Phenotype Instability in *Botrytis cinerea* in the Absence of Benzimidazole and Dicarboximide Fungicides

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ABSTRACT

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Stability of phenotypes of isolates of *Botrytis cinerea* that were sensitive or resistant to benzimidazole and dicarboximide fungicides was examined in the absence of fungicides in laboratory and growth room experiments. Twelve greenhouse isolates of *B. cinerea* were subcultured on potato dextrose agar (PDA) for 20 generations and on geranium seedlings for 15 generations. Three isolates of each of the following four phenotypes were used: sensitive to the fungicides thiophanate-methyl (a benzimidazole) and vinclozolin (a dicarboximide) (S^TS^V), resistant to both fungicides (R^TR^V), resistant to thiophanate-methyl and sensitive to vinclozolin (R^TS^V), and sensitive to thiophanate-methyl and resistant to vinclozolin (S^TR^V). In three trials on PDA, 36 populations were subcultured; 8 populations changed phenotypes by the end of 20 generations, as determined by conidium germination on fungicide-amended medium. Five of the eight initially were S^TR^V ; the resulting phenotypes were S^TS^V , R^TS^V , and R^TR^V . Populations from eight other isolates exhibited temporary changes in phenotype during intermediate generations on PDA but reverted to initial phenotypes by the twentieth generation; five of these populations changed to phenotype R^TR^V . In two geranium seedling trials, each of the 12 greenhouse isolates was inoculated onto a set of three

seedlings for each generation, and diseased tissue that developed was used to initiate the next generation. Therefore, a total of 72 populations of *B. cinerea* were subcultured in the two trials; 5 of these populations changed phenotype at the end of 15 generations. Three of the five initially were S^TR^V ; these changed to phenotypes S^TS^V or R^TR^V . In each of the two trials on geranium seedlings, a population subcultured from one S^TS^V isolate changed phenotype one to phenotype R^TR^V and one to phenotype R^TS^V . In all trials, no population resistant to thiophanate-methyl changed to a thiophanate-methyl-sensitive phenotype, and no population changed to phenotype S^TR^V . Random amplified polymorphic DNA (RAPD) fingerprints were generated with the 12 initial isolates and 49 isolates subcultured on PDA or geranium seedlings. Cluster analyses of RAPD markers showed that subcultured isolates exhibiting the same phenotype clustered together and that subcultured isolates derived from a common greenhouse isolate but with different phenotypes were in different clusters. Some populations that did not change phenotype exhibited considerable differences in RAPD marker patterns. The results of this study indicate that, in the absence of fungicides, sensitive populations of *B. cinerea* can develop resistance to thiophanate-methyl and vinclozolin, and this resistance can be maintained in populations through multiple generations. Populations resistant only to vinclozolin (S^TR^V) exhibited a high frequency of phenotype change, and populations resistant to both fungicides (R^TR^V) were stable.

Botrytis cinerea Pers.:Fr. is an important and notorious pathogen of a variety of crops worldwide, including many economically important greenhouse-grown ornamental crops (8,19). Managing *Botrytis* blight in production greenhouses is a challenge, and an integrated approach to disease management is recommended (8). Resistance to *B. cinerea* is not available for most ornamental crops so, in addition to cultural practices like environmental manipulation and sanitation, fungicides play an important role in managing diseases caused this pathogen (8,19,23).

Resistance in populations of *B. cinerea* to benzimidazole and dicarboximide fungicides has been studied almost since the introduction of these fungicides in the 1960s and 1970s, respectively. Resistance to both classes of fungicides was reported soon after their introduction, and strategies to prolong efficacies of these products were initiated (5). Benzimidazole fungicides (e.g., thiophanate-methyl and benomyl) were very effective for managing diseases caused by *B. cinerea* and many other plant pathogenic fungi, but the development of high levels of resistance reduced effectiveness of these compounds. Recommendations for man-

agement of *Botrytis* diseases have been revised to limit or exclude the use of benzimidazoles (25). Likewise, the effectiveness of dicarboximide fungicides (e.g., vinclozolin and iprodione) also has been compromised by the development of resistance in populations of *B. cinerea*, one of the primary pathogens for which these fungicides were developed (18,28,29). The mechanism for resistance of *B. cinerea* to benzimidazoles has been reported (39), and the mechanism for resistance to dicarboximides has been speculated (27).

The development and stability of fungicide resistance in populations of *B. cinerea* is of concern to both growers, who have a limited arsenal of fungicides to manage diseases caused by this pathogen, and agricultural chemical companies, who develop and market fungicides. Frequently, isolates resistant to dicarboximides also are resistant to benzimidazoles (17,20,29,40); the high frequencies of resistance in field populations of *B. cinerea* suggest that fungicide-resistant phenotypes are stable. Conversely, the relatively infrequent occurrence of isolates resistant to dicarboximides but sensitive to benzimidazoles may indicate an unstable genome for this phenotype. For example, in a previous study, 67% of 325 isolates of *B. cinerea* from commercial greenhouses in South Carolina were resistant to both thiophanate-methyl and vinclozolin, whereas only 2% of the isolates were sensitive to thiophanate-methyl and resistant to vinclozolin (40).

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In a companion study (41), random amplified polymorphic DNA (RAPD) fingerprints of wild-type greenhouse isolates of *B. cinerea* that were sensitive and resistant to thiophanate-methyl and vinclozolin revealed that several sensitive isolates from different geographic locations had similar RAPD marker patterns but that isolates resistant to one or both fungicides exhibited different marker patterns. In the same study, laboratory-produced variants that were sensitive to thiophanate-methyl and resistant to vinclozolin had RAPD marker patterns that were similar to those of their fungicide-sensitive parents. These variants exhibited both low and high levels of resistance to vinclozolin (i.e., 50% effective concentration [EC₅₀] values for mycelium growth were 1 to 10 and ≥ 100 μ g/ml, respectively). Variants resistant to thiophanate-methyl diverged greatly from parent isolates in cluster analyses.

B. cinerea is an anamorph species that has exceptional potential for genetic variation; cells in hyphae and conidia can be multinucleate and heterokaryotic, and nuclei can vary in ploidy (6,16,22). This inherent characteristic could affect the stability of fungicide-sensitivity phenotypes in populations of *B. cinerea*. Although research has been conducted on genetic characterization of resistance to benzimidazole and dicarboximide fungicides in *B. cinerea* (10–13,41), the stability of fungicide-sensitivity phenotypes in the absence of fungicide selection pressure has not been investigated to any extent. Therefore, research was conducted to address this issue. Our primary objective was to determine the stability of fungicide-sensitivity phenotypes of *B. cinerea* when isolates were subcultured for multiple generations in the absence of benzimidazole and dicarboximide fungicides. A secondary objective was to determine the relationships among generations of these subcultured populations using RAPD fingerprint analysis.

