

Pathogenicity and Benzimidazole Resistance in *Penicillium* Species Recovered from Flotation Tanks in Apple Packinghouses

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ABSTRACT

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Three hundred and sixty-eight isolates of *Penicillium* were recovered from water collected from flotation tanks in four apple packinghouses in southeastern New York. The 345 isolates, which caused blue mold in inoculated apples (*Malus domestica*), were identified to species and tested for sensitivity to methyl 2-benzimidazolecarbamate (MBC) and diphenylamine (DPA). Sixty-five isolates were *P. expansum*, 277 were *P. aurantiogriseum*, two were *P. citreonigrum*, and one was *P. dendriticum*. Thirty-one isolates of *P. expansum* were sensitive to MBC, 27 were resistant to MBC but sensitive to DPA, and seven were resistant to both MBC and DPA when tested in vitro. Ninety-five percent of the 277 isolates of *P. aurantiogriseum* were resistant to both MBC and DPA. *P. aurantiogriseum* was the predominant species recovered on all five sampling dates (November to March). Seventy-four percent of the isolates of *P. expansum* were recovered in the November and December sampling dates.

Additional keywords: negative cross-resistance, *P. solitum*

In the United States, a benzimidazole fungicide is commonly applied to apples (*Malus domestica* Borkh.) after harvest to prevent storage decays caused by *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr. Strains of *P. expansum* resistant to benomyl and/or methyl 2-benzimidazolecarbamate (MBC) have been found in many areas of the United States (1,3,15,21). However, benzimidazole fungicides are still used for postharvest treatment of apples because no effective alternative fungicides are available and because storage and packinghouse operators believe benzimidazole treatments remain effective despite the presence of strains resistant to MBC. Actually, some benzimidazole-resistant strains of *P. expansum* are probably being controlled by diphenylamine (DPA), a storage scald inhibitor commonly applied with a benzimidazole fungicide in postharvest drenches (14).

DPA inhibits the growth of strains of *P. expansum* that are highly resistant to benzimidazole fungicides, but it has less effect on strains that are sensitive or moderately resistant to these fungicides

(14). When a benzimidazole fungicide and DPA are applied together, the benzimidazole controls strains sensitive to benzimidazoles and DPA controls strains highly resistant to benzimidazoles, but strains with moderate resistance to benzimidazoles are not controlled. Thus, incidence of strains in the latter group could affect the performance of the benzimidazole/DPA treatments used by packinghouse operators.

Our objective in this study was to determine levels of benzimidazole resistance in populations of *P. expansum* found in apple packinghouses. During the course of the study, we discovered that *P. aurantiogriseum* Dierckx was prevalent in apple packinghouses. Therefore, we proceeded to study both *Penicillium* species. A preliminary report of this study has been published (13).

MATERIALS AND METHODS

Collecting isolates. Water samples were collected on five dates from flotation tanks in each of four commercial apple packinghouses located in Ulster County, NY. Collection dates were 14 November and 12 December 1985 and 10 January, 14 February, and 21 March 1986. The packinghouse operators changed the water in the flotation tanks at the end of each week, so water samples were collected on Fridays when the largest spore populations were likely to be present. None of the packinghouse operators used chlorine or any other biocides in their flotation tanks.

For each water sample, 0.1 ml of a 1:100 dilution was spread on each of six plates containing acidified potato-dextrose agar. Three plates were similarly inoculated with a 1:10 dilution. Plates were incubated at 20 C. Developing colonies of *Penicillium* were counted on days 3, 4, and 5. Colony morphology was used to differentiate nonsporulating colonies of *Penicillium* from other contaminants on the plates. These initial colony identifications were verified after colonies started sporulating. Randomly selected nonsporulating colonies of *Penicillium* were transferred from the plates to potato-dextrose agar (PDA) slants in test tubes. After 2-3 wk, spores were collected from the agar slants and the cultures were stored on silica gel (18) until needed for further testing. For all but the first collection date, the number of spores of *Penicillium* per milliliter of flotation water was calculated using colony counts from both the 1:10 and 1:100 dilution plates.

Evaluating pathogenicity. Pathogenicity of each isolate was tested on Empire apples that had received no late-season or postharvest fungicide treatments. The apples were kept in controlled atmosphere storage until needed for pathogenicity tests later in the winter. A scalpel was used to remove a pyramidal (approximately 4 mm per side) section of tissue from opposite faces of each fruit. A loopful of dry spores (about 10^7 conidia on a 4-mm-diameter loop) was transferred from a 4- to 7-day-old culture to each of the two wounds on a fruit. Inoculations were repeated on a second fruit only for isolates that produced inconsistent results on the two wounds on the first fruit. Inoculated fruit were immediately enclosed in individual plastic bags and incubated on the lab bench at 18-23 C. The diameter of the decayed area was measured after 7 and 12 days.

