# Benzimidazole- and Dicarboximide-Resistant *Botrytis cinerea* from Pennsylvania Greenhouses

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**ABSTRACT**

Moorman, G. W., and Lease, R. J. 1992. Benzimidazole- and dicarboximide-resistant *Botrytis cinerea* from Pennsylvania greenhouses. Plant Dis. 76:477-480.

*Botrytis cinerea* was isolated from infected plants collected in 13 different greenhouses in Pennsylvania. The linear growth rates of the isolates and sensitivities to the benzimidazole fungicide, benomyl, and the dicarboximide fungicide, vinclozolin, were assessed in vitro. The area under the disease progress curve and sensitivity to benomyl and vinclozolin were assessed on geranium leaf disks (*Pelargonium* × *hortorum* cv. Red Elite). Isolates with resistance to benomyl were found in all 13 facilities, and double resistance to benomyl and vinclozolin was detected in isolates from six greenhouses. All isolates with $EC_{50}$ values greater than 70 μg/ml for benomyl or approximately 1.0 μg/ml for vinclozolin in vitro overcame the presence of those fungicides and caused disease on geranium leaf disks. One isolate with an $EC_{50}$ value near 0.1 μg/ml for vinclozolin infected vinclozolin-treated leaf disks, but nine others with $EC_{50}$ values between 0.1 and 0.4 μg/ml did not infect treated disks. Neither the area under the disease progress curve nor linear growth rates were correlated with vinclozolin resistance.

*Botrytis cinerea* Pers.:Fr. infects a wide range of greenhouse plants including lettuce, tomatoes, and numerous floricultural crops (7). Infections can occur at almost any stage of plant development, either as a result of conidia germinating on and infecting susceptible tissue or from mycelium growing from infected tissue that has fallen onto healthy tissue. Benzimidazole fungicides (benomyl, thiophanate-methyl, and thiophanate-ethyl), dicarboximides (vinclozolin and iprodione), dicloran, cupric hydroxide, and mancozeb are available for controlling gray mold in Pennsylvania greenhouses, and these must be used repeatedly to protect the crops. When populations of *B. cinerea* are repeatedly exposed to site-specific fungicides, resistant strains can be readily selected. The result of this selection is documented in cases in which benzimidazole or dicarboximide fungicides have been used extensively in greenhouse production (8,10,15,18,19). Resistance to benomyl was first observed with *B. cinerea* infecting *Cyclamen* in a greenhouse (3). It has been found that benomyl resistance in *Botrytis* populations stabilizes at a very high percent in the greenhouse (13) and persists for many years after benomyl use ceases (6). Double resistance to dicarboximides and benzimidazoles was first reported in 1979 (21), and other reports followed (13,20). Although work has been done in several other countries (9,14,16,19), little is known about the fungicide sensitivity of *Botrytis* populations in greenhouses in the United States.

In Pennsylvania and most northeastern states, primary propagators start plants from seed or by vegetative propagation and sell these to other growers, who finish the plants for final sale or serve as secondary propagators and increase the number of plants before final sale. During the production of crops including poinsettias, geraniums, and cyclamens, plants are usually grown in two or more different greenhouse facilities, each facility with its own fungicide-use regime. In 1989 it was found that over 90% of 135 Pennsylvania growers surveyed use benzimidazoles, and over 30% use dicarboximides, on a regular basis (Moorman, *unpublished*). Although routine weekly application of fungicides is common, grower experience indicates that *Botrytis* accompanies plants as they are shipped from one greenhouse to another. Even if individual growers limit the use of fungicides that are at risk to the development of resistance, it is probable that *Botrytis* is repeatedly exposed to benzimidazoles and dicarboximides during production, since fungicide-application records are not passed along with the plants at the time of sale. Although no disease-control failures in Pennsylvania greenhouses as a result of *Botrytis* resistance to fungicides have been documented, it is not a common grower practice to evaluate gray mold incidence before and after a fungicide application; only a dramatic control failure would be noticed. In the absence of such control failures in greenhouses, work has not been done in the northeastern United States to ascertain the prevalence of fungicide resistance in *Botrytis*. The research reported herein was conducted to determine whether benzimidazole or dicarboximide resistance occurs in *Botrytis* isolates found in Pennsylvania green-

Accepted for publication 26 November 1991 (submitted for electronic processing).

houses and to quantify the relative fitness parameters of these isolates.