MATERIALS AND METHODS

Many of the procedures and methods used in this study are reported in detail in two previous papers (40,41). Consequently, such procedures will be summarized and the previous papers are referenced.

Fungicides. Commercial flowable formulations of thiophanate-methyl (3336F, 46% a.i. by weight; W. A. Cleary Corporation, Somerset, NJ) and vinclozolin (Curalan, 41.3% a.i. by weight; BASF Corporation, Research Triangle Park, NC) were used throughout this study. Fungicides were stored at 15°C in the dark to preserve fungicide activity.

TABLE 1. Fungicide-sensitivity phenotypes and 50% effective concentration (EC₅₀) values for 12 greenhouse isolates of *Botrytis cinerea* that were used to produce subcultured populations on potato dextrose agar and geranium seedlings^a

Phenotype ^b	Isolate	Thiophanate-methyl		Vinclozolin	
		EC ₅₀ -germ	EC ₅₀ -growth	EC ₅₀ -germ	EC ₅₀ -growth
S ^T S ^V	CG-2-a	1.9	0.1	0.9	0.2
	CH-5-a	0.2	0.2	1.4	0.3
	PP-4-b	0.4	0.1	2.7	0.2
R ^T R ^V	BU-3-b	>500	>500	>500	1.9
	DD-4-b	>500	>500	>500	2.2
	MD-3-a	>500	>500	>500	1.8
R ^T S ^V	CH-5-b	>500	>500	0.7	0.4
	DD-9-b	>500	>500	1.6	0.3
	HK-1-b	>500	>500	1.1	0.3
S ^T R ^V	A-8-a	0.4	0.1	>500	1.9
	CH-6-b	0.9	0.1	>500	2.0
	H-3-a	0.4	0.1	>500	1.7

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

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

Isolates of *B. cinerea*. For this study, 12 isolates of *B. cinerea* from 9 different greenhouses were used. These were selected from a collection of 325 isolates recovered from ornamental plants in 35 greenhouses in South Carolina (40). Of these 12 isolates, 3 isolates had each of the following four fungicide-sensitivity phenotypes: sensitive to both thiophanate-methyl and vinclozolin (S^TS^V), resistant to both fungicides (R^TR^V), resistant to thiophanate-methyl and sensitive to vinclozolin (R^TS^V), and sensitive to thiophanate-methyl and resistant to vinclozolin (S^TR^V) (Table 1). Phenotype designations and EC₅₀ values previously were determined by conidium germination and mycelium growth on fungicide-amended minimal medium, and virulence was determined on geranium seedlings (40). For this study, phenotypes were verified by retesting conidia of all 12 isolates for germination on fungicide-amended medium as done previously (41). All isolates were stored in an ultralow-temperature freezer (–80°C) as conidia suspended in 15% glycerol in 2-ml cryogenic vials (Nalgene Corporation; Rochester, NY) and were retrieved from storage as described previously (40). Frozen conidia were used to start cultures for DNA extraction and RAPD analyses (41) and for phenotype verification based on germination of conidia on fungicide-amended agar medium (40).

Isolates subcultured on potato dextrose agar. Each of the 12 greenhouse isolates was subcultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit) for 20 consecutive generations. To produce the first generation, frozen conidia of each greenhouse isolate were suspended in a sterile solution of 0.01% Tween 80 and standardized to approximately 2 to 3×10^4 conidia per ml with a hemacytometer; then, 30- μ l aliquots were transferred to fresh PDA in disposable petri dishes (15 $\times$ 100 mm). After 10 to 14 days, conidia produced by the first generation of subcultured isolates were collected, suspended in sterile 0.01% Tween 80, and diluted to the standard concentration; 20- μ l aliquots were transferred to fresh PDA to initiate the second generation. Conidia from subcultured isolates also were evaluated for sensitivity to thiophanate-methyl and vinclozolin based on conidium germination (41). The remaining conidia from each subcultured isolate were stored in 15% glycerol at –80°C to preserve isolates for RAPD analyses. The transfer of conidia to produce a subsequent generation and phenotype determinations were conducted for each of the 20 generations. This experiment was conducted three times.

Isolates subcultured on geranium seedlings. Each of the 12 greenhouse isolates also was subcultured on geranium seedlings for 15 generations. Geranium seedlings (cv. Orbit, Salmon; Ball Seed Co., W. Chicago) were grown in 72-cell seedling trays filled with a soilless container mix (Peat-Lite; Fafard Inc., Anderson, SC) in a greenhouse. When cotyledons were fully expanded, but before the first true leaf appeared, individual cells were cut from the seedling trays and placed in empty seedling trays or in 90-ml plastic cups to hold individual plants upright. Seedling trays and cups were placed on inverted bedding plant trays in plastic tubs containing 5 cm of water to help keep the relative humidity high when tubs were covered. Tubs were placed in a walk-in growth chamber with a 14-h photoperiod and a daily temperature range of 17 to 20°C.

Conidium suspensions for all 12 greenhouse isolates were prepared as described above, except conidia were suspended in 0.01% Tween 80 supplemented with 0.1 M glucose (26,40), and suspensions were standardized to approximately 1 to 2×10^6 conidia per ml. Individual seedlings were inoculated by dipping sterile, 6-mm-diameter filter paper disks (Whatman No. 1) into a conidium suspension and placing one disc onto each cotyledon (40). Each isolate was inoculated onto a separate set of three seedlings. Five additional noninoculated seedlings served as controls, receiving discs dipped in the sterile Tween 80-glucose solution. All 41 seedlings for one generation (i.e., 36 inoculated and 5 control seedlings) were completely randomized within a plastic

tub. Each seedling was covered with an inverted plastic cup to keep it isolated from all the others, and the entire plastic tub was covered with a tight-fitting lid to maintain high relative humidity. Once each day for the first 2 days, water from a hand sprayer was misted into the tubs over the covered seedlings to provide additional humidity.

After 4 to 6 days, diseased seedlings with conidia of *B. cinerea* were placed individually into sterile microcentrifuge tubes and seedlings were crushed with sterile forceps to provide inocula for the next generation. Control seedlings were examined for evidence of Botrytis blight and were discarded; a lack of symptoms confirmed that subcultured isolates remained isolated on individual seedlings. To start the next generation, a small piece of crushed plant material was placed on each cotyledon of a healthy seedling. The inoculum was covered with a sterile 6-mm filter paper disk that had been dipped into the sterile Tween 80-glucose solution. Both cotyledons of one healthy seedling were inoculated with diseased plant material from one seedling of the previous generation. Fresh noninoculated control seedlings also were included at each generation. After inocula were transferred to healthy seedlings, each microcentrifuge tube containing the remaining portion of a diseased plant was filled with 500 µl of 0.01% Tween 80 and 500 µl of 30% glycerol and stored at -80°C to preserve subcultured isolates for RAPD analyses. This procedure was continued for 15 generations, and the experiment was conducted twice.

