Postharvest Fungicides for Apples: Development of Resistance to Benomyl, Vinclozolin, and Iprodione

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ABSTRACT

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The mean incidence of vinclozolin-resistant and iprodione-resistant spores from four isolates of *Penicillium expansum* was 1.96×10^{-5} and 1.71×10^{-6}, respectively. All of 25 isolates tested that were resistant to iprodione were also resistant to vinclozolin. However, only 9 of 16 isolates that were resistant to vinclozolin were also resistant to iprodione. In a postharvest fungicide test with McIntosh fruit, two wild-type isolates were more pathogenic than the two isolates resistant to vinclozolin and the two resistant to iprodione. Mean incidence of decay in fruit not treated with fungicides was 65% for wild-type isolates compared with 0.4% for isolates resistant to vinclozolin and iprodione. Benomyl and CGA 64251 were effective against all these isolates.

Additional key words: cross-resistance, fungicide resistance

Penicillium expansum. Lk. ex Thom., the pathogen causing blue mold in apples, is effectively controlled by the benzimidazole fungicides benomyl and thiabendazole, but isolates resistant to benomyl have recently been reported in Australia (10) and in several states in the United States (1,2,6). The dicarboximide fungicides vinclozolin and iprodione have been tested as alternatives for benomyl in postharvest treatments (2,8). However, isolates of *Monilinia fructicola* (Wint.) Honey, *Botrytis cinerea* Pers. ex Fr., and *P. expansum* resistant to the dicarboximides can easily be selected when spores of these fungi are plated on fungicide-amended agar (4,5,8,9). The pathogenicity and ecologic fitness of these resistant isolates have not been determined.

The objectives of this study were to determine the incidence of spores of *P. expansum* resistant to vinclozolin and iprodione in vitro, the incidence of cross-resistance and the effects of fungicide concentrations on growth of resistant isolates, and the pathogenicity of resistant isolates under apple storage conditions.

MATERIALS AND METHODS

Incidence of resistant spores. Spores from four isolates of *P. expansum* originally isolated from decayed apples were seeded on potato-dextrose agar (PDA) amended with vinclozolin (VPDA) or iprodione (IPDA) to determine the incidence of spores resistant to these compounds. Two (P-8, P-24) of the four isolates were resistant to benomyl and two (P-26, P-28) were susceptible. All four isolates, hereafter called "parent wild-type isolates," were pathogenic to apple fruit when mycelial plugs were used to inoculate wounded apples in the lab.

All fungicide-amended agars used in this study were made by adding the commercially formulated fungicides to PDA immediately after autoclaving. Fungicide concentrations are given as micrograms of active ingredient per milliliter. Vinclozolin (Ronilan 50W, BASF Wyandotte Corporation, Parsippany, NJ 07054) and iprodione (Rovral 50W, Rhone-Poulenc, Inc., Monmouth Junction, NJ 08852) were added to PDA to produce concentrations of 75, 150, 300, 600, and 1,200 μg/ml. Spore suspensions of each of the four parent wild-type isolates were made by flooding 8-day-old cultures with sterile distilled water. The spore suspensions were adjusted to $1.4–1.5 \times 10^7$ spores per milliliter by hemacytometer counts, and 0.1 ml of the suspensions was spread on PDA plates amended with vinclozolin or iprodione at 1,200 μg/ml. We assured adequate separation between resistant colonies on plates by diluting spore suspensions 1:1 and 1:4 in water before inoculating plates amended with 300 and 600 and with 75 and 150 μg of fungicide per milliliter, respectively. Three replicate platings were made for each of the parent wild-type isolates on each of the five concentrations of two fungicides. Plates were incubated at room temperature on the laboratory bench and were observed for growth after 25 days.

To test for resistance selection in vivo under commercial apple storage conditions, we isolated *P. expansum* from 228 decayed fruits that had been treated with vinclozolin or iprodione at 600–1,200 μg/ml in the postharvest fungicide tests included in this study or reported elsewhere (7). Fruits in these postharvest tests were stored at 0–3 C in commercial apple storages. Isolates were recovered from decayed fruits by plating small bits of decayed flesh on PDA. We tested spores from the resulting colonies for resistance to vinclozolin and iprodione by streaking them across divided petri plates containing PDA on one side and VPDA or IPDA, with fungicide at 150 μg/ml, on the other side.

