Sensitivity of *Botrytis cinerea* from Connecticut Greenhouses to Benzimidazole and Dicarboximide Fungicides

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ABSTRACT

LaMondia, J. A., and Douglas, S. M. 1997. Sensitivity of *Botrytis cinerea* from Connecticut greenhouses to benzimidazole and dicarboximide fungicides. Plant Dis. 81:729-732.

Botrytis cinerea was isolated from infected plants in six greenhouses in Connecticut. Forty-five isolates were evaluated in vitro to determine fungicide sensitivity to benzimidazole (benomyl and thiophanate-methyl) and dicarboximide fungicides (vinclozolin and iprodione). *B. cinerea* isolates with fungicide resistance were recovered from each greenhouse sampled. Benzimidazole resistance was more common than dicarboximide resistance (74 to 76% versus 36 to 43%, respectively). Multiple fungicide resistance was common. Nineteen isolates were resistant to both a benzimidazole and a dicarboximide fungicide. The level (EC_{50}) of resistance to dicarboximides was low compared with resistance to benzimidazoles. Isolate growth rate was not correlated to fungicide sensitivity or EC_{50}. Fungicide resistance was apparently unrelated to the patterns of fungicide use in greenhouses sampled.

Additional keywords: gray mold

Botrytis cinerea Pers.:Fr. is an important pathogen of many greenhouse-grown crops. Current recommendations for control of this fungus suggest an integrated approach combining environmental modification to reduce inoculum, humidity, and leaf wetness with the use of several fungicides, including the site-specific benzimidazole and dicarboximide fungicides. In many cases, *B. cinerea* populations are repeatedly exposed to site-specific fungicides, creating selection pressure for the development of fungicide resistance. Resistance (insensitivity) to both fungicide classes has been described from a number of crops in the greenhouse and in the field (1–4,6,8–13). Resistance to these fungicides was first reported in 1979 (12) and was found in Pennsylvania greenhouses in 1992 (10). Fungicide resistance has been assumed to occur but has not been reported from New England.

Fungicides are routinely used for *Botrytis* control in Connecticut greenhouses, often with variable results. No dramatic control failures have been documented, but control measures are usually integrated, and monitoring for *Botrytis* incidence, efficacy of control, and the relative control achieved by different fungicides has not been done. As a result, *B. cinerea* fungicide sensitivity and fungicide efficacy are unknown, and both benzimidazole and dicarboximide use continue to be widespread and common. This study was initiated, in part, to address grower concerns regarding the presence and prevalence of fungicide-resistant isolates of *B. cinerea* in the state. The objectives of this research were (i) to determine if fungicide resistance exists in Connecticut greenhouses, and (ii) to determine the levels of sensitivity of *B. cinerea* isolates from several Connecticut greenhouses to benzimidazole and dicarboximide fungicides.

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Accepted for publication 17 April 1997.

Publication no. D-1997-0516-05R

MATERIALS AND METHODS

Isolates of *B. cinerea* were recovered from a number of flowering or foliar ornamental greenhouse-grown crops from six greenhouses in three counties (Hartford, New Haven, and New London) in Connecticut (Table 1). Greenhouse managers were surveyed for previous fungicide use in these crops. Fungicides reported for each greenhouse were used in multiple applications.

B. cinerea was either isolated from sections of lesions in infected plant tissues on water agar, or conidia were transferred to water agar from individual, sporulating lesions in moist chambers. All isolates were single-spored and transferred to potato dextrose agar (PDA, Difco). Single-conidium isolates were maintained on one-half strength PDA.