At the end of the fifteenth generation, subcultured isolates from all 36 inoculated seedlings were assessed for sensitivity to thiophanate-methyl and vinclozolin. A piece of each diseased seedling was placed in a petri dish containing PDA amended with chloramphenicol (PDA+C) at 200 µg/ml. When cultures sporulated, conidia were collected and phenotypes were determined using the conidium germination assay described above; subsamples of all conidium suspensions were stored at -80°C to preserve subcultured isolates. When a subcultured isolate from the fifteenth generation exhibited a phenotype that was different from that of the initial generation, selected generations of that population also were assessed for sensitivity to the two fungicides.

DNA extraction. DNA was extracted from young, actively growing mycelia of the 12 greenhouse isolates and some of the isolates subcultured from them. Gamborg's B-5 basal broth with vitamins and minimal organic compounds (Sigma BioSciences;

St. Louis) was prepared according to the label; glucose and K₂HPO₄ were added to give final concentrations of 0.3% and 0.25 g/liter, respectively, and a final pH of 6.6. Broth (20 ml) was dispensed into sterile petri dishes, and 0.5 ml of a conidium suspension containing approximately 1 to 2 × 10⁷ conidia per ml was added to each dish. Dishes were placed at 20°C in the dark and, after 3 days, mycelia were harvested. Mycelium mats were washed with sterile, distilled water and blotted to remove excess liquid. For each isolate, 300 to 500 µl of mycelium was placed into a sterile microcentrifuge tube and stored in a freezer at -80°C. The procedure for extracting DNA was modified from the method of Möller et al. (24) and was described previously (41).

RAPD procedures. In all, 12 primers were used: 11 arbitrary-base 10-mer primers (OPB-9, -11, -19, and -20; OPC-3, -9, and -14; and OPD-4, -6, -9, and -10) from Operon primer kits (Operon Technologies Inc.; Alameda, CA) and primer no. 162 (5' AACTTACCGC 3'), which was supplied by Y. Wang and R. A. Dean (Clemson University Genomics Institute). Polymerase chain reaction (PCR) methods and subsequent procedures to produce RAPD fingerprints were identical to those reported previously (41).

A total of 12 greenhouse isolates and 49 isolates subcultured from 7 of these greenhouse isolates (Table 2) were used to generate two independent sets of RAPD fingerprints. Each set of fingerprints was comprised of 12 gels, one for each of the selected primers. Fingerprint set 1 (Fig. 1) was generated to investigate relationships among greenhouse isolates and subcultured isolates from different populations; a population here is defined as all isolates subcultured from one greenhouse isolate in a single trial. Set 1 contained 36 isolates: all 12 greenhouse isolates, 23 subcultured isolates from nine different populations, and 1 fungicide-resistant variant (CH-5-a/T6 [R^{TSV}]) that was selected from agar medium amended with thiophanate-methyl (41). Fingerprint set 2 (Fig. 2) was generated to investigate relationships among subcultured isolates within a population and contained 35 isolates: 3 greenhouse isolates (H-3-a, A-8-a, and CH-6-b) and 32 subcultured isolates from PDA trials. Twenty-three subcultured isolates came from three populations that changed phenotype (one population from each of the greenhouse isolates used in this set), and nine subcultured isolates came from three populations that did not change phenotype. Three greenhouse isolates and six subcultured isolates were common to both sets.

TABLE 2. Sources and fungicide-sensitivity phenotypes of 49 subcultured isolates of *Botrytis cinerea* that were used to produce random amplified polymorphic DNA fingerprints^a

Isolate	Phenotype ^b	Source ^c	Phenotype/generation no. ^d
CH-5-a	S ^{TSV}	Geranium 1-1	S ^{TSV} /15
		Geranium 1-3	S ^{TSV} /2, R ^{TSV} /9, R ^{TSV} /15
BU-3-b	R ^{TRV}	PDA 3	R ^{TRV} /1, R ^{TRV} /10, R ^{TRV} /20
DD-4-b	R ^{TRV}	PDA 2	R ^{TRV} /1, R ^{TRV} /10, R ^{TRV} /20
CH-5-b	R ^{TSV}	PDA 2	R ^{TSV} /1, R ^{TSV} /10, R ^{TSV} /20
A-8-a	S ^{TRV}	PDA 1	S ^{TRV} /2, S ^{TRV} ?/3, S ^{TSV} /4, S ^{TSV} /5, R ^{TSV} /6, R ^{TSV} /20
CH-6-b	S ^{TRV}	PDA 1	S ^{TRV} /2, S ^{TSV} /6, S ^{TSV} /20
		PDA 2	S ^{TRV} /2, S ^{TRV} ?/3, R ^{TSV} /4, R ^{TSV} /5, R ^{TSV} /20
		PDA 3	S ^{TRV} /20
		Geranium 2-3	S ^{TSV} /7
H-3-a	S ^{TRV}	PDA 1	S ^{TRV} /2, S ^{TRV} /3, R ^{TSV} ?/4, R ^{TSV} /5, R ^{TRV} ?/10, R ^{TRV} ?/11, R ^{TSV} ?/12, R ^{TSV} /13, R ^{TSV} /16, R ^{TRV} ?/17, R ^{TRV} /18, R ^{TRV} /19
		PDA 2	S ^{TRV} /20
		PDA 3	S ^{TRV} /2, R ^{TSV} /6, R ^{TSV} /20
		Geranium 1-2	S ^{TRV} /15
		Geranium 1-3	S ^{TRV} /2, S ^{TRV} /12, R ^{TRV} /15

^a Isolates resulted from subculturing seven greenhouse isolates on potato dextrose agar (PDA) for 20 generations and on geranium seedlings for 15 generations.

^b Initial phenotypes of greenhouse isolates before subculturing; isolates were sensitive (S) or resistant (R) to thiophanate-methyl (°) or vinclozolin (°). Isolates were designated as resistant if normal germination of conidia occurred on agar medium amended with thiophanate-methyl or vinclozolin at 5 µg/ml.

^c Isolates were subcultured on PDA in three independent trials (i.e., PDA 1, 2, and 3) and on geranium seedlings in two independent trials (Geranium 1 and 2). In geranium seedling trials, each isolate was subcultured on a set of three individual seedlings at each generation; subcultured isolates on individual seedlings were designated Geranium 1-1, 1-2, and 1-3 for trial 1 and Geranium 2-1, 2-2, and 2-3 for trial 2.

^d Fungicide-sensitivity phenotypes of subcultured isolates and the generation number in which each subculture was produced. A question mark indicates an unusual conidium germination response.

Each fingerprint set was generated twice, and data were analyzed separately. Results from repeated sets were nearly identical; therefore, data from the two replicate sets were combined. Binary matrices were constructed according to the presence or absence of comigrating bands. Cluster analyses were conducted by the unweighted pair group method using arithmetic average (UPGMA) (14) with the software program Phylogenetic Analysis Using Parsimony (PAUP), version 4.0b2 (33). 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 (in parentheses).

