusters with high bootstrap values occurred, they were composed of isolates with the same phenotype. In GH set 1, 197 characters were informative and were used to construct a cladogram detailing relationships among isolates (Fig. 1A); in GH set 2, 179 characters were used to construct a cladogram (Fig. 1B). One primer, OPD-6, gave a strong amplification product of approximately 450 bp in all isolates of *B. cinerea* examined. This has been suggested as a possible marker for *B. cinerea* (20,39).

Ten and eleven isolates sensitive to both fungicides (S^{TSV}) were included in GH sets 1 and 2, respectively, with four isolates (B-14-b, DC-5, GA-1, and HL-7-1) common to both sets. In GH set 1 (Fig. 1A), 6 of 10 sensitive isolates were clustered together, with a bootstrap value of 91%. These six isolates included three (B-14-b, GA-1, and HL-7-1) of the four sensitive isolates that were common to both fingerprint sets. Two other S^{TSV} isolates clustered together (DC-5 and UG-2), and the remaining two S^{TSV} isolates (BW-3-b and CC-6-b)

did not cluster with any other isolate. In GH set 2 (Fig. 1B), 9 of 11 S^{TSV} isolates formed a single large cluster—including B-14-b, GA-1, and HL-7-1—and the other 2 S^{TSV} isolates were not closely associated with other S^{TSV} isolates in the set.

In contrast, in both GH sets 1 and 2, isolates that were resistant to one or both fungicides either did not cluster with other isolates or clustered with other resistant isolates of the same phenotype. In GH set 1 (Fig. 1A), the following clusters of similar phenotypes occurred: R^{TSV}: HK-3-a and HH-9-b, H-3-b and A-7-b; S^{TRV}: CH-6-b and BT-3-b, A-8-a and CC-4-a; and R^{TRV}: BW-1-b and B-5-b, UG-9 and GO-1-b, CN-2-b and CG-1-b. The remaining isolates did not cluster. In GH set 2 (Fig. 1B), the following clusters of resistant isolates occurred: R^{TSV}: HK-1-b and HH-9-b, C-3 and GG-4-b; S^{TRV}: CC-4-a and A-8-a, BT-3-b and CH-6-b (as in GH set 1); and R^{TRV}: HC-1-b and PB-1-a, GO-1-b and CC-6-a, BU-3-b and MD-3-a.

Variant isolates. A total of 148 variant isolates resistant to thiophanate-methyl, vinclozolin, or both was selected in the laboratory from 14 S^{TSV} parents that had been placed on media amended with either thiophanate-methyl or vinclozolin. Of these, 57 variants (R^{TSV}, S^{TRV}, and R^{TRV}) were selected for further study genetic variation and relationships among isolates with known parentage (Table 2). Three RAPD fingerprints sets (Variant sets 1, 2, and 3) were generated with the 57 variants, 14 parent, and 11 resistant isolates recovered from greenhouses. In all three sets, all isolates had a strong amplification product of approximately 450 bp from reactions primed by OPD-6, which was consistent with results for isolates of *B. cinerea* in GH sets 1 and 2.

EC₅₀ values for mycelium growth were greater than 500 µg of thiophanate-methyl per ml for all but one of the variants resistant to thiophanate-methyl (Table 2). Variants resistant to thiophanate-methyl and vinclozolin (R^{TRV}) had EC₅₀ values ranging from 1.0 to 9.1 µg of vinclozolin per ml. Variants resistant to thiophanate-methyl and sensitive to vinclozolin (R^{TSV}) had EC₅₀ values ranging from 0.1 to 0.2 µg of vinclozolin per ml. Variants sensitive to thiophanate-methyl and resistant to vinclozolin (S^{TRV}) had EC₅₀ values ranging from 0.03 to 0.4 µg of thiophanate-methyl per ml and 1.8 to >100 µg of vinclozolin per ml.