Each of 45 isolates was tested in vitro for fungicide sensitivity to the benzimidazoles, benomyl (Benlate 50 WP, DuPont Agricultural Products, Wilmington, DE) and thiophanate-methyl (Domain FL, The Scotts Company, Marysville, OH), or to the dicarboximides, vinclozolin (Ronilan 50W BASF Corp., Research Triangle, NC) and iprodione (Chipco 26019, Rhone-Poulenc, Research Triangle, NC). Commercially formulated fungicides were added to molten, sterile, one-half strength PDA to create stock solutions of 10,000 µg a.i./ml (ppm a.i.). Fungicide concentrations of 0, 1, 10, 100, and 1,000 µg a.i./ml were prepared by dilution of the stock solution into additional sterile molten agar and by using unamended media. Media were poured into 15 × 100 mm petri plates, and a 2 mm^2 plug of mycelium cut from the edge of an actively growing culture on one-half strength PDA was inverted and placed in the center of each fungicide-amended plate. Each of 45 isolates was transferred to two plates of each of five concentrations of four fungicides. Plates were incubated in the dark at 20°C, and the experiment was done twice. Colony diameter was measured daily from day 3 to day 7, and mean growth rate (mm per day) was calculated. Growth rate was normalized as a percentage of isolate growth on unamended media (0 µg a.i./ml), and regression was used to determine the EC_{50} (µg a.i./ml concentration that suppressed growth to one-half that of the fungus on fungicide-free media) for each fungicide–isolate combination. Isolates were considered resistant to benzimidazoles and dicarboximides if EC_{50} values were greater than 10 µg a.i./ml or 1 µg a.i./ml, respectively.

RESULTS

Fungicide-resistant *B. cinerea* isolates were recovered from a number of different crops in six greenhouses in Connecticut (Table 1). Benomyl resistance was the most common (occurring among 75.6% of isolates sampled) (Table 2). Resistance to thiophanate-methyl was also common (74.4%), and all but one of the benzimidazole-resistant isolates were resistant to both benomyl and thiophanate-methyl. EC_{50} levels for benomyl-resistant isolates ranged from 25 to 251 µg a.i./ml (mean = 101 µg a.i./ml). EC_{50} levels for thiophanate-methyl–resistant isolates were much higher than for benomyl, greater than 1,000 µg a.i./ml for 26 of the 45 isolates.

The incidence of resistance to dicarboximide fungicides was lower, 43.2 and 35.6% for vinclozolin and iprodione, respectively. EC_{50} values for vinclozolin-resistant isolates were also lower and ranged from 2.7 to 30.5 µg a.i./ml (mean = 11.1). EC_{50} values for iprodione-resistant isolates ranged from 4.3 to 13.9 µg a.i./ml (mean = 7.1). Resistance to more than one fungicide was common, as 19 isolates were resistant to both benzimidazole and dicarboximide fungicides. Ten of the 45 isolates evaluated were sensitive to all fungicides. The level of resistance (EC_{50}) to dicarboximides was low compared with resistance to benzimidazoles.

The growth rate of fungicide-sensitive, benzimidazole-resistant, or multiple fungicide-resistant isolates did not differ (Table 3). Fungicide EC_{50} was not correlated with growth rate for any fungicide examined (benomyl: $r = 0.139$, $P = 0.36$; thiophanate-methyl: $r = -0.042$, $P = 0.79$; vinclozolin: $r = -0.015$, $P = 0.92$; and iprodione: $r = 0.159$, $P = 0.30$), and there was no relationship between fungicide resistance or EC_{50} and previous fungicide use in the crop (Table 1).

DISCUSSION

Resistance or insensitivity to fungicides has been defined in several ways. We consider the use of fungicide EC_{50} as the most meaningful in vitro test. Resistance to benzimidazoles such as benomyl has consistently been defined as EC_{50} values greater than 10 µg a.i./ml (2,10,11). Resistance to dicarboximide fungicides such as iprodione has been defined as EC_{50} values greater than 1 or 2 µg a.i./ml (7,10,11,15).

Our results demonstrate that *B. cinerea* isolates with resistance to benzimidazole and dicarboximide fungicides are common and widespread in greenhouses in Connecticut. Most isolates resistant to benomyl were also resistant to thiophanate-methyl, and most isolates resistant to vinclozolin were also resistant to iprodione. While they were cross-resistant, we found that the levels of resistance may be quite different for different fungicides in the same class. In addition, a number of these isolates were resistant to both benzimidazole and dicarboximide fungicides. Fungicide resistance was not absolute, however, as over 20% of the isolates recovered were sensitive to both benzimidazole and dicarboximide fungicides.