RESULTS

Isolates subcultured on PDA. In all three trials combined, 36 populations of *B. cinerea* were produced from 12 greenhouse isolates subcultured on PDA. Sixteen populations (44%) from

Fig. 1. Fingerprint set 1. Results of cluster analysis of random amplified polymorphic DNA (RAPD) markers among 36 isolates of *Botrytis cinerea* that were resistant (R) or sensitive (S) to thiophanate-methyl (^T) or vinclozolin (^V), including 12 greenhouse isolates, which are designated by isolate number and fungicide-sensitivity phenotype; isolate CH-5-a/T6 (^TS^V), a resistant variant that previously was selected in the laboratory on agar medium amended with thiophanate-methyl; and 23 isolates that resulted from subculturing three greenhouse isolates (CH-5-a, CH-6-b, and H-3-a). Subcultured isolates are designated by initial greenhouse isolate number, the generation and trial numbers in which they were produced (in parentheses), and phenotype. Greenhouse isolates were subcultured on potato dextrose agar for 20 generations in three independent trials (i.e., P1, P2, and P3) or on geranium seedlings for 15 generations in two independent trials (i.e., G1, G2). On geranium seedlings, each isolate was subcultured on a set of three separate seedlings in each generation (e.g., G1-1, G1-2, and G1-3). The cladogram was constructed by the software program PAUP (version 4.0b2) and mean distances and bootstrap values (in parentheses) were labeled at the nodes. The control was the RAPD pattern of polymerase chain reactions without template DNA.

11 isolates exhibited at least one change in phenotype during the 20 generations; eight, five, and three populations changed phenotypes in trials 1, 2, and 3, respectively (Table 3). Eight populations (22%) subcultured from six greenhouse isolates changed to and remained different phenotypes by the twentieth generation: A-8-a, DD-9-b, and HK-1-b (trial 1); PP-4-b (trial 2); CH-6-b (trials 1 and 2); and H-3-a (trials 1 and 3). Five of the eight subcultured populations that changed phenotypes initially were sensitive to thiophanate-methyl and resistant to vinclozolin (^TR^V) and changed to one of the three other phenotypes: one to ^TS^V, one to ^TR^V, and three to ^TS^V (Table 3). Of the three other subcultured populations that changed phenotypes, one initially was ^TS^V (PP-4-b) and two initially were ^TR^S (DD-9-b and HK-1-b); all three of these populations changed to ^TR^V.

Six other isolates produced eight subcultured populations (22%) that changed phenotypes during intermediate generations, but these exhibited unstable changes in conidium germination rates (Tables 3 and 4). These populations reverted to original phenotypes before the twentieth generation. The three ^TR^V populations exhibited a temporary change to the ^TS^V phenotype, one ^TS^V isolate temporarily became ^TR^V, and two ^TS^V populations changed temporarily to ^TR^V.

Phenotypes of sensitive (^TS^V) and thiophanate-methyl resistant (^TR^V or ^TS^V) populations changed to other phenotypes at vari-

Fig. 2. Fingerprint set 2. Results of cluster analysis of random amplified polymorphic DNA (RAPD) markers among 35 isolates of *Botrytis cinerea* that were resistant (R) or sensitive (S) to thiophanate-methyl (^T) or vinclozolin (^V), including three greenhouse isolates (A-8-a, CH-6-b, and H-3-a), which are designated by isolate number and fungicide-sensitivity phenotype; 23 isolates subcultured from these three isolates; and nine isolates subcultured from three other greenhouse isolates (BU-3-b, CH-5-b, and DD-4-b). Subcultured isolates are designated by initial greenhouse isolate number, the generation and trial numbers in which they were produced (in parentheses), and phenotype; question marks indicate some uncertainty about phenotype designations based on a conidium germination assay. Greenhouse isolates were subcultured on potato dextrose agar for 20 generations in three independent trials (i.e., P1, P2, P3). The cladogram was constructed by the software program PAUP (version 4.0b2) and mean distances and bootstrap values (in parentheses) are labeled at the nodes. The control was the RAPD pattern of polymerase chain reactions without template DNA.

ous generations during repeated subculturing, but S^TR^V populations consistently changed phenotypes beginning in the third, fourth, or fifth generations (Tables 3 and 5). Populations of two of the three isolates with an initial S^TR^V phenotype changed more than once in the 20 generations. In trial 1, A-8-a changed to S^TS^V at the fourth generation and changed again to R^TS^V after two more generations (Table 5). Also in trial 1, H-3-a changed phenotype four times during the 20 generations, alternating be-

tween R^TS^V and R^TR^V , and remained R^TR^V by the twentieth generation (Tables 3 and 5).

In the three PDA trials, each of the four fungicide-sensitivity phenotypes initially was represented by nine populations. Two S^TS^V populations changed phenotypes by the twentieth generation; one was only a temporary change. Five R^TR^V populations changed phenotypes, but all were temporary changes that reverted to the original phenotype. Four R^TS^V populations changed phenotypes,

TABLE 3. Initial, intermediate, and final fungicide-sensitivity phenotypes of 12 populations of *Botrytis cinerea* that resulted from subculturing greenhouse isolates for 20 generations on potato dextrose agar in three independent trials and the generation numbers in which changes in phenotype occurred

Isolate	Initial ^a	Trial 1			Trial 2			Trial 3		
		Intermed. ^b	No. ^c	Final ^d	Intermed.	No.	Final	Intermed.	No.	Final
CG-2-a	S^TS^V	nc	...	S^TS^V	nc	...	S^TS^V	nc	...	S^TS^V
CH-5-a	S^TS^V	R^TR^V	12–15	S^TS^V	nc	...	S^TS^V	nc	...	S^TS^V
PP-4-b	S^TS^V	nc	...	S^TS^V	R^TR^V	4–7	R^TR^V	nc	...	S^TS^V
BU-3-b	R^TR^V	R^TS^V	14–17	R^TR^V	R^TS^V	6–8	R^TR^V	nc	...	R^TR^V
DD-4-b	R^TR^V	R^TS^V	14–17	R^TR^V	nc	...	R^TR^V	nc	...	R^TR^V
MD-3-a	R^TR^V	nc	...	R^TR^V	R^TS^V	9–10	R^TR^V	R^TS^V	6–8	R^TR^V
CH-5-b	R^TS^V	R^TR^V	8–15	R^TS^V	nc	...	R^TS^V	nc	...	R^TS^V
DD-9-b	R^TS^V	R^TR^V	14–17	R^TR^V	nc	...	R^TS^V	nc	...	R^TS^V
HK-1-b	R^TS^V	nc	...	R^TS^V	R^TR^V	18	R^TS^V	R^TR^V	9	R^TR^V
A-8-a	S^TR^V	S^TS^V	4–5	R^TS^V	nc	...	S^TR^V	nc	...	S^TR^V
CH-6-b	S^TR^V	S^TS^V	4–5	S^TS^V	R^TS^V	3	R^TS^V	nc	...	S^TR^V
H-3-a	S^TR^V	R^TS^V	4	R^TR^V	nc	...	S^TR^V	R^TS^V	5	R^TS^V
		R^TR^V	10	...	...	...	...	...	...	...
		R^TS^V	13	...	...	...	...	...	...	...
		R^TR^V	17	...	...	...	...	...	...	...