Eight parent isolates produced at least one variant isolate resistant to both fungicides (R^{TRV}) even though parent isolates were exposed to only one fungicide in the selection procedure. Parent isolate CH-5-a produced 29 clonal derivatives on thiophanate-methyl-amended medium and 28 clonal derivatives on vinclozolin-amended medium in two independent selection procedures. All of these isolates were resistant to both fungicides, except one R^{TSV} variant selected on thiophanate-methyl-amended medium. Nine of the variants from parent CH-5-a were used in this study.

Variant set 1. RAPD fingerprints were made with 36 isolates—4 S^{TSV} parent and 32 resistant variants (i.e., 24 R^{TRV}, 5 R^{TSV}, and 3 S^{TRV}). A cladogram (Fig. 2A) was constructed from a data set with 169 informative characters. The four S^{TSV} parent isolates and three S^{TRV} variants, all from parent isolate HH-5-a, clustered with a bootstrap value of 100%. In contrast, variants resistant to thiophanate-methyl (either R^{TRV} or R^{TSV}) clustered independently from their S^{TSV} parent isolates—including the only R^{TRV} clonal derivative of HH-5-a. In addition, most variants derived from the same parent with the same phenotype clustered together with high bootstrap values, but these variants did not cluster with clonal derivatives from the same parent with different phenotypes. RAPD profiles of all eight R^{TRV} clonal derivatives from parent CH-5-a were nearly identical and clustered with a bootstrap value of 100%. However, variant CH-5-a/T6, was R^{TSV} and was the only clonal derivative of CH-5-a that did not cluster with the R^{TRV} variants. All four R^{TSV} clonal derivatives from parent FL-4-b clustered together with a bootstrap value of 100%; these four isolates clustered independently, however, from the seven R^{TRV} clonal derivatives from this same parent.

Variant set 2. RAPD fingerprints from a second set of variant isolates were analyzed to further investigate the genetic relation-

TABLE 2. Phenotypes of and EC₅₀ values for thiophanate-methyl and vinclozolin for 57 variant isolates of *Botrytis cinerea*^a