Cross-resistance of isolates. Sixteen isolates resistant to vinclozolin (VR) and 25 resistant to iprodione (IR) were subcultured from fungicide-amended plates by the removal of colonies before they sporulated. The colonies were transferred to VPDA or IPDA slants (containing the fungicide at 150 μg/ml)

Table 1. Incidence of vinclozolin-resistant spores from four isolates of *Penicillium expansum* as influenced by the concentration of vinclozolin in potato-dextrose agar (PDA)

Isolate	Vinclozolin (μg/ml) in amended PDA					Means for isolates[w]
	75	150	300	600	1,200	
P-8	3.26[x]	5.67	3.21	3.44	0.33	3.18
P-24	1.79	1.79	1.48	0.94	0.13	1.23
P-26	1.37	2.65	2.02	1.83	0.20	1.61
P-39	1.95	2.31	2.71	...[y]	0.22	1.81
Means for concentrations[z]	2.09	3.11	2.37	2.02	0.22	

[w] LSD ($P = 0.05$) between means for isolates = 0.95×10^{-5}.
[x] Numbers ($\times 10^{-5}$) are means for three plates. The least significant difference (LSD) for isolate-concentration comparisons ($P = 0.05$) = 1.47×10^{-5}.
[y] Missing data.
[z] LSD ($P = 0.05$) between means for concentrations = 0.93×10^{-5}.

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to prevent germination of any of the original, ungerminated *P. expansum* spores that might have been picked up with the resistant colonies. Spores produced by the resistant isolates on the fungicide-amended PDA slants were streaked across plates of PDA and across plates of seven fungicide-amended PDAs to test for cross-resistance. The PDA amendments used were benomyl at 250 μg/ml and vinclozolin and iprodione each at 75, 300, and 1,200 μg/ml. Each isolate was tested on three plates of each fungicide-amended PDA. Cross-resistance was determined by observing growth and sporulation of the streaks after 7 days.

Pathogenicity of resistant isolates and decay control with fungicides. A factorial experiment involving six isolates of *P. expansum* and seven postharvest treatments was designed to determine the pathogenicity of VR and IR isolates under several storage conditions. Two IR isolates (PIR-5, PIR-6), two VR isolates (PVR-4, PVR-7), and two wild-type isolates (P-34, P-39) were selected for this experiment. The two wild-type isolates and the two VR isolates were susceptible to benomyl; the two IR isolates were resistant. A spore suspension of each isolate was prepared by flooding 12-day-old cultures with 18 ml of sterile water containing a drop of Tween 80. The suspensions of each isolate were adjusted with a hemacytometer to provide a final volume of 6–8 L containing 50,000 spores per milliliter. Germination of spores in each suspension was tested by plating on PDA and ranged from 86–94% for the six isolates.

A single face on McIntosh apples harvested 25 September 1979 was wounded to a depth of 2–3 mm using three 6-d finishing nails spaced 1 cm apart in a triangle and mounted in a cork. Apples were placed 12 per bag in plastic mesh bags, dipped in inoculum for 10 sec, allowed to dry for 2 hr, and then dipped in fungicides or in a water check for 20 sec. Fresh fungicide solutions were used for each of the six isolates to avoid cross-contamination of isolates. After the postharvest treatments had dried, apples were removed from the bags and placed with the wounded face up on tray packs. Ten replicates of 12 apples each were used for each isolate-postharvest treatment combination. Five replicates were kept at 16 C and observed after 14 and 20 days, and five replicates were kept in cold storage (0 C) from the day after inoculation (28 September) until 3 January 1980.

The seven postharvest treatments consisted of a water check, benomyl at 300 μg/ml, both vinclozolin and iprodione at 600 and 1,200 μg/ml, and CGA 64251 10W (1-[[2-(2,4-dichlorophenyl)-4-ethyl-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole; Ciba-Geigy Corporation, Greensboro, NC 27409) at 45 μg/ml.

RESULTS

Incidence of resistant spores. A few *P. expansum* colonies were visible on fungicide-amended plates after 3 days, but colonies grew slowly and new colonies continued to appear until plates were discarded after 4 wk. Most resistant colonies were erumpent, rugose, produced a red orange pigment in agar, ranged in color from reddish yellow to tan and brown, and produced spores only after 3–4 wk.

Because colonies were well separated on the plates, we assumed that each colony developed from a single resistant spore. The number of colonies visible after 25 days and the original inoculum load were used to calculate the incidence of resistant spores on each plate, and a 4 × 5 factorial analysis was used to determine significant differences attributable to the four isolates and the five fungicide concentrations tested. On VPDA, the concentration of vinclozolin had a significant ($P = 0.05$) effect on the incidence of resistant colonies, and isolate P-8 produced significantly more resistant colonies than other isolates (Table 1). The incidence of resistant spores was much lower on IPDA, with no significant differences because of fungicide concentration. Means ($\times 10^{-6}$) for P-8, P-24, P-26, and P-39 on IPDA were 4.53, 0.63, 0.59, and 1.11, respectively, with a least significant difference ($P = 0.05$) of 2.86 $\times 10^{-6}$. The mean incidence of resistant spores for all isolates and fungicide concentrations was 1.96×10^{-5} and 1.71×10^{-6} for vinclozolin and iprodione, respectively.