^a Phenotypes of greenhouse isolates before subculturing; isolates were sensitive (S) or resistant (R) to thiophanate-methyl (^T) or vinclozolin (^V). Isolates were designated as resistant if normal germination of conidia occurred on agar medium amended with thiophanate-methyl or vinclozolin at 5 µg/ml.

^b Intermediate phenotype. Populations of some isolates changed phenotypes in intermediate generations; however, some isolates had no change (nc) in phenotype during 20 generations of subculturing.

^c Intermediate generation numbers in which changes in phenotype occurred.

^d Final phenotypes of populations after being subcultured for 20 generations on potato dextrose agar.

TABLE 4. Conidium germination rates (percentage) on fungicide-amended and nonamended agar media for eight populations of *Botrytis cinerea* that changed phenotypes and then reverted to original phenotypes when greenhouse isolates were subcultured on potato dextrose agar for 20 generations^a

Isolate	Phenotype ^b			Medium ^c	Rates (%) in generation no.																
	Initial	Interm.	Trial		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CH-5-a	S ^T S ^V	R ^T R ^V	1	Control	...	...	...	...	...	...	...	99	99	97	99	97	99	92	98	98	98
				TM	...	...	...	...	...	...	...	0	40	60	4	79	1	0	0	0	0
				Vin	...	...	...	...	...	...	...	0	36	61	0	20	0	0	0	0	0
BU-3-b	R ^T R ^V	R ^T S ^V	1	Control	...	...	...	...	...	...	...	...	...	99	98	98	97	98	96	100	98
				TM	...	...	...	...	...	...	...	...	...	98	98	97	98	98	97	99	98
				Vin	...	...	...	...	...	...	...	...	...	97	4	9	3	5	96	100	98
		R ^T S ^V	2	Control	98	99	97	98	98	97	98	...	...	...	...	...	...	...	...	...	99
				TM	97	99	98	98	98	99	98	...	...	...	...	...	...	...	...	...	99
				Vin	92	86	0	0	60	96	98	...	...	...	...	...	...	...	...	...	98
DD-4-b	R ^T R ^V	R ^T S ^V	1	Control	...	...	...	...	...	...	...	...	...	97	98	96	99	95	99	98	98
				TM	...	...	...	...	...	...	...	...	...	98	98	96	98	96	98	100	99
				Vin	...	...	...	...	...	...	...	...	...	95	9	16	3	2	71	96	99
MD-3-a	R ^T R ^V	R ^T S ^V	2	Control	...	...	...	...	98	98	97	98	99	...	...	...	...	...	...	...	100
				TM	...	...	...	...	97	98	98	99	99	...	...	...	...	...	...	...	100
				Vin	...	...	...	...	97	29	9	82	99	...	...	...	...	...	...	...	99
		R ^T S ^V	3	Control	98	97	98	98	93	98	...	...	...	...	...	...	...	...	...	...	98
				TM	99	98	99	98	95	99	...	...	...	...	...	...	...	...	...	...	98
				Vin	97	81	23	38	12	98	...	...	...	...	...	...	...	...	...	...	97
CH-5-b	R ^T S ^V	R ^T R ^V	1	Control	...	...	...	97	96	98	99	98	98	99	98	95	98	96	98	97	100
				TM	...	...	...	98	98	98	99	75	13	8	97	86	98	95	100	97	100
				Vin	...	...	...	0	66	39	83	56	81	88	1	7	0	0	0	0	0
HK-1-b	R ^T S ^V	R ^T R ^V	2	Control	...	...	...	...	...	...	...	...	...	...	...	...	98	97	100	100	100
				TM	...	...	...	...	...	...	...	...	...	...	...	...	97	93	83	98	100
				Vin	...	...	...	...	...	...	...	...	...	...	...	...	0	0	75	0	0

^a Conidium germination rates are means of two replicates of 100 conidia per replicate in each of three independent trials; ... indicates a consistent trend with previous or subsequent germination rates. Germination rate for the twentieth (final) generation is provided for all populations.

^b Phenotypes of isolates before (initial) and during (intermediate) subculturing; isolates were sensitive (S) or resistant (R) to thiophanate-methyl (^T) or vinclozolin (^V). Isolates were designated as resistant if normal germination of conidia occurred on agar medium amended with thiophanate-methyl or vinclozolin at 5 µg/ml.

^c Conidium germination was evaluated on agar medium without fungicide amendment (Control) or on agar medium amended with either thiophanate-methyl (TM) or vinclozolin (Vin) at 5 µg/ml.

with two being temporary changes, and five S^{TRV} populations changed phenotypes by the twentieth generation. No subcultured population shifted to the S^{TRV} phenotype in any of the three trials, and no population that initially was resistant to thiophanate-methyl (R^{TRV} or R^{TSV}) became sensitive (S^{TSV}).

Isolates subcultured on geranium seedlings. The 12 greenhouse isolates also were subcultured on geranium seedlings for 15 generations to determine whether interaction with a host affected the tendency of some populations of *B. cinerea* to change phenotype. Only isolates that had changed phenotype at the end of 15 generations were tested for changes in phenotype during earlier generations. Therefore, populations that underwent temporary changes but reverted to initial phenotypes were not detected.

In all, 72 subcultured populations were produced on geranium seedlings, but only 5 of these populations (7%) had changed phenotype at the end of 15 generations (Table 6). Two populations were subcultured from S^{TSV} isolate CH-5-a, and three populations were subcultured from the three S^{TRV} isolates (A-8-a, CH-6-b, and H-3-a), one from each isolate. Both of the populations from CH-5-a became resistant to thiophanate-methyl; one was R^{TSV} and one was R^{TRV}. Populations from the three S^{TRV} isolates changed to phenotypes R^{TRV} (two populations) and S^{TSV} (one population). In trial 1, one population of HK-1-b was not recovered when plant tissue was placed on PDA+C after the ninth generation, and crushed tissue from the ninth generation did not cause disease on a healthy seedling (Table 6). Throughout the 15 generations in both trials, all control plants remained free of disease symptoms for the 4- to 6-day period that constituted a generation cycle.

RAPD fingerprints. Two sets of RAPD fingerprints were analyzed with 12 greenhouse isolates, 49 subcultured isolates, and variant CH-5-a/T6 (Table 2). Cladograms constructed from fingerprint sets 1 and 2 had 163 and 109 informative characters, respectively. All isolates gave a strong amplification product of approximately 450 bp with primer OPD-6 in RAPD fingerprints;

this band has been reported as a reputed species-specific marker for *B. cinerea* (36) and also was observed previously (41).