In this study, *B. cinerea* insensitivity to benzimidazoles was greater than for dicarboximide fungicides. The average EC_{50} of *B. cinerea* isolates resistant to benomyl was approximately 100 µg a.i./ml, similar to levels described by Moorman and Lease (10) in 1992. However, we found that EC_{50} values for thiophanate-methyl–resistant isolates were much higher, generally greater than 1,000 µg a.i./ml. The consistently higher level of insensitivity of *B. cinerea* isolates to thiophanate-methyl when compared with benomyl may be an indication of the continued selection pressure associated with benzimidazole use over time.

The EC_{50} of dicarboximide-resistant isolates was in the 1 to 10 µg a.i./ml range. This level of resistance has been associated with the ability to cause disease in plants treated with label rates of fungicide (approximately 600 µg a.i./ml vinclozolin) (10,15). While the percentage of isolates resistant to vinclozolin was similar to levels reported in 1992 (10), the EC_{50} values that we observed for dicarboximide-resistant isolates were several times the values previously reported (10). The level of dicarboximide resistance may be increasing over time and should continue to be monitored in the future after additional selection.

All fungicide resistance in our experiments was naturally occurring acquired resistance, with no mutation or previous selection for resistance performed. The assumption that fungicide resistance is closely related to fungicide use patterns does not hold, as we were not able to correlate fungicide resistance with fungicide use in a particular greenhouse. Fungicide-resistant isolates were recovered from greenhouses that had no recent history of fungicide use. For example, while resistance to both fungicide classes was very common in greenhouse F, which had previously been treated with both fungicide types, greenhouses A and B had no recent fungicide use and also had a high proportion of resistant isolates. Conversely, isolates from greenhouses C and D, treated only with dicarboximides, were resistant to benzimidazoles but not to dicarboximides. This may be due to the persistence of resistance genes in *Botrytis* populations over time, to the movement of fungicide-resistant *B. cinerea* between different greenhouses with infected plant material (10), or both.

There has been some disparity concerning the fitness of *B. cinerea* isolates with resistance to fungicides. Several researchers concluded that dicarboximide resistance was correlated with reduced growth

Table 1. Host plant, previous fungicide exposure, and radial growth rate on PDA (20°C) of *Botrytis cinerea* isolates from Connecticut greenhouses

Greenhouse	Isolate	Host plant	Previous fungicide use	Growth (mm/day)
A	1	Begonia	None	12.8
	2	Begonia	None	13.8
	3	Lamium	None	9.5
	4	Lamium	None	11.7
	5	Salvia	None	11.9
	6	Salvia	None	13.3
	7	Strawberry	None	10.7
	8	Strawberry	None	9.3
B	9	Swedish ivy	None	12.2
	10	Swedish ivy	None	12.0
	11	Pelargonium	None	9.7
	12	Pelargonium	None	12.1
	13	Pelargonium	None	9.8
	14	Pelargonium	None	11.6
	15	Pelargonium	None	5.5
	16	Pelargonium	None	10.0
C	17	*Hebe* sp.	Dicarboximides	12.6
	18	*Hardenbergia* sp.	Dicarboximides	12.6
	19	*Plumbago* sp.	Dicarboximides	12.1
	20	*Chrysanthemum* sp.	Dicarboximides	11.1
	21	*Veronica* sp.	Dicarboximides	11.9
	22	*Oxalis* sp.	Dicarboximides	12.0
	23	*Carissa* sp.	Dicarboximides	11.3
	24	*Thunbergia* sp.	Dicarboximides	11.1
	25	*Pelargonium* sp.	Dicarboximides	11.6
D	26	Boulevard cypress	Dicarboximides	9.6
	27	Boulevard cypress	Dicarboximides	11.4
	28	Boulevard cypress	Dicarboximides	12.6
	29	Boulevard cypress	Dicarboximides	12.2
	30	Boulevard cypress	Dicarboximides	12.8
E	31	Tomato	Benzimidazoles & dicarboximides	6.2
	32	Tomato	Benzimidazoles & dicarboximides	8.1
	33	Tomato	Benzimidazoles & dicarboximides	11.0
	34	Tomato	Benzimidazoles & dicarboximides	13.7
	35	Tomato	Benzimidazoles & dicarboximides	5.9
F	36	Impatiens	Benzimidazoles & dicarboximides	9.5
	37	Impatiens	Benzimidazoles & dicarboximides	10.3
	38	Impatiens	Benzimidazoles & dicarboximides	9.8
	39	Impatiens	Benzimidazoles & dicarboximides	11.9
	40	Impatiens	Benzimidazoles & dicarboximides	9.2
	41	Impatiens	Benzimidazoles & dicarboximides	8.3
	42	Impatiens	Benzimidazoles & dicarboximides	11.5
	43	Impatiens	Benzimidazoles & dicarboximides	11.5
	44	Impatiens	Benzimidazoles & dicarboximides	10.6
	45	Impatiens	Benzimidazoles & dicarboximides	9.3