None of the 228 *P. expansum* isolates recovered from 104 decayed fruits treated with iprodione or from 124 treated with vinclozolin showed resistance to these compounds when tested in vitro.

Cross-resistance of isolates. All of the 25 IR isolates tested for cross-resistance were resistant to vinclozolin at 75 and 300 μg/ml, but only 9 of the 16 VR isolates were cross-resistant to iprodione (Table 2). Vinclozolin at 1,200 μg/ml inhibited growth of more of the resistant isolates than did iprodione at 1,200 μg/ml. With the exception of VR isolates on IPDA and of both VR and IR isolates on VPDA at 1,200 μg/ml, sporulation of resistant isolates was more common on fungicide-amended than on unamended PDA.

Benomyl resistance or susceptibility of the parent wild-type isolates was retained by the 16 VR isolates tested. However, 2 of 17 IR isolates from benomyl-resistant parent isolates (P-8 and P-24) were not benomyl resistant, and 2 of 8 IR isolates from benomyl-susceptible parent isolates (P-26 and P-39) were benomyl resistant.

Pathogenicity of resistant isolates and decay control with fungicides. Because the results of fungicide treatments differed markedly between the wild-type and the VR and IR isolates, results for the two groups were analyzed separately.

Table 2. Cross-resistance and effects of fungicide concentration on germination and growth of spores from 25 iprodione-resistant (IR) and 16 vinclozolin-resistant (VR) isolates of *Penicillium expansum* as determined by streaking spores across fungicide-amended potato-dextrose agar (PDA)

	VR isolates		IR isolates	
PDA	Growing	Sporulating	Growing	Sporulating
Unamended PDA	16	10	25	12
Vinclozolin				
75 μg/ml	16	11	25	24
300 μg/ml	16	15	25	21
1,200 μg/ml	7	2	18	3
Iprodione				
75 μg/ml	9	5	25	22
300 μg/ml	9	8	25	23
1,200 μg/ml	9	8	25	20

Table 3. Effects of seven postharvest treatments and of two wild-type isolates of *Penicillium expansum* on percentage of decayed McIntosh fruits following incubation at 16 or 0 C

		Decayed fruit (%)				
		16 C[x]		Cold storage (0 C)[y]		
Treatment[w] or isolate	Rate (μg/ml)	14 days	20 days	0 days	8 days	14 days
Water check	···	34.9 c[z]	90.8 d	64.6 b	81.9 c	95.1 c
Benomyl	300	4.1 b	4.1 bc	0.0 a	0.3 ab	2.0 a
CGA 64251	45	0.0 a	0.0 a	0.0 a	0.0 a	1.0 a
Vinclozolin	600	0.5 ab	4.6 bc	0.8 a	4.8 b	25.3 b
Vinclozolin	1,200	0.8 ab	0.8 ab	1.5 a	3.6 ab	20.2 b
Iprodione	600	3.4 b	6.5 c	1.4 a	5.9 b	19.8 b
Iprodione	1,200	0.3 ab	4.7 bc	0.9 a	4.6 ab	17.3 b
P-34	···	9.4 a[z]	13.2 a	4.6 a	9.6 a	24.6 a
P-39	···	3.9 a	7.0 a	3.0 a	7.4 a	20.8 a

[w] Treatments were applied 27 September to wounded, inoculated fruit by dipping in fungicide or water check for 20 sec.

[x] Fruits held at 16 C were evaluated 14 days and again 20 days after inoculation.

[y] Fruits were kept in cold storage from 28 September 1979 to 3 January 1980 and were evaulated upon removal (0 day) and after an additional 8 and 14 days at 16 C.

[z] Numbers are grand means of percentage of decayed fruit for treatments and isolates from 2 × 7 factorial analyses with five replicates of 12 apples in each treatment-isolate cell. The arc sin transformation was used for statistical analysis. Means within columns for treatments or for isolates followed by the same letter are not significantly different (LSD, $P = 0.05$).

Grand means for the 2 × 7 factorial analyses of the two wild-type isolates are presented in Table 3, and grand means for the 4 × 7 factorial analyses of the four VR and IR isolates are presented in Table 4.

The two wild-type isolates did not differ significantly in their pathogenicity as determined by