RAPD fingerprint set 1. RAPD fingerprints were made and a cladogram was constructed (Fig. 1) to investigate relationships among greenhouse isolates and subcultured isolates from different populations. Isolates clustered primarily by phenotype; within each major phenotype cluster, isolates subcultured from the same greenhouse isolate (i.e., from the same and different populations) usually clustered together. There was one large S^{TSV} cluster (composed of three greenhouse isolates and subcultured isolates from four populations) and one R^{TRV} cluster (composed of four isolates, each from a separate population). There were two S^{TRV} clusters (one containing isolates from five populations of H-3-a and one containing isolates from three populations of CH-6-b) and three R^{TSV} clusters (each composed of isolates from multiple populations). Of the 12 greenhouse isolates, 3 did not cluster with other isolates.

For isolates from the same population, mean distances were small when phenotypes were the same and were large when phenotypes were different. For example, isolates subcultured from H-3-a in PDA trial 1 did not cluster when the phenotype changed from S^{TRV} in the second generation to R^{TSV} in the fifth generation (mean distance of 0.166). Similarly, an isolate from the fifth generation (R^{TSV}) did not cluster with one from the nineteenth generation (R^{TRV}) (mean distance of 0.188). In PDA trial 3, subcultured isolates of H-3-a from the second (S^{TRV}) and sixth generations (R^{TSV}) had a mean distance of 0.171, whereas those from the sixth and the twentieth generations when no phenotype change occurred had a mean distance of only 0.014. A similar trend occurred among other subcultured isolates from the same population (e.g., isolates of CH-5-a and CH-6-b).

RAPD fingerprint set 2. A second set of RAPD fingerprints was analyzed and a cladogram was constructed (Fig. 2) to investigate relationships among isolates within the same populations. For populations that changed phenotype during 20 generations on

TABLE 5. Conidium germination rates (percentage) on fungicide-amended and nonamended agar media for eight populations of *Botrytis cinerea* that changed phenotypes when greenhouse isolates were subcultured on potato dextrose agar for 20 generations^a

Isolate	Phenotype ^b			Trial	Med. ^c	Rates (%) in generation no.																			
	Initial	Final	2			3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
PP-4-b	S ^T S ^V	R ^T R ^V	2	Cont.	98	99	97	99	96	97	97	...	...	...	...	...	...	...	...	...	...	...	...	99	
				TM	0	5	35	98	80	75	97	...	...	...	...	...	...	...	...	...	...	...	...	97	
				Vin	0	0	50	37	27	52	96	...	...	...	...	...	...	...	...	...	...	...	...	99	
DD-9-b	R ^T S ^V	R ^T R ^V	1	Cont.	...	...	...	...	...	...	...	...	...	...	...	99	98	96	98	97	98	98	98		
				TM	...	...	...	...	...	...	...	...	...	...	...	98	98	95	98	96	99	97	97		
				Vin	...	...	...	...	...	...	...	...	...	...	0	7	23	28	17	96	98	96			
HK-1-b	R ^T S ^V	R ^T R ^V	3	Cont.	57	68	87	92	56	71	59	99	98	99	...	...	...	...	...	...	...	...	100		
				TM	58	70	86	89	57	72	56	100	99	99	...	...	...	...	...	...	...	...	100		
				Vin	0	0	0	0	0	0	0	77	98	99	...	...	...	...	...	...	...	...	...	100	
A-8-a	S ^T R ^V	R ^T S ^V	1	Cont.	99	100	98	96	98	97	97	...	...	...	...	...	...	...	...	...	...	...	100		
				TM	1	2	0	2	88	99	98	...	...	...	...	...	...	...	...	...	...	...	...	100	
				Vin	99	98	11	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	...	0	
CH-6-b	S ^T R ^V	S ^T S ^V	1	Cont.	99	100	98	84	98	98	...	...	...	...	...	...	...	...	...	...	...	...	98		
				TM	1	3	2	3	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0	
				Vin	99	99	56	52	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0	
	R ^T S ^V	2	Cont.	98	95	99	92	98	99	98	96	60	98	95	79	99	100	...	...	...	...	...	100		
			TM	0	11	98	79	99	98	99	96	69	97	97	76	99	98	...	...	...	...	...	100		
			Vin	99	82	0	0	0	0	0	0	0	0	0	0	0	0	...	...	...	...	...	...	0	
H-3-a	S ^T R ^V	R ^T R ^V	1	Cont.	98	98	99	96	99	97	97	96	96	87	97	98	98	86	98	94	97	98	98		
				TM	1	0	38	96	99	98	98	98	96	87	99	98	98	83	98	57	76	95	77		
				Vin	100	99	34	0	0	0	0	0	50	66	32	0	0	0	0	9	97	97	95		
	R ^T S ^V	3	Cont.	98	98	97	98	99	96	...	...	...	...	...	...	...	...	...	...	...	...	...	100		
			TM	6	5	5	50	98	97	...	...	...	...	...	...	...	...	...	...	...	...	...	99		
			Vin	98	99	92	3	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		

^a Conidium germination rates are means of two replicates of 100 conidia per replicate in each of three independent trials; ... indicates a consistent trend with previous or subsequent germination rates. Germination rate for the twentieth (final) generation is provided for all populations.

^b Phenotypes of isolates before (initial) and after (final) subculturing; isolates were sensitive (S) or resistant (R) to thiophanate-methyl (T) or vinclozolin (V). Isolates were designated as resistant if normal germination of conidia occurred on agar medium amended with thiophanate-methyl or vinclozolin at 5 µg/ml.

^c Conidium germination was evaluated on agar medium (Med.) without fungicide amendment (Cont.) or on agar medium amended with either thiophanate-methyl (TM) or vinclozolin (Vin) at 5 µg/ml.

PDA, only isolates with the same phenotype clustered together, and these clusters, in all cases but one, consisted of isolates from the same population; the exception was a cluster of three R^TS^V isolates subcultured from A-8-a, CH-6-b, and H-3-a. These results are consistent with those from fingerprint set 1. Changes in phenotype between generations always resulted in large mean distances in cladograms. For example, S^TR^V isolates from the initial and second generations of isolate A-8-a were separated by a mean distance of 0.011, and S^TS^V isolates from the fourth and fifth generations of A-8-a were separated by a mean distance of 0.026. In contrast, a mean distance of 0.160 occurred between isolates from the third and fourth generations when the phenotype changed from S^TR^V to S^TS^V. Likewise, another phenotype change occurred between generations five (S^TS^V) and six (R^TS^V), and these isolates were separated by a mean distance of 0.145. Similar results occurred with isolates subcultured from S^TR^V isolates CH-6-b and H-3-a (i.e., mean distances were small only among isolates with the same phenotype).