No. ^c	Isolate	Year ^e	EC ₅₀ -growth ^b	
			Thiophanate-methyl	Vinclozolin
B-14-b/T1	R ^{TRV}	1997	>500	1.1
B-14-b/T2	R ^{TRV}	1997	>500	3.7
B-14-b/T3	R ^{TRV}	1997	>500	9.1
B-14-b/T4	R ^{TRV}	1997	>500	7.7
B-14-b/V1	R ^{TRV}	1997	>500	2.3
B-14-b/V2	R ^{TRV}	1997	>500	4.2
B-14-b/V3	R ^{TRV}	1997	>500	3.2
B-14-b/V4	R ^{TRV}	1997	>500	3.2
BW-3-b/V1	S ^{TRV}	1997	<0.1	>100
BW-3-b/Va	S ^{TRV}	1998	0.1	2.2
BW-3-b/Vb	S ^{TRV}	1998	0.2	25.2
CC-6-b/T1	R ^{TRV}	1997	>500	1.6
CC-6-b/T2	R ^{TRV}	1997	>500	4.5
CC-6-b/V1	R ^{TRV}	1997	>500	1.2
CH-5-a/T1	R ^{TRV}	1997	>500	1.1
CH-5-a/T6	R ^{TSV}	1997	>500	0.1
CH-5-a/T20	R ^{TRV}	1997	>500	1.5
CH-5-a/Tb	R ^{TRV}	1998	>500	1.5
CH-5-a/Tj	R ^{TRV}	1998	>500	1.5
CH-5-a/V1	R ^{TRV}	1997	>500	1.8
CH-5-a/V9	R ^{TRV}	1997	>500	1.6
CH-5-a/Va	R ^{TRV}	1998	>500	1.6
CH-5-a/Vh	R ^{TRV}	1998	>500	1.3
DC-5/V1	R ^{TRV}	1997	>500	1.0
DC-5/V2	S ^{TRV}	1997	0.2	>100
DD-10-a/V1	S ^{TRV}	1997	0.1	>100
DD-10-a/V2	S ^{TRV}	1997	0.1	>100
FL-4-b/T1	R ^{TRV}	1997	>500	1.4
FL-4-b/T2	R ^{TRV}	1997	>500	1.6
FL-4-b/T3	R ^{TRV}	1997	>500	1.4
FL-4-b/T4	R ^{TRV}	1997	>500	1.2
FL-4-b/T5	R ^{TSV}	1997	>500	0.1
FL-4-b/Tb	R ^{TSV}	1998	>500	0.2
FL-4-b/Tff	R ^{TSV}	1998	>500	0.1
FL-4-b/Tg	R ^{TSV}	1998	241.1	0.1
FL-4-b/V1	R ^{TRV}	1997	>500	1.2
FL-4-b/V3	R ^{TRV}	1997	>500	1.4
FL-4-b/V4	R ^{TRV}	1997	>500	3.3
GA-1/T1	R ^{TSV}	1997	>500	0.1
GA-1/V1	S ^{TRV}	1997	0.1	2.6
GA-1/V2	R ^{TRV}	1997	>500	1.2
GA-1/Va	S ^{TRV}	1998	0.1	>100
GA-1/Vb	S ^{TRV}	1998	0.2	>100
H-4-b/T1	R ^{TSV}	1997	>500	0.1
HC-2-a/Va	S ^{TRV}	1998	0.1	1.8
HC-2-a/Vb	S ^{TRV}	1998	0.2	2.1
HH-5-a/V1	S ^{TRV}	1998	0.2	>100
HH-5-a/V2	S ^{TRV}	1998	0.2	>100
HH-5-a/V3	R ^{TRV}	1998	>500	1.3
HH-5-a/V4	S ^{TRV}	1998	0.2	>100
HL-7-1/Vc	S ^{TRV}	1998	0.1	57.4
HL-7-1/Vd	S ^{TRV}	1998	0.1	19.2
MD-6-a/Vb	S ^{TRV}	1998	0.4	>100
MD-6-a/Vf	S ^{TRV}	1998	0.2	>100
MD-6-a/Vh	S ^{TRV}	1998	0.3	>100
MD-6-a/Vi	S ^{TRV}	1998	0.2	>100
UG-2/V1	R ^{TRV}	1997	>500	1.4

^a Selected in 1997 or 1998 from fungicide-amended media. Conidium suspensions of isolates sensitive to both fungicides (S^{TSV}) were spread on minimal agar medium amended with a single fungicide (thiophanate-methyl, 10 µg/ml, or vinclozolin, 5 µg/ml) and placed at 20°C in the dark for 5 days. Colonies that appeared normal were subcultured on the same fungicide-amended medium from which they had been selected and then were transferred to unamended potato dextrose agar for continued growth and conidium production.

^b EC₅₀ values were calculated as the effective concentration of a fungicide that inhibited mycelium growth by 50% on fungicide-amended medium.

^c Isolate numbers consisted of arbitrary uppercase letters to indicate the geographic site of collection (Table 1); a number corresponding to an individual piece of infected plant tissue; a lowercase letter, a or b, when two isolates were collected from the same piece of infected tissue; and a variant designation, based on whether variants were produced on thiophanate-methyl (T)- or vinclozolin (V)-amended medium and a selection number or letter.

^d Phenotypes were sensitive (S) or resistant (R) to thiophanate-methyl (T) or vinclozolin (V); EC₅₀ values ≥1 µg of fungicide per ml. indicated resistant isolates.

^e The year variant isolates were selected.