Determining sensitivity to MBC and DPA. Isolates were tested for their ability to grow on PDA and on PDA amended with DPA at 10 $\mu\text{g/ml}$ and with MBC at 5, 30, and 100 $\mu\text{g/ml}$. Appropriate quantities of MBC and freshly prepared DPA stock solutions were added to autoclaved PDA that had been cooled to 47 C in a water bath. The MBC stock

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Table 1. Volume of apples (in t) packed before collection of water samples from packinghouse dumps on four dates during the 1985–1986 packing season and mean numbers of *Penicillium* spores per milliliter of dump water as determined by dilution plating

Packinghouse	Sample 2: 12 December		Sample 3: 10 January		Sample 4: 14 February		Sample 5: 21 March	
	Apples packed (t)	<i>Penicillium</i> spores ^a (no./ml)	Apples packed (t)	<i>Penicillium</i> spores (no./ml)	Apples packed (t)	<i>Penicillium</i> spores (no./ml)	Apples packed (t)	<i>Penicillium</i> spores (no./ml)
1	38.1	2,000	49.5	1,330	53.3	2,166	ND	ND
2	57.1	670	57.1	5,666	25.7	1,666	22.9	1,166
3	ND ^b	1,670	57.1	9,170	ND	4,333	22.9	9,333
4	22.9	100	33.1	2,833	35.8	166	44.6	10,166
Means	39.4	1,110	49.2	4,750	38.3	2,083	30.1	6,888

^aWater collected from flotation tanks was diluted 1:10 and 1:100 with sterile distilled water. Acidified potato-dextrose agar plates were inoculated with 0.1 ml of the diluted solutions, incubated at 20 C, and observed for developing colonies of *Penicillium* after 3–5 days. Numbers are means calculated from colony counts on three 1:10 and six 1:100 dilution plates.

^bND = No data available.

solution (1,000 µg/ml) was prepared by the method described by Wilde (19). Technical DPA was dissolved in absolute ethanol. To compensate for possible effects of ethanol and Tween 20 added to agars via the stock solutions, appropriate quantities of these chemicals were added to all agars to bring the final concentrations of ethanol and Tween 20 to 0.1 and 0.01% v/v, respectively.

Spore suspensions were prepared from 4- to 7-day-old cultures. A 4-mm-diameter wire loop was used to transfer dry conidia from the culture to 5-ml screw cap tubes containing 0.5 ml of 1% water agar and 0.01% Tween 20. For each isolate, a drop of spore suspension was streaked on two plates of the PDA and on two plates of each of the MBC- and DPA-amended agars. The width of the growth along the streaks was measured after the plates had been incubated at 20 C for 3 days (MBC) or 10 days (DPA).

Isolates were rated as sensitive to MBC if no growth appeared on PDA amended with 5 µg of MBC per milliliter, slightly resistant if growth appeared at 5 but not 30 µg of MBC per milliliter, moderately resistant if growth on media amended with 100 µg of MBC per milliliter was 10–90% of that on unamended medium, and highly resistant if growth on 100 µg of MBC per milliliter was 90–100% of that on unamended medium. Isolates considered sensitive to DPA failed to produce growth on the DPA-amended agar within 10 days, whereas those producing visible growth within 10 days were considered resistant.

Identifying isolates. Isolates were separated into groups based on the color and gross morphology of 7- and 14-day-old colonies on Czapek's medium and malt extract agar. Representative isolates from each group were sent to the USDA Northern Regional Research Laboratory in Peoria, IL, where they were identified to species with the keys and methods of Pitt (10) and Williams and Pitt (20).