## MATERIALS AND METHODS

Leaves or flowers infected with *Botrytis* were collected from crops in 13 greenhouses selected at random from widely separated locations in Pennsylvania. Isolates 4, 5, and 17 were obtained from the same greenhouse complex on three dates approximately 1 yr apart (Table 1). Isolates 10, 11, and 12 were obtained on the same date from one greenhouse complex. Isolate 18 was from a plant purchased from a retail florist. All other isolates came from different greenhouses. Infected tissue was plated on half-strength potato-dextrose agar (PDA), and hyphal tips were transferred to fresh PDA.

The linear growth rate of each isolate was determined by transferring a plug of mycelium (5 mm in diameter), cut with a sterile cork borer from the leading edge of a 24- to 48-hr culture on PDA, to the center of each of five petri plates containing PDA and incubating it for approximately 72 hr in the dark (at 20 C). Growth was measured from the edge of the inoculum to the leading edge of the colony along four radii at right angles to each other, and the growth rate in millimeters per hour was calculated. This experiment was conducted twice, and data were subjected to an analysis of variance and the Bonferroni (Dunn) *t* test (25).

To test the fungicide sensitivity of each isolate in vitro, commercially formulated fungicides were measured and suspended in molten, sterile PDA and poured into 15- × 100-mm petri plates. Two plates per fungicide concentration were inoculated, incubated, and measured as outlined above. Each isolate was tested four times, and the average $EC_{50}$ (concentration of active ingredient that suppresses the growth rate to half that of the fungus on fungicide-free agar) was calculated from the data subjected to probit analysis (25). Benomyl (Benlate 50WP) was incorporated directly into molten agar. Because of the small amounts required, vinclozolin (Ornalin 50WP) and dicloran (Botran 75WP) were first suspended in sterile distilled water and then added to molten agar.

The pathogenicity of each isolate was tested on excised geranium (*Pelargonium* × *hortorum* L. H. Bailey) leaf tissue as follows. Red Elite geraniums were grown from seed in a walk-in growth chamber for 12 wk at 20–25 C (16-hr photoperiod). Disks, 10 mm in diameter, were cut from the fifth leaf down from the stem apex. Leaf number one was arbitrarily defined as the top-most leaf, 1 cm in diameter. Ten disks in each of five petri plates per isolate were inoculated in each of two replicate tests. Disks were incubated on moist filter paper in Parafilm-sealed petri plates at 20 C for 10 days (16 hr photoperiod supplied by 20W cool-white fluorescent lights at 5.8 $W/m^2$). To inoculate the excised tissue, spores were first harvested from a 10- to 20-day-old PDA culture grown at 20 C (for a 16-hr photoperiod) using a vacuum device. A vacuum pump was fitted with a hose, the distal end of which had a plastic tubing connector attached. A 1-cm-square piece of 1-μm-mesh nylon macrofilter fabric (Spectrum Medical Industries, Inc., Los Angeles, CA) was held in place over the tubing connector opening with a truncated disposable plastic pipet tip. The filter fabric with spores and the disposable pipet tip were placed into a test tube containing sterile 0.1 M dextrose. The spores were counted with the aid of a hemacytometer, and the suspension was adjusted to 4,000 spores per milliliter with sterile 0.1 M dextrose. To the adaxial side of each leaf disk 100 spores were applied in 0.025 ml of 0.1 M dextrose. Control disks each received 0.025 ml of sterile 0.1 M dextrose. Isolates were deemed pathogenic on geranium if the leaf disks developed browning, characteristic of *Botrytis* infection, by the end of the incubation period.

