Benzimidazole-Resistant Mutant Derived from a Single Ascospore Culture of Botryotinia fuckeliana

F. J. Polach and W. T. Molin

Assistant Professor and Research Assistant, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456.

Approved by the Director as Journal Series Paper No. 2161, New York State Agricultural Experiment Station, Geneva.

Accepted for publication 18 March 1975.

ABSTRACT

A benomyl-resistant mutant of *Botrytis cinerea* was obtained by plating conidia derived from single ascospore isolates of *Botryotinia fuckeliana* on PDA plates supplemented with 10 μg/ml benomyl. Fifty percent reduction in linear mycelial growth of the wild-type and benomyl-resistant isolates occurred at 0.05 to 0.10 and 350 μg/ml benomyl, respectively. Some growth and limited sporulation of the resistant isolate occurred even at 1,000 μg/ml benomyl, the highest level tested. The resistant isolate produced conidia at all levels of benomyl tested and consistently sporulated more profusely than the sensitive isolate. Germination of conidia produced on 1,000 μg/ml benomyl was almost 100% on water agar, and 98% of these conidia formed colonies and subsequently sporulated when transferred from water agar to 500 μg/ml benomyl. The resistant isolate was also resistant to other benzimidazole fungicides tested, whereas both benomyl-resistant and benomyl-sensitive isolates were sensitive to dichloran. Both resistant and sensitive isolates were pathogenic on bean leaves and pods, although the sensitive isolate was slightly more aggressive.

Phytopathology 65:902-904

Additional key words: *Phaseolus vulgaris*, gray mold.

Gray mold, incited by *Botrytis cinerea* Pers. ex. Fr., is an important disease of beans (*Phaseolus vulgaris* L.) in New York State where 115,000 acres are planted annually. The disease is effectively controlled by benomyl [1-(butylcarbamoyl)-2-benzimidazole-carbamate] which is used widely by growers. Because of reports of resistance of *B. cinerea* to benomyl (1,7) and the numerous occurrences of resistance to benomyl in other fungi (2,3,4,6,8), an attempt was made to determine if benomyl-resistant mutants of *B. cinerea* could arise from benomyl-sensitive isolates obtained from bean fields.

This paper reports the isolation of a benomyl-resistant mutant (3R) from a benomyl-sensitive (3S) isolate of *B. cinerea*; the influence of benomyl on linear mycelial growth and sporulation of the resistant and sensitive isolates; the reaction of the benomyl-resistant isolate to other benzimidazole fungicides; and a comparison of the pathogenicity of the resistant and sensitive isolates to beans.

MATERIALS AND METHODS.—*B. cinerea* cultures used in this study were derived from single ascospore isolates of *Botryotinia fuckeliana* (De Bary) Whetz. obtained from a bean field near Alton, New York, in 1973 (5). The cultures were maintained on potato-dextrose agar (PDA) and single-spore cultures were reisolated periodically from artificially infected bean tissues. In the search for benomyl-resistant mutants, conidia were plated on PDA amended with 10 μg/ml benomyl. Survivors were transferred to PDA plates. Benomyl-resistant mutants occurred at a frequency of 1 in 5×10^7 conidia. One of these benomyl-resistant mutants (3R) and a benomyl-sensitive isolate (3S) from which the mutant arose were selected for further experimentation.

The linear mycelial growth of 3R and 3S isolates was compared on benomyl-amended PDA after 72 hours' incubation under 14 hours of fluorescent light and 10 hours of dark at 24 ± 2 C. Benomyl was added to PDA prior to autoclaving in final concentrations of 0, .05, .10, .25, .50, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 75.0, 100.0, 200.0, 350.0, 500.0, 750.0, and 1,000.0 μg/ml. Each plate was centrally seeded with a 4-mm agar-mycelium disk cut from the actively-growing margin of a 3-day-old PDA culture. Each treatment consisted of five replicates, and the experiment was repeated three times. The in vitro responses of 3R and 3S isolates to other benzimidazoles, methyl [[[1-(5-cyanopentyl) amino] carbonyl]-1*H*-benzimidazole-2-yl]carbamate (Bay Dam 18654), thiophanate methyl (Topsin M), and thiabendazole (Mertect), were examined at rates similar to those given above. A nonbenzimidazole fungicide, dichloran (Botran), was also tested.