Phenotypes of isolates subcultured from BU-3-b (R^TR^V), DD-4-b (R^TR^V), and CH-5-b (R^TS^V) did not change over the course of 20 generations, but differences in RAPD marker patterns between isolates in generations 1, 10, and 20 varied considerably. For example, the mean distance between isolates from generations 1 and 10 of isolate BU-3-b was 0.260 and between isolates from generations 10 and 20 was 0.194; the mean distance between isolates from generations 1 and 10 of isolate CH-5-b was 0.220 but that between isolates from generations 10 and 20 was only 0.012; and the mean distances between isolates from generations 1 and 10 and between those from generations 10 and 20 of isolate DD-4-b were 0.064 and 0.246, respectively.

DISCUSSION

In the absence of fungicide selection pressure, some populations of *B. cinerea* changed fungicide-sensitivity phenotypes when isolates were subcultured for 20 generations on PDA or 15 generations on geranium seedlings. In our experiments, resistance to thiophanate-methyl was a very stable characteristic, whereas response to vinclozolin was an unstable characteristic. No population that initially was resistant to thiophanate-methyl became sensitive to this fungicide during repeated subculturing, but nine sensitive populations developed resistance. In contrast, changes in vinclozolin response occurred in 17 of the 21 populations that changed phenotype; 7 populations switched from sensitive to resistant and 10 populations switched from resistant to sensitive.

Previously, we suggested that major genetic changes were associated with resistance to thiophanate-methyl but relatively minor changes were involved in vinclozolin resistance (41). Results presented here are consistent with this hypothesis.

Overall, isolates resistant to both fungicides (R^TR^V) had the most stable phenotype. All populations that initially were R^TR^V retained this phenotype after 20 generations on PDA or 15 generations on geranium seedlings. Some R^TR^V populations temporarily became sensitive to vinclozolin (R^TS^V) during intermediate generations on PDA, but these populations always maintained resistance to thiophanate-methyl; the occurrence of temporary changes during intermediate generations on geranium seedlings was not examined. In addition, populations with other phenotypes changed to the R^TR^V phenotype during subculturing. Populations subcultured from the 12 greenhouse isolates used in this study exhibited 13 stable changes in phenotype: 8 changes at the end of PDA trials and 5 changes at the end of geranium seedling trials. Of these 13 phenotype changes, 7 (54%) were to the R^TR^V phenotype; 4 (31%) were to the R^TS^V phenotype; and 2 (15%) were to the S^TS^V phenotype, both by populations that initially had the S^TR^V phenotype.

This trend in phenotype instability was similar for populations subcultured on PDA and on geranium seedlings. However, populations subcultured on geranium seedlings appeared to change phenotype less frequently than populations subcultured on PDA, but the number of generations subcultured on seedlings was less than that on PDA. This might reflect differences between growth on nutrient-rich PDA and growth on living plant tissue where infection and pathogenesis are involved. A study to investigate the effect of in vitro and in planta culture on phenotype stability may be useful.

Populations sensitive to thiophanate-methyl and resistant to vinclozolin (S^TR^V) were the most unstable and had the highest incidence of change to a different phenotype among the four fungicide-sensitivity phenotypes tested in this study. No S^TR^V greenhouse isolate underwent a temporary change that reverted; instead, all changes from this phenotype remained stable at the end of various trials. In addition, none of the populations initially exhibiting one of the other three phenotypes (i.e., S^TS^V, R^TR^V, or R^TS^V) changed to the S^TR^V phenotype in any of the trials on PDA or geranium seedlings. The instability of the S^TR^V phenotype may help explain why this phenotype rarely was found among isolates of *B. cinerea* collected from ornamental crops in South Carolina greenhouses in a previous study (40). In contrast to our results, others have found that some isolates of *B. cinerea* resistant to

TABLE 6. Changes in fungicide-sensitivity phenotypes in 72 populations of *Botrytis cinerea* that resulted from subculturing 12 greenhouse isolates for 15 generations on geranium seedlings in two independent trials and the generation number in which changes occurred^a

Isolate	Initial phenotype ^b	Trial 1 (change/generation no.)			Trial 2 (change/generation no.)		
		Seedling 1	Seedling 2	Seedling 3	Seedling 1	Seedling 2	Seedling 3
CG-2-a	S ^T S ^V	nc	nc	nc	nc	nc	nc
CH-5-a	S ^T S ^V	nc	nc	R ^T S ^V /7	R ^T R ^V /12	nc	nc
PP-4-b	S ^T S ^V	nc	nc	nc	nc	nc	nc
BU-3-b	R ^T R ^V	nc	nc	nc	nc	nc	nc
DD-4-b	R ^T R ^V	nc	nc	nc	nc	nc	nc
MD-3-a	R ^T R ^V	nc	nc	nc	nc	nc	nc
CH-5-b	R ^T S ^V	nc	nc	nc	nc	nc	nc
DD-9-b	R ^T S ^V	nc	nc	nc	nc	nc	nc
HK-1-b	R ^T S ^V	nc	nc	...	nc	nc	nc
A-8-a	S ^T R ^V	nc	nc	R ^T R ^V /10	nc	nc	nc
CH-6-b	S ^T R ^V	nc	nc	nc	nc	nc	S ^T S ^V /6
H-3-a	S ^T R ^V	nc	nc	R ^T R ^V /13	nc	nc	nc

^a Each greenhouse isolate was inoculated onto a set of three geranium seedlings for each generation, and each seedling was maintained independently. For many isolates, no change (nc) in phenotype was detected at the fifteenth generation; isolates in intermediate generations were not examined for temporary phenotype changes for populations that exhibited no change at the fifteenth generation; ... indicates one population of isolate HK-1-b that was not recovered at the ninth generation.

^b Phenotypes of greenhouse isolates before subculturing; isolates were sensitive (S) or resistant (R) to thiophanate-methyl (^T) or vinclozolin (^V). Isolates were designated as resistant if normal germination of conidia occurred on agar medium amended with thiophanate-methyl or vinclozolin at 5 µg/ml.

vinclozolin maintained this resistance after being subcultured in the absence of fungicides (9,35,37), but they did not report if these isolates also were resistant to benzimidazole fungicides. Katan (20) found that 36 of 37 vinclozolin-resistant isolates retained resistance to vinclozolin after successive conidium transfers to PDA, but all of these isolates also were resistant to the benzimidazole fungicide benomyl.

Phenotype changes that were identified in a conidium germination assay were corroborated by changes in RAPD fingerprint patterns. These results are consistent with those from a previous study (41) in that usually only isolates with the same phenotype had similar RAPD marker patterns and clustered together. Likewise, we demonstrated here that isolates from the same population with different phenotypes had different RAPD marker patterns and were not clustered together. In addition, isolates from different populations that were resistant to thiophanate-methyl (R^{TSV} or R^{TRV}) either did not cluster with other isolates or formed clusters with relatively large mean distances between pairs of isolates. This also supports the hypothesis that significant genetic changes are associated with resistance to this fungicide (41).