RESULTS

Populations in flotation tanks. Populations of *Penicillium* in water from flotation tanks varied from the minimum

Table 2. Incidence of *Penicillium* species recovered from water dumps in four apple packinghouses during the 1985–1986 storage season and segregated by sensitivity to methyl 2-benzimidazole carbamate (MBC) and diphenylamine (DPA)

<i>Penicillium</i> species	Sensitivity		Isolates collected (no.)					Percent of total collection ^c
	MBC ^a	DPA ^b	Packinghouse				Total	
			1	2	3	4		
<i>P. expansum</i>	+	—	7	10	1	13	31	9.0
	—	—	0	0	0	1	1	0.3
	---	—	0	2	0	4	6	1.7
<i>P. aurantiogriseum</i>	----	+	5	1	21	0	27	7.8
	+	—	1	0	3	3	7	2.0
	----	+	1	1	4	0	6	1.7
	---	—	3	7	10	6	26	7.6
	----	—	38	80	62	58	238	69.0

^aIsolates were rated as MBC-sensitive (+) if no growth appeared on PDA amended with 5 g of MBC per milliliter, slightly resistant (—) if growth appeared at 5 but not 30 g of MBC per milliliter, moderately resistant (---) if growth on media amended with 100 g of MBC per milliliter was 10–90% of that on unamended medium, and highly resistant (----) if growth on 100 g of MBC per milliliter was 90–100% of that on unamended medium.

^bIsolates considered sensitive to DPA (+) failed to produce growth on the DPA-amended agar within 10 days, whereas those producing visible growth within 10 days were considered resistant (—).

^cA total of 342 isolates of *P. expansum* and *P. aurantiogriseum* were collected and tested during the survey.

Table 3. Comparisons by collection date showing the proportions of the total *Penicillium* population attributable to the major fungicide sensitivity subgroups for *P. expansum* and *P. aurantiogriseum*

<i>Penicillium</i> species	Sensitivity		Percent of total collection for each date				
	MBC ^a	DPA ^b	14 Nov. 1985	12 Dec. 1985	10 Jan. 1986	28 Feb. 1986	21 March 1986
<i>P. expansum</i>	+	—	20	17	2	10	1
	----	+	34	0	4	0	0
<i>P. aurantiogriseum</i>	---	—	1	12	7	10	7
	----	+	39	56	81	73	87
Total number of isolates collected			71	41	82	76	75

^aIsolates were rated as MBC-sensitive (+) if no growth appeared on PDA amended with 5 g of MBC per milliliter, moderately resistant (---) if growth on media amended with 100 g of MBC per milliliter was 10–90% of that on unamended medium, and highly resistant (----) if growth on 100 g of MBC per milliliter was 90–100% of that on unamended medium.

^bIsolates considered sensitive to DPA (+) failed to produce growth on the DPA-amended agar within 10 days, whereas those producing visible growth within 10 days were considered resistant (—).

detection level of 10² to more than 10⁴ conidia per milliliter in the 15 samples tested (Table 1). Twenty-two to 60 t of fruit had been processed through each flotation tank before a water sample was collected.

Pathogenicity of isolates. Twenty-

three of the 368 isolates collected from dilution plates failed to cause lesions in apple fruit. Sixty-five of the 345 pathogenic isolates produced lesion diameters of 50–70 mm within 12 days, whereas the diameters of the remainder were <30 mm. None of the isolates caused lesions

of 30–50 mm.

Resistance to MBC and DPA. Eighty-nine percent of the pathogenic isolates were moderately or highly resistant to MBC. However, 280 of the isolates caused only small decays. The strain that accounted for 69% of the total collection was weakly aggressive, highly resistant to MBC, and resistant to DPA. The second most common strain (9% of the population) was highly aggressive, sensitive to MBC, and resistant to DPA. Representatives of the six most common strains were recovered from at least three of the four packinghouses (Table 2). The only unusual distribution was for the strain that was highly aggressive, highly resistant to MBC, and sensitive to DPA. For this strain, 21 of 27 isolates were recovered from a single packinghouse.

Species identification. All 65 of the highly aggressive isolates were identified as *P. expansum*. Most were recovered early in the packing season. Fifty of the 65 isolates were recovered from flotation tanks where most of the fruit processed had not received any postharvest fungicide treatment.

All but three of the 280 weakly aggressive isolates were *P. aurantiogriseum*. The proportion of *P. aurantiogriseum* in the population increased from 40% in November to 94% in March (Table 3). Twenty representative isolates of *P. aurantiogriseum* were deposited in the Agricultural Research Culture Collection as NRRL A-27610-27619, NRRL A-27622-27624, NRRL A-27590, and NRRL A-27592-27597.

A single isolate of *P. dendriticum* Pitt (NRRL A-27591) was recovered in November, and two isolates of *P. citreonigrum* Dierckx (NRRL A-27620 and NRRL A-27621) were recovered from the same packinghouse in December. All three isolates were less aggressive than most isolates of *P. aurantiogriseum* and caused very limited decays when initially inoculated into apple fruit.