rates (8,14), while others suggested that there were no differences in growth rate, sporulation, or pathogenicity (1,6,10). Benomyl has not been available for greenhouse use in the United States since 1991. Moorman and Lease (10) speculated that the long-term persistence of benomyl-resistant isolates for years after the fungicide use was discontinued (3) was due to the fitness of isolates. While our level (EC$_{50}$) of benomyl resistance continues to be similar several years after benomyl application was stopped, we suggest that this may also be due to further selection by application of other selective cross-resistant benzimidazole fungicides such as thiophanate-methyl, or to the continued use of benomyl on other crops for control of this ubiquitous and nonspecific pathogen. The heterokaryotic nature of *B. cinerea* may also add to the persistence of fungicide resistance. The multinucleate hyphae and conidia of *B. cinerea* allow nuclei with fungicide resistance genes to remain in populations at low frequencies long after selection has ceased, with little genetic cost to the fungus (5). Our results did not demonstrate any relationship between growth rate and fungicide EC$_{50}$ for any fungicide tested.

Information convcerning the status of *B. cinerea* fungicide resistance in Connecticut or in a particular greenhouse will aid in gray mold management by incorporating modified patterns for fungicide use to better deal with resistance in *B. cinerea* populations. Control failures under greenhouse conditions have been and will continue to be difficult to document. Growers commonly attribute unsatisfactory control to weather, application method, or frequency and rate of application, and quickly follow up with another control tactic.

In the absence of knowledge about fungicide resistance, growers select fungicides for *B. cinerea* control based on factors such as cost, re-entry interval, efficacy (or residual), range of plants on the label, presence–absence of visible residue, tank-mix compatibility, and potential for phytotoxicity. As a result, it is not uncommon for a grower to use the same fungicide for the entire production cycle of a particular crop, thereby encouraging the development of resistance in the *B. cinerea* population. Discussions with growers indicated that while most were aware of the phenomenon

Table 2. Benzimidazole and dicarboximide fungicide sensitivity (EC$_{50}$ on fungicide-amended potato dextrose agar for 7 days at 20°C) of *Botrytis cinerea* isolates from Connecticut greenhouses