There is concern among growers and researchers that reduced efficacy of benzimidazole and dicarboximide fungicides is a permanent condition. The results of this study support this concern and suggest that, even in the absence of fungicides, some sensitive populations may change to resistant ones; resistant populations can be maintained for many generations, and isolates resistant to both fungicides can be stable. This helps to explain results from a previous study (40) in which a high percentage of isolates of *B. cinerea* (83%) found on ornamental crops in commercial greenhouses in South Carolina were resistant to thiophanate-methyl, vinclozolin, or both.

Other studies have indicated that field isolates of *B. cinerea* resistant to dicarboximides frequently were also resistant to benzimidazole fungicides (17,20,29,40). One explanation for this has been that the extensive and continued use of benzimidazoles throughout the world has selected for benzimidazole resistance in populations of *B. cinerea* prior to their exposure to dicarboximide fungicides (20). Yet, the prevalence of doubly resistant isolates in areas with no history of prior benzimidazole use suggests that additional explanations need to be considered (29). We demonstrated here that populations that acquired resistance to both fungicides (R^{TRV}) were not likely to become sensitive. In addition, we consistently observed a pattern of phenotype instability in the absence of fungicides (i.e., populations sensitive to thiophanate-methyl and resistant to vinclozolin [S^{TRV}] frequently changed phenotype, populations sensitive to vinclozolin [S^{TSV} and R^{TSV}] occasionally changed phenotype, and populations with the R^{TRV} phenotype were stable). Consequently, resistance to benzimidazole and dicarboximide fungicides may develop naturally in some populations of *B. cinerea*, even when these fungicides are not used.

Analyses of crosses between ascospores of the teleomorph of *B. cinerea*, *Botryotinia fuckeliana*, resulted in detection of two major genes, *Mbc1* and *Daf1*, that conferred benzimidazole and dicarboximide resistance, respectively, and tetrad analyses showed a probable linkage of these genes (10,13). A loose linkage of these genes also was found by Weeds et al. (38). This relationship was manifested by independent assortment of these genes in some sexual crosses but not in others (10,36). Our findings support the premise of a possible linkage between these two genes. In the present study, S^{TSV} populations became resistant to both fungicides concurrently during subculturing in the absence of fungicides. In a previous study, we reported that isolates resistant to both fungicides were selected on agar medium amended with just one of the fungicides (41).

We have speculated that one explanation for the apparent genetic similarity among S^{TSV} parent isolates and their S^{TRV} variant progeny, which were selected from fungicide-amended medium, and the genetic dissimilarity among parent isolates and R^{TSV} and

R^{TRV} variant progeny is that thiophanate-methyl resistance is conferred by multiple copies of genes for resistance to benzimidazoles and that vinclozolin resistance is conferred by a single copy of the gene for dicarboximide resistance (41). The results of this study support this hypothesis. *B. cinerea* is an asexual fungus with multinucleate, heterokaryotic cells that vary in ploidy (6,16,22). Multiple nuclei may affect phenotype stability. The number of nuclei per conidium of *B. cinerea* ranges from 3 to 18, with greater variation among vegetative cells of hyphae (16,22,30,32). Büttner et al. (6) reported that treatment with benomyl reduced the ploidy of a reference isolate of *B. cinerea* (possibly a triploid). In their study, subcultured isolates initially had increased numbers of nuclei per conidium compared with the parent isolate; but, after 2 months of subculturing, conidia of these isolates had reduced numbers of nuclei per cell.

The S^{TRV} phenotype may reflect a population with a nucleus having a single copy of the allele for dicarboximide resistance and a low number of nuclei with alleles for resistance to benzimidazoles. If additional nuclei were incorporated into the population (e.g., by the parasexual cycle), it is possible that the number of alleles for benzimidazole resistance would be sufficient to effect a change of phenotype to thiophanate-methyl resistance (i.e., a change to R^{TRV}). Phenotype changes from S^{TRV} to S^{TSV} could be the result of loss of the single resistant allele, whereas changes to the R^{TSV} phenotype might be explained by the concurrent loss of the dicarboximide resistance allele and the gain of multiple copies of benzimidazole resistance genes.

The nature of dominance of nuclei or alleles of *Mbc1* and *Daf1* has not been determined for *B. cinerea*. In addition, the widespread use of benzimidazoles may have irrevocably increased the number of alleles for resistance to benzimidazoles throughout the world's populations of *B. cinerea*. It is not known if recessive genes for resistance to benzimidazoles were present in populations of *B. cinerea* prior to the introduction of benzimidazole compounds, as has been hypothesized for some nematode populations (4,15). In ascospore crosses of *Aspergillus nidulans*, mutants cross-resistant to dicarboximides were found to be genetically diverse, with resistance alleles either dominant, codominant, or recessive to the wild-type sensitive alleles (7).

Faretra and Pollastro (11,12) suggested that the instability of mutants of *Botryotinia fuckeliana* grown on fungicide- and salt-amended media was a result of heterokaryons containing both sensitive and resistant nuclei and that nuclear ratios for sensitive or resistant phenotypes may have been modified under selection pressure. In our study, isolates resulting from sequential subculturing were not exposed to fungicides although the possibility of rearrangement of nuclei in the absence of fungicides exists. Some of the isolates that did not change phenotypes during the 20 generations subcultured on PDA exhibited considerable variation in RAPD marker patterns.

Even in the absence of fungicide selection pressure, a pathogen may be subject to environmental or genetic signals that cause changes that could result in phenotype shifts. Mycelia, interconnected networks of cells that form reproductive organs, can respond to an array of environmental signals (21). Mechanisms for the transfer of genetic information have not been studied sufficiently in *B. cinerea* although the multinucleate and polyploid characteristics may be important factors (6,16,22). Akutsu et al. (1–3) reported that stable benomyl- and vinclozolin-resistant isolates of *B. cinerea* were obtained by exposing a sensitive isolate to hyphae of a resistant isolate. Furthermore, they reported that dicarboximide-resistant isolates were produced from exposure to culture filtrates from other dicarboximide-resistant isolates. Movement of nuclei by anastomoses of hyphae and germ tubes is known (16,22), but development of resistance by noncellular mechanisms has not been confirmed by others.

In summary, several populations of *B. cinerea* changed sensitivities to thiophanate-methyl and vinclozolin during the course of

subculturing multiple generations on PDA and geranium seedlings in the absence of fungicides. Consequently, the high incidence of fungicide-resistant isolates of *B. cinerea* found on numerous crops worldwide may, in some cases, be attributed to the inherent instability in the genome of this fungus. Populations may develop resistance to benzimidazoles and dicarboximides in the absence of fungicide use, and these resistant phenotypes can be stable. This phenomenon should be considered for the newer fungicides being used to manage *B. cinerea* on ornamental and other crops that may be prone to resistance development (e.g., cyprodinil, fenhexamid, and fludioxonil). Researchers working on fungicide chemistry should be alerted to this potential cause of reduced efficacy.

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