Fig. 1. Genetic relationships among 56 isolates of *Botrytis cinerea* resistant (R) or sensitive (S) to thiophanate-methyl (^T) or vinclozolin (^V) collected from ornamental crops in commercial greenhouses in South Carolina. Isolates were divided into two sets for random amplified polymorphic DNA (RAPD) fingerprint analyses: **A**, Greenhouse set 1 had 35 isolates, and **B**, Greenhouse set 2 had 36 isolates; 15 isolates were common to both sets (*). Table 1 explains isolate numbers. Phenotype designations follow numbers. Mean character difference values and bootstrap confidence values [in ()] are labeled above branches. The control was the RAPD profile of polymerase chain reactions without template DNA. Cladograms were constructed by PAUP software, version 4.0b2.

ships among isolates resistant to thiophanate-methyl and included 6 S^TS^V parent, 24 resistant variants (i.e., 19 R^TR^V, and 5 R^TS^V), and 6 greenhouse isolates resistant to thiophanate-methyl (i.e., 4 R^TR^V and 2 R^TS^V). A cladogram was constructed (Fig. 2B) from a data set with 184 informative characters. Isolates in this set that also were used in other fingerprint sets clustered similarly. For example, S^TS^V isolates B-14-b, CH-5-a, and FL-4-b were common to Variant sets 1 and 2 and clustered together in both sets with a bootstrap value of 100%. They were among the 12 S^TS^V isolates that were clustered similarly in GH sets 1 and 2 (Fig. 1A and 1B, respectively). As in Variant set 1, fungicide-sensitive parent isolates clustered separately from thiophanate-methyl-resistant variants derived from them except for isolate CC-6-b, which clustered with one of its own R^TR^V clonal derivatives and one R^TR^V greenhouse isolate from the same location and away from other S^TS^V parent isolates. Again, thiophanate-methyl-resistant variants with the same phenotype and same parent tended to cluster together, if at all, and clonal derivatives from the same parent with different phenotypes clustered independently. Three of the six resistant greenhouse isolates clustered with unrelated isolates with similar phenotypes, and the other three greenhouse isolates did not cluster.

Variant set 3. RAPD fingerprints of this set were examined to further investigate the relationships of fungicide-sensitive parent isolates (S^TS^V) with variants that remained sensitive to thiophanate-methyl but were resistant to vinclozolin (S^TR^V). This set included 8 S^TS^V parent isolates, 23 variants (i.e., 20 S^TR^V, 2 R^TR^V, and 1 R^TS^V), and 5 S^TR^V greenhouse isolates, which also were included in GH sets 1 and 2 (Fig. 1A and 1B, respectively). A cladogram for these isolates (Fig. 2C) was constructed from a data set with 196 informative characters. Variant set 3 corroborated the overall relationship between S^TS^V parents and S^TR^V variants that was observed in Variant set 1. In fact, the genetic relationship among S^TS^V parents and their S^TR^V clonal derivatives was stronger than the relationship among unrelated S^TS^V parents. All eight S^TS^V parent isolates clustered with at least some of their S^TR^V clonal derivatives. A cluster of 16 isolates that included 5 S^TS^V parent isolates and their 11 S^TR^V variants clustered with a bootstrap value of 98%. Within this group were five clusters containing individual S^TS^V parent isolates and their S^TR^V clonal derivatives with bootstrap values ranging from 87 to 99%. For example, S^TS^V parent isolate DD-10-a was clustered with its two S^TR^V clonal derivatives with a bootstrap value of 96%. The relationship of S^TS^V parent HH-5-a and its three S^TR^V clonal derivatives corroborated results observed in Variant set 1 (Fig. 2A). S^TR^V variants also were more similar to their S^TS^V parents than to the five wild-type S^TR^V isolates collected from greenhouses and included in this fingerprint set. Only one S^TR^V greenhouse isolate, CC-4-a, clustered with a variant isolate of the same phenotype, BW-3-b/V1.

As in other fingerprint sets, variants with resistance to thiophanate-methyl were genetically very distant from their parents. Parent isolate GA-1 clustered with its three S^TR^V clonal derivatives but not with its two thiophanate-methyl-resistant clonal derivatives, GA-1/T1 (R^TS^V) and GA-1/V2 (R^TR^V). Similarly, parent isolate DC-5 clustered with its only S^TR^V clonal derivative, DC-5/V2, but not with its R^TR^V clonal derivative, DC-5/V1. Interestingly, the two R^TR^V variant isolates GA-1/V2 and DC-5/V1 clustered together.