	Benomyl		Thiophanate-methyl		Vinclozolin		Iprodione	
Isolate	**EC$_{50}$** [a]	**SE**	**EC$_{50}$**	**SE**	**EC$_{50}$**	**SE**	**EC$_{50}$**	**SE**
1	115.2	1.4	607.6	1.8	10.1	2.0	5.9	2.0
2	<1.0	… [b]	<1.0		<1.0		<1.0	
3	127.8	1.4	>1,000	7.5	<1.0		<1.0	
4	251.8	2.5	>1,000	4.5	12.0	2.0	8.0	1.8
5	177.8	1.5	552.0	2.8	8.9	2.0	<1.0	
6	124.9	1.6	>1,000	5.3	7.6	2.1	3.6	2.0
7	159.2	1.5	409.9	1.9	NT [c]		<1.0	
8	<1.0		<1.0		<1.0		<1.0	
9	131.6	1.7	>1,000	88.8	11.0	1.8	5.9	2.3
10	209.9	1.5	27.6	2.9	21.9	1.3	5.5	1.8
11	162.6	1.7	>1,000	2.9	<1.0		<1.0	
12	76.0	1.4	>1,000		8.7	2.1	6.8	1.9
13	88.2	1.6	811.8	2.1	30.5	1.8	13.9	2.4
14	53.7	1.6	>1,000	24.9	10.0	2.0	5.0	2.0
15	27.1	1.5	>1,000	3.9	11.8	2.0	<1.0	
16	63.8	1.4	>1,000		11.5	2.1	4.6	2.3
17	<1.0		<1.0		<1.0		<1.0	
18	<1.0		<1.0		<1.0		<1.0	
19	76.7	1.6	>1,000	29.4	<1.0		<1.0	
20	100.5	1.3	868.3	32.8	<1.0		<1.0	
21	25.4	2.0	<1.0		<1.0		<1.0	
22	<1.0		<1.0		<1.0		<1.0	
23	<1.0		<1.0		<1.0		<1.0	
24	65.4	1.6	>1,000		<1.0		<1.0	
25	41.3	1.7	>1,000	2.3	<1.0		<1.0	
26	<1.0		<1.0		<1.0		<1.0	
27	141.5	2.3	>1,000	93.6	<1.0		<1.0	
28	95.6	1.7	>1,000	12.8	<1.0		<1.0	
29	188.5	1.9	>1,000	295.1	<1.0		<1.0	
30	<1.0		<1.0		<1.0		<1.0	
31	<1.0		NT		3.2	2.2	4.3	2.6
32	67.0	2.1	NT		<1.0		<1.0	
33	<1.0		<1.0		<1.0		<1.0	
34	31.9	1.2	>1,000	13.2	<1.0		<1.0	
35	<1.0		<1.0		<1.0		<1.0	
36	47.7	1.5	>1,000	2.9	12.3	1.9	<1.0	
37	90.7	2.1	>1,000	13.6	16.8	1.5	5.0	2.1
38	154.5	2.0	>1,000	6.1	16.3	1.5	5.7	2.0
39	68.8	1.8	>1,000	12.5	<1.0		<1.0	
40	120.3	1.4	>1,000	11.7	3.7	2.3	<1.0	
41	27.7	1.6	>1,000	9.3	3.6	2.2	<1.0	
42	85.4	1.6	>1,000	28.5	<1.0		11.3	2.0
43	89.2	1.6	>1,000	13.9	2.7	2.3	11.3	2.0
44	125.8	1.6	>1,000	21.4	8.1	1.9	11.5	2.0
45	29.1	1.4	>1,000	5.1	<1.0		5.5	2.3

[a] EC$_{50}$ is the concentration of fungicide a.i. (µg/ml) that suppresses the growth rate to half that of the fungus on fungicide-free agar, calculated from the regression of normalized growth rate and log µg/ml fungicide.
[b] Regression not reported, no significant regression relationship; EC$_{50}$ determined directly from data.
[c] NT = not tested.

Table 3. Growth rate of *Botrytis cinerea* isolates sensitive or resistant to benzimidazole and dicarboximide fungicides

Isolate type[a]	Number of isolates	Growth rate (mm/day)	Standard error
Sensitive to all fungicides	10	10.83	0.56
Resistant to benzimidazoles	12	11.23	0.51
Resistant to benzimidazoles & dicarboximides	19	10.96	0.41
ANOVA, $P = 0.86$			

[a] Resistant isolates with EC_{50} > 10 µg a.i./ml for benzimidazoles, and EC_{50} > 1 µg a.i./ml for dicarboximides.

of fungicide resistance, only a limited number actually incorporated resistance management into their disease management programs (S. M. Douglas, *unpublished*). This included several of the greenhouses in the present study found to harbor resistance to benzimidazole and dicarboximide fungicides.

Documentation of the presence of *B. cinerea* populations with fungicide resistance also has important implications for nongreenhouse crops in Connecticut. For example, resistance to benzimidazole and dicarboximide fungicides can be important for small fruit and vegetable production. Some current recommendations for commercial growers (e.g., New England Vegetable Pest Management Guide 1996–97) do not mention fungicide resistance or management; whereas others (New England Small Fruit Pest Management Guide and New England Greenhouse Floriculture Crop Pest Management and Growth Regulator Guide) briefly allude to the potential for resistance. Given the ubiquitous and nonselective nature of *B. cinerea*, movement of resistant populations to and from field and greenhouse crops would not be surprising.

ACKNOWLEDGMENTS

We thank Jane Canepa-Morrison, Robert Ballinger, and Timothy Ciesielski for technical assistance.

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