In comparative pathogenicity tests of 3R and 3S isolates, leaves and pods of greenhouse-grown beans (cultivar 'Cascade') were artificially wounded with a sterile needle, and each was inoculated with a 4-mm agar-mycelium disk cut from the advancing margin of a 3-day-old PDA culture. Another set was inoculated without wounding. Each treatment consisted of five replicates and was repeated four times. Inoculated leaves and pods were placed on screen trays in closed plastic boxes lined with moist paper towel and with 1 cm of water in the bottom. The boxes were kept in the laboratory under 14 hours of fluorescent light and 10 hours of dark at 24 ± 2 C. Lesion diameters were recorded at 72 hours.

Sporulation of the two isolates was determined by counting the number of conidia produced on the various benomyl concentrations after 8 days' incubation. Culture plates were flooded with 10 ml of sterile distilled water and swirled with a glass rod to free the spores. The resultant spore suspension was decanted into sterile test tubes and diluted for counting with a hemacytometer. Percentage germination was determined by plating the conidia on water agar and counting a minimum of 100 conidia in each of five different fields. In addition, spores of the 3R isolate harvested from plates amended with 1,000 μg/ml benomyl were removed singly from the water agar plates and placed on PDA plates amended with 500 μg/ml benomyl to determine if resistance was retained in the spores.

RESULTS.—The mean colony diameters of *B. cinerea* isolates on benomyl-amended PDA are shown in Fig. 1. The linear mycelial growth of the 3S isolate was reduced by 50% between 0.05 and 0.10 μg/ml benomyl and by 100% at 2.0 μg/ml. Linear mycelial growth of isolate 3R was reduced by 50% at approximately 350 μg/ml and by 77% at 1000 μg/ml, the highest level tested.

Conidial production at the various levels of benomyl concentrations was determined after 8 days of incubation as shown in Table 1. Isolate 3R consistently sporulated more profusely than 3S in the presence or absence of benomyl. Isolate 3S did not sporulate on PDA containing more than 0.1 μg/ml benomyl, whereas 3R sporulated heavily at 100 μg/ml and lightly at 1,000 μg/ml. Germination of these conidia approached 100% on water agar plates. When colonies from conidia formed on 1,000 μg/ml plates were transferred from the water agar plates to 500 μg/ml benomyl plates, 98% of them formed colonies and subsequently produced conidia.

Isolate 3R was resistant to the other benzimidazoles tested (Table 2) although the levels of sensitivity varied somewhat, whereas 3S was sensitive to these compounds. Isolate 3R was more sensitive to thiabendazole than to the other benzimidazoles, with 50% reduction in linear mycelial growth between 50 and 75 μg/ml active ingredient, and no measurable growth at 200 μg/ml. On medium containing dichloran, the only nonbenzimidazole fungicide tested, the two isolates were equally sensitive to the fungicide. Linear mycelial growth of both 3R and 3S isolates was reduced approximately 50% at 1 μg/ml dichloran, and no growth of either isolate occurred at 7.5 μg/ml.

In the pathogenicity tests (Table 3), isolates 3S was somewhat more pathogenic on both detached pods and leaves, although the lesions induced by isolate 3R exhibited more sporulation. Lesions were slightly larger when the tissue was wounded before inoculation.

DISCUSSION.—The benomyl-resistant isolate reported in this study was derived from a benomyl-sensitive isolate under laboratory conditions. However, the fact that the mutant was obtained from a field-collected, single-ascosporic isolate which had not been treated with mutagenic agents, and which produced primarily benomyl-sensitive conidia, suggests that benomyl-resistant mutants might arise under field conditions.