The area under the disease progress curve (AUDPC) for selected isolates was determined by inoculating 10 leaf disks in each of five separate petri plates and incubating them in a lighted growth chamber for 14 days, as described for pathogenicity tests. Disks were examined daily for the characteristic browning. The cumulative number of browned disks was

**Table 1.** Linear growth rates, pathogenicity, and fungicide sensitivity of *Botrytis cinerea* isolates from Pennsylvania greenhouses, and the $EC_{50}$ of fungicides in vitro

| Isolate | Original host | Mean growth rate (mm/hr at 20 C)[t] | AUDPC[u] | Infected vinclozolin-treated leaf disks[v] | $EC_{50}$ (μg/ml)[w] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Benomyl | Vinclozolin | Dicloran |
| 1 | Geranium | 0.396 a | 51.8 | + | 73.9 ± 9.8 | NT | 2.1 ± 0.2 |
| 2 | Geranium | 0.367 b | 71.1 | + | 225.4 ± 17.6 | 1.2 ± 0.05 | 1.8 ± 0.2 |
| 4[x] | Begonia | 0.393 a | NT | + | 164.9 ± 11.4 | 1.3 ± 0.08 | 2.7 ± 0.2 |
| 8 | Geranium | 0.318 ef | 37.6 | + | 183.9 ± 16.1 | 1.0 ± 0.16 | 2.4 ± 0.2 |
| 10[y] | Geranium | 0.281 hi | NT | + | 371.5 ± 77.7 | 1.5 ± 0.09 | 3.9 ± 0.5 |
| 11[y] | Petunia | 0.264 i | 64.6 | + | 175.8 ± 14.9 | 1.0 ± 0.05 | 2.2 ± 0.2 |
| 17[x] | Geranium | 0.374 b | NT | + | 256.4 ± 17.8 | 1.3 ± 0.09 | 2.3 ± 0.1 |
| 18[z] | Cyclamen | 0.298 gh | 68.4 | + | 298.7 ± 28.6 | 1.3 ± 0.07 | 2.7 ± 0.2 |
| 3 | Miniature rose | 0.318 ef | 64.8 | + | 163.1 ± 12.8 | 0.1 ± 0.02 | 0.7 ± 0.05 |
| 5[x] | Geranium | 0.211 j | NT | − | 1.5 ± 2.0 | 0.4 ± 0.04 | 1.2 ± 0.1 |
| 6 | Geranium | 0.318 ef | 71.5 | − | 178.8 ± 17.5 | 0.1 ± 0.01 | 1.0 ± 0.1 |
| 7 | Geranium | 0.367 b | 62.3 | − | 77.4 ± 11.0 | 0.1 ± 0.01 | 0.9 ± 0.04 |
| 9 | Geranium | 0.342 cd | 62.9 | − | 840.9 ± 1,013.1 | 0.1 ± 0.01 | 1.1 ± 0.1 |
| 12[y] | Fuchsia | 0.273 i | 65.5 | − | 207.0 ± 16.6 | 0.1 ± 0.01 | 0.8 ± 0.1 |
| 13 | Peperomia | 0.379 ab | 68.6 | − | 247.1 ± 18.1 | 0.2 ± 0.01 | 0.9 ± 0.05 |
| 14 | Geranium | 0.338 cd | 68.8 | − | 134.9 ± 21.0 | 0.1 ± 0.01 | 0.8 ± 0.04 |
| 15 | Geranium | 0.326 de | 56.9 | − | 357.7 ± 116.6 | 0.2 ± 0.01 | 0.7 ± 0.1 |
| 16 | Geranium | 0.304 fg | 64.6 | − | 199.4 ± 14.4 | 0.1 ± 0.01 | 0.8 ± 0.1 |

[t] Numbers followed by the same letter are not significantly different according to the Bonferroni (Dunn) *t* test ($P > 0.05$).
[u] Area under the disease progress curve when fungicide-free leaf disks were inoculated and incubated 14 days. NT = not tested.
[v] Ornalin 50WP (0.6 g of vinclozolin per liter) was applied to whole, 12-wk-old Red Elite geraniums the day before tissue was excised and inoculated; + = infected; − = did not infect.
[w] Average concentration of active ingredient that suppresses the growth rate to half that of the fungus on fungicide-free agar ($EC_{50}$) calculated from the data subjected to probit analysis. Benomyl (Benlate 50WP), vinclozolin (Ornalin 50WP), or dicloran (Botran 75WP) was suspended in half-strength potato-dextrose agar. NT = not tested.
[x] Isolates 4, 5, and 17 came from one greenhouse but were collected on different dates.
[y] Isolates 10, 11, and 12 came from one greenhouse and were collected on the same date.
[z] Isolate 18 came from a plant purchased at a retail florist shop.

recorded, and the AUDPC values were calculated (2). The average AUDPC values, based on two replications of the experiment, are reported.