DISCUSSION

Genetic mechanisms for the development of resistance by many organisms to fungicides and other pesticides have been the subject of theoretical and experimental investigations for many years (26). Isolates of *B. cinerea* associated with ornamental crops that are resistant to the popular and commonly used fungicides thiophanate-methyl and vinclozolin occur widely on various crops around the world (2,4,16); however, the genetic variation associated with this resistance has not been investigated fully. In this study, analyses

of RAPD marker polymorphisms were used to study genetic variation and relatedness among isolates of *B. cinerea* sensitive and resistant to fungicides. Twelve primers provided a sufficient number of informative characters to construct cladograms and identify relationships among the isolates. RAPD profiles were consistent between trials with the same isolates, relationships among isolates

common to different fingerprint sets were similar, and relationships identified with one set of isolates were confirmed and validated with other sets of independent isolates.

Cluster analyses of 56 greenhouse isolates of *B. cinerea*, from diverse geographic areas in South Carolina, were analyzed as 2 independent RAPD fingerprint sets and revealed a wide range of

Fig. 2. Genetic relationships among 82 isolates, including 57 variant and 25 greenhouse isolates, of *Botrytis cinerea* that were resistant (R) or sensitive (S) to thiophanate-methyl (^T) or vinclozolin (^V). Isolates were separated into three sets of 36 isolates for random amplified polymorphic DNA (RAPD) fingerprint analyses: **A**, Variant set 1 had 32 variant isolates and 4 sensitive parent isolates (S^TS^V); **B**, Variant set 2 had 24 variant, 6 S^TS^V parent, and 6 resistant greenhouse isolates (*) (4 R^TR^V, 2 R^TS^V); and **C**, Variant set 3 had 23 variant, 8 S^TS^V parent, and 5 S^TR^V greenhouse isolates (*). All variant isolates were produced in the laboratory from S^TS^V greenhouse isolates (Table 2). Phenotype designations follow isolate numbers. Mean character difference values and bootstrap confidence values (in parentheses) are labeled above branches. The control was the RAPD profile of polymerase chain reactions without template DNA. Cladograms were constructed by PAUP software, version 4.0b2.

genetic differences. RAPD profiles were very similar among isolates sensitive to both thiophanate-methyl and vinclozolin (S^{TS^V}), usually with high bootstrap confidence values assigned to the nodes. Greenhouse isolates resistant to one or both fungicides were more diverse genetically and frequently were not clustered with other isolates, suggesting greater diversity among genotypes of fungicide-resistant isolates. However, when these isolates were clustered together, isolates within a cluster had the same phenotype.

To further investigate and elucidate genetic relationships observed among greenhouse isolates, fungicide-resistant variant isolates derived from fungicide-sensitive greenhouse isolates (S^{TS^V}) were selected, and RAPD fingerprints for three sets of variant isolates were generated and subjected to cluster analyses. Clonal derivatives sensitive to thiophanate-methyl but resistant to vinclozolin (S^{TR^V}) were very similar genetically to their S^{TS^V} parents. This suggests that only minor genetic changes were involved in conferring vinclozolin resistance. Resistance to dicarboximides, such as vinclozolin, is believed to be encoded by *Daf1* (5), and it is possible that the similarity among genotypes of S^{TS^V} parents and S^{TR^V} clonal derivatives is the result of a single copy of a resistant allele.

In a previous study (5), wild-type isolates resistant to dicarboximides nearly always exhibited a low level of resistance (i.e., EC_{50} for mycelium growth from 1 to 3 $\mu\text{g/ml}$) while variants selected in the laboratory commonly had high levels of resistance (i.e., EC_{50} for mycelium growth $>100 \mu\text{g/ml}$). Of the variant isolates produced in this study, many of those resistant only to vinclozolin (S^{TR^V}) exhibited high levels of resistance to this fungicide, but none of the isolates resistant to both fungicides (R^{TR^V}) were highly resistant to vinclozolin. In addition, few of the greenhouse isolates studied previously (42) had high levels of resistance to vinclozolin. In cladograms produced in this study, the close relationship of S^{TS^V} isolates and their S^{TR^V} clonal derivatives occurred regardless of the level of vinclozolin resistance among the S^{TR^V} variants. Consequently, there appears to be little genetic difference between isolates with low or high levels of resistance to vinclozolin.