In the absence of benzimidazoles, the rate of linear mycelial growth of the 3R mutant did not differ from that of 3S at $P = 0.05$; however, isolate 3R consistently sporulated more profusely than 3S. Isolates 3R and 3S were similar in pathogenicity, although 3R appeared somewhat less aggressive. However, it has been reported (1,8) that benomyl-resistant isolates of *B. cinerea* and *Verticillium malthousei* were considerably less pathogenic, and thus probably less competitive, than the wild-type isolates.

Other workers (1,2,4) have reported that strains of fungi resistant to benomyl are resistant to other benzimidazoles as well. This was also true of the 3R isolate which displayed resistance to Bay Dam 18654, thiophanate methyl, and thiabendazole as well as to benomyl. Both 3R and 3S were sensitive to dichloran, a nonbenzimidazole fungicide. However, both isolates produced increased numbers of conidia on increasing levels of dichloran, which may indicate why it has not adequately controlled gray mold of bean previously.

That the benomyl-resistant isolate studied here was obtained without mutagenic treatment, and compared favorably with the wild-type isolate in linear mycelial growth rate, sporulation and pathogenicity demonstrates

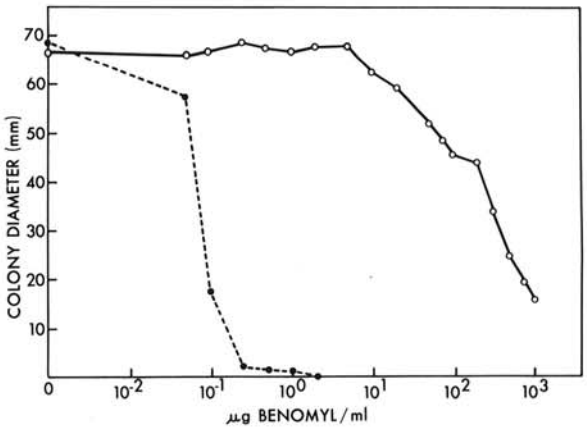

Fig. 1. Mean colony diameter of a benomyl-sensitive (•- - - -•) and a benomyl-resistant (o————o) isolate of *Botrytis cinerea* incubated for 72 hours at 24 ± 2 C on potato-dextrose agar amended with various concentrations of benomyl.

TABLE 1. Conidial production of benomyl-resistant (3R) and benomyl-sensitive (3S) isolates of *Botrytis cinerea* on benomyl-amended PDA

	Concentration of benomyl (μg/ml)						
Isolate	0	.1	1	10	100	500	1,000
Resistant (3R)	74.0[a]	74.0	76.0	74.0	78.0	22.8	0.5
Sensitive (3S)	9.9	7.8	0	0	0	0	0

[a]Millions of conidia per plate after 8 days.

TABLE 2. Effect of four benzimidazole fungicides incorporated in PDA on linear mycelial growth of a benomyl-resistant isolate (3R) of *Botrytis cinerea*

	Concentration of active ingredient (μg/ml)								
	0	1	10	50	75	100	200	500	1,000
(Bay Dam 18654)[a]	67[b]	67	63	...	...	44	...	36	26
Benomyl	67	67	62	...	...	45	...	24	16
Thiobendazole	67	...	63	37	29	19	0	...	...
Thiophanate methyl	67	65	62	...	...	45	...	28	16

[a]Methyl [1-[[(5-cyanopentyl) amino] carbonyl]-1 *H*-benzimidazole-2-yl] carbamate.

[b]Colony diameters were measured in mm after 72 hours of incubation at 24 ± 2 C. Each figure represents the means of three experiments with five replicates each.

TABLE 3. Pathogenicity of benomyl-resistant (3R) and benomyl-sensitive (3S) isolates of *Botrytis cinerea* to detached, injured and uninjured bean leaves and pods

	Lesion diameter (mm)			
	Bean leaves		Bean pods	
	Injured	Uninjured	Injured	Uninjured
Resistant (3R)	27.8[a]	25.3	26.8	20.2
Sensitive (3S)	30.1	28.5	29.4	22.7

[a]Lesion diameters were measured in mm after 72 hours of incubation at 24 ± 2 C. Each figure represents the means of four experiments with five replicates each.

the necessity of developing methods of control which would prevent a resistant strain from becoming established.

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