To test the sensitivity of each isolate to benomyl on plant tissue, Benlate 50WP was applied at the manufacturer-recommended rate (0.3 g a.i/L) to whole, 12-wk-old Red Elite geraniums on the day before tissue was excised, inoculated, and incubated as described above for the pathogenicity tests. A hand-held, $CO_2$-powered sprayer (0.21 kPa) with a flat-fan nozzle was used to apply the fungicide to run-off. Vinclozolin (Ornalin 50WP) was tested similarly at the manufacturer-recommended concentration (0.6 g a.i./L). Five petri plates, each containing 10 leaf disks, were used for each isolate as described above for pathogenicity tests. Control leaf disks from fungicide-treated plants received only 0.1 M dextrose and no spores. These sensitivity experiments were replicated twice for each isolate. An isolate was defined as being resistant if it infected fungicide-free and fungicide-treated leaf disks similarly in both replications.

## RESULTS

Isolates with benomyl resistance were found in every greenhouse visited, and double resistance to benzimidazoles and dicarboximides was found in six of the greenhouses surveyed (Table 1). Isolate 18, resistant to both benomyl and vinclozolin, was obtained from a plant purchased at a retail flower store.

All isolates were pathogenic on excised geranium leaf disks. Uninoculated leaf disks remained green or slowly yellowed during the 10-day incubation period. None of the leaf disks browned unless inoculated with *Botrytis*. All isolates except isolate 5 infected benomyl-treated leaf disks. Isolate 5 did not infect benomyl-treated disks, had the slowest rate of growth in culture, did not form sclerotia in culture, sporulated very sparsely in culture, and had an in vitro $EC_{50}$ value of 1.5 $\mu$g/ml for benomyl (Table 1). All other isolates had $EC_{50}$ values greater than 70 $\mu$g/ml for benomyl. Isolates 4 and 17, from the same greenhouse complex as isolate 5, were among the fastest-growing and were resistant to both benomyl and vinclozolin on leaf disks. These three isolates were collected on different dates.

Eight of the isolates infected vinclozolin-treated leaf disks and had $EC_{50}$ values of approximately 1.0 $\mu$g/ml for vinclozolin in vitro (Table 1). These vinclozolin-resistant isolates had $EC_{50}$ values of approximately 2 $\mu$g/ml or higher for the aromatic hydorcarbon fungicide, dicloran. Isolate 3, which had $EC_{50}$ values of 0.1 $\mu$g/ml for vinclozolin and 0.7 $\mu$g/ml for dicloran, also infected vinclozolin-treated leaf disks.

Isolates from a variety of greenhouse floricultural crops (Table 1), exhibited variation in the mean growth rate at 20 C and average AUDPC values. AUDPC and resistance to vinclozolin on leaf disks did not appear to be related, since vinclozolin-resistant isolates had AUDPC values ranging from 37.6 to 71.1, and vinclozolin-sensitive isolates had AUDPC values ranging from 56.9 to 71.5.

## DISCUSSION

*Botrytis cinerea* isolates from Pennsylvania greenhouses exhibited wide variation in growth rate and AUDPC values, characteristics commonly used as indicators of fitness, suggesting that the resistant isolates tested are no more or less fit than sensitive isolates. While Beever and Brien (1) found no obvious differences in growth rate, sporulation, or sclerotia production among *Botrytis* isolates with differing levels of dicarboximide resistance, Gullino, Romano, and Garibaldi (9) found that the dicarboximide-resistant and dicarboximide-sensitive isolates in their study grew about the same on agar, but that resistant isolates produced more sclerotia. Others (12,15,26) have reported statistical correlations between reduced growth rates and fungicide resistance. The persistence of benomyl-resistant isolates of *Botrytis* in greenhouses for more than 10 yr after benomyl use ceased (6) indicates that benomyl resistance is probably not detrimental to fitness in *Botrytis* populations.