DISCUSSION

At least 11 species of *Penicillium* have been reported to cause apple blue mold (2,7,9,11,12). Although *P. expansum* generally has been the predominant species found in pome fruits, *P. aurantiogriseum* was the most prevalent species in our survey. *P. aurantiogriseum* may predominate in flotation water because *P. expansum* is effectively suppressed by postharvest treatment with a benzimidazole plus DPA, whereas *P. aurantiogriseum* is not. A similar phenomenon occurred in Israel when imazalil treatment controlled *P. expansum* but resulted in an unexpectedly high incidence of *P. crustosum* Thom in apple fruit (11).

P. aurantiogriseum, *P. dendriticum*, and *P. citreonigrum* are unlikely to cause major losses in apple storages because isolates of these species were much less aggressive in inoculated apples than were

isolates of *P. expansum*. The isolates of *P. dendriticum* and *P. citreonigrum* we recovered from flotation tanks caused very small lesions in our initial pathogenicity tests and were nonpathogenic in freshly harvested Empire fruit when retested in 1989 (D. A. Rosenberger, unpublished). Despite the prevalence of *P. aurantiogriseum* in flotation tanks, *P. expansum* may still be the predominant cause of fruit decays. Further research with *P. aurantiogriseum* is warranted, however, because this species was frequently isolated from decayed fruit collected from apple packinghouses in a subsequent storage survey (D. A. Rosenberger, unpublished).

The strains we identified as *P. aurantiogriseum* might eventually be re-assigned to another species as the taxonomy of the terverticillate *Penicillia* is revised. Cruickshank and Pitt (4) distinguished *P. solitum* Westling from *P. aurantiogriseum* based on distinctly different pectic zymogram patterns. One of our isolates (NRRL A-27592), identified here as *P. aurantiogriseum* sensu Pitt 1979 (10), was sent to R. H. Cruickshank for identification. It produced a pectic zymogram pattern identical to that of *P. solitum* (R. H. Cruickshank, personal communication). Additional isolates in our collection might be identified as *P. solitum* if pectic zymograms were used as the basis for identification.

The change in the incidence of *P. expansum* in flotation water as the storage season progressed may reflect the effects of postharvest treatment. In New York, most apples kept in regular cold storage are not given postharvest treatments, whereas a benzimidazole/DPA postharvest treatment is applied to fruit intended for controlled-atmosphere storage. Apples packed in November and December and some of those packed in January came from regular cold storages. About 77% of the isolates of *P. expansum* came from flotation tanks used to process this mostly untreated fruit. Only 15 of the 65 isolates of *P. expansum* we recovered came from flotation tanks used to process treated fruit from controlled-atmosphere storage.

The isolates resistant to MBC collected from flotation water used to process untreated fruit may have originated from selection pressure in apple storages or in the field. Hudson Valley growers, including most of those supplying fruit to the storages surveyed here, commonly used benomyl or thiophanate-methyl in field sprays to control apple scab, sooty blotch, and fly speck. Spotts and Cervantes (17) have suggested field use of benzimidazoles may increase the prevalence of MBC-resistant *P. expansum* in storages. Only 4–31% of the isolates of *P. expansum* that they collected were resistant to benzimidazoles compared with 52% in our survey.

One proposed method for controlling fungicide-resistant populations of plant pathogens is to apply combinations of products to which the target organisms exhibit negative cross-resistance (5). Negative cross-resistance is defined as resistance to one fungicide being linked to sensitivity to a second fungicide. Partner compounds evaluated for use with benzimidazoles have controlled only the pathogen strains that are highly resistant to benzimidazoles. Moreover, pathogen strains with slight or moderate benzimidazole resistance are common in most host-pathogen systems investigated (6,8,16,19), and these strains are not adequately inhibited by either the benzimidazole or the partner compounds. MBC-resistance in *P. expansum* appears unique in that relatively few isolates with slight or moderate resistance were found in storages where benzimidazole/DPA combinations have been used for nearly 15 yr. Unknown factors in the apple/*P. expansum* postharvest system apparently inhibit development of large populations of *P. expansum* with slight to moderate benzimidazole resistance. As a result, benzimidazole/DPA postharvest treatments remain effective against the common strains of *P. expansum* in packinghouses. The apple/*P. expansum* postharvest system represents a commercial situation where negative cross-resistance has been exploited successfully for managing resistance to a fungicide.

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