In all three sets of variant isolates studied here, thiophanate-methyl-resistant isolates clustered independently from their fungicide-sensitive parents, except for one instance in Variant set 2. For thiophanate-methyl-resistant variants from the same parents, isolates with the same phenotypes (R^{TR^V} or R^{TS^V}) were genetically similar whereas isolates with different phenotypes (R^{TR^V} and R^{TS^V}) were genetically different. Consequently, genetic changes associated with resistance were more important than clonal ties in determining genetic relationships among these isolates. Thiophanate-methyl-resistant variants, either R^{TR^V} or R^{TS^V} , from different parents also were genetically distinct. Therefore, among the thiophanate-methyl-resistant isolates examined in this study, both the inherent variability among isolates from different thalli and genetic changes associated with thiophanate-methyl resistance were important determinants of genetic relationships.

The genetic changes associated with thiophanate-methyl resistance appeared to be major, based on the lack of relatedness among resistant variants and sensitive parents. This might indicate the involvement of multiple copies of alleles of *Mbc1*. Source and magnitude of genetic variability within *B. cinerea* have been attributed to the multinucleate character of this fungus, parasexual exchange of nuclei between hyphae, or possible differences in ploidy level among isolates (3,11,25,35,36). Other explanations for the large genetic changes associated with fungicide resistance that we observed may be selection for modifier genes (22) such as in dicarboximide-resistant isolates of *Neurospora crassa* (10) and benzimidazole-resistant isolates of *Fusarium oxysporum* (28) or extragenic suppressor mutations of β -tubulin mutants (41) that caused reversion to benomyl sensitivity in *Coprinus cinereus* (17,18).

To produce fungicide-resistant variants for this study, fungicide-sensitive greenhouse isolates (S^{TS^V}) were placed on agar medium amended with only one fungicide—either thiophanate-methyl or vinclozolin. Unexpectedly, when variants were selected from a fungicide-amended medium, instead of finding variants resistant to only the fungicide used in the medium (R^{TS^V} or S^{TR^V}), some variants were resistant to both fungicides (R^{TR^V}). Ordered tetrad analysis of ascospores from *Botryotinia fuckeliana*, the teleomorph of *B. cinerea*, indicated that the two genes involved in resistance to benzimidazole and dicarboximide fungicides, *Mbc1* and *Daf1*, respectively, probably were linked; the calculated map distance between them was approximately 47 map units (6). A loose linkage also was observed by Weeds et al. (40). This linkage may explain why some parent isolates sensitive to both fungicides (S^{TS^V}) generated doubly resistant clonal derivatives (R^{TR^V}) when exposed to a medium amended with only one fungicide. Cluster analysis of R^{TR^V} clonal derivatives from the same fungicide-sensitive parent isolates did not appear to differentiate isolates on the basis of exposure to thiophanate-methyl or vinclozolin or the year in which resistant variants were selected (i.e., 1997 or 1998).

The high incidence of resistance to either benzimidazole or dicarboximide fungicides that occurs in populations of *B. cinerea* worldwide has been explained by the constant selection pressure created by the continued use of these fungicides over many years. However, some researchers studying *B. cinerea* have speculated that benzimidazole resistance might somehow promote the acquisition of resistance to dicarboximide fungicides (13,31) and, therefore, the occurrence of isolates resistant to both fungicides (R^{TR^V}). Based on our research, it is possible that R^{TR^V} variants can develop after sensitive wild-type isolates of *B. cinerea* are exposed to either thiophanate-methyl or vinclozolin—which may provide another explanation for the high incidence of R^{TR^V} isolates found in agricultural fields and greenhouses around the world.

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