Dicarboximide-resistant and dicarboximide-sensitive isolates were pathogenic and sporulated well on geranium tissue in this study. No relationship between resistance and AUDPC value was apparent. Resistant isolates were as variable in their pathogenic ability as sensitive isolates. These results agree with those of Davis and Dennis (5), who found that isolates resistant or sensitive to dicarboximide were very similar in their infection of strawberries. That report and our results contrast with others that state that dicarboximide-resistant isolates tend to be less virulent and generally sporulate less than sensitive isolates (22,23). These differing results reflect the high variability among isolates of *Botrytis cinerea*.

The benomyl $EC_{50}$ value of 70 $\mu$g/ml that was associated with isolates resistant to benomyl may be a useful indicator of concentration for routine testing. Isolate 5 and other isolates with $EC_{50}$ values well below 70 $\mu$g/ml from other sources (Moorman and Lease, *unpublished*) were not resistant to benomyl on leaf disks. These $EC_{50}$ values for benomyl-resistant and benomyl-sensitive isolates agree with other reports (4,29). A benomyl concentration of 2.5 $\mu$g/ml is employed for routine use in radial growth studies for separation of resistant and sensitive isolates (27). Among isolates found to be vinclozolin-resistant on leaf disks, in vitro $EC_{50}$ values were approximately 1 $\mu$g/ml. This finding agrees with other studies (11,12,17, 20,21,28). However, isolate 3, vinclozolin-resistant on treated leaf disks, had an $EC_{50}$ value of 0.1 $\mu$g of vinclozolin per milliliter. Therefore, regardless of in vitro results, it should be standard practice to verify putative in vitro fungicide resistance by using tests on treated plant tissue.

In some studies (12), *Botrytis* isolates with resistance to vinclozolin have been found to be cross-resistant to the aromatic hydrocarbon fungicide dicloran. Others (17) have found the vinclozolin-dicloran cross-resistance association to be inconsistent. We did not test the sensitivity of our isolates to dicloran on leaf disks. However, all isolates studied here with resistance to vinclozolin on leaf disks, except isolate 3, had $EC_{50}$ values of approximately 2 $\mu$g/ml or higher for dicloran, and vinclozolin-sensitive isolates had dicloran $EC_{50}$ values of approximately 1 $\mu$g/ml or lower. This possible cross-resistance should be explored further with additional isolates both in vitro and on plant tissue.

All 13 greenhouses surveyed were found to harbor *Botrytis* strains resistant to the benzimidazole fungicide, benomyl. Six greenhouses harbored *Botrytis* with double resistance to benomyl and vinclozolin. Since it is common for plant material to be shipped from one commercial greenhouse to another as the plants proceed through various stages of production, and since conidia of *Botrytis* are known to have the ability to remain dormant on plant tissue for weeks (24), *Botrytis* strains with resistance to fungicides are probably being spread from facility to facility via plant shipment. Therefore, even if greenhouse operators have not used benzimidazoles or dicarboximides, *Botrytis* resistant to these fungicides may be present on the plants they purchase. Once present in a greenhouse, the fungicide-resistant isolates may persist for many years, even if the chemical to which it is resistant is not applied (6), thus making that fungicide ineffective. For these reasons, primary and secondary propagators who sell plants to other growers should be particularly careful to use fungicides that are at risk to the development of resistance in a manner that minimizes resistance selection. In order for all growers in the greenhouse industry to minimize selecting for resistance, however, it must become standard practice for fungicide application records to accompany the treated plants throughout the production cycle.

Because of the prevalence of benomyl resistance found in this study, benzimidazoles are no longer recommended for the control of gray mold in Pennsylvania greenhouses. A test that growers

can employ to detect the presence of fungicide resistance in *Botrytis* is currently needed, so that sound decisions can be made concerning the use of dicarboximides.


## ACKNOWLEDGMENTS

This research was funded by the American Floral Endowment, the Pennsylvania Flower Growers, The Fred C. Gloeckner Foundation, The Pennsylvania Department of Agriculture, the USDA National Northeast Regional Pesticide Impact Assessment Program, and the Pennsylvania State University Agricultural Experiment Station. We thank Karen Harper for her excellent technical assistance and Janice Derr and Yldiz Akin for their help in statistical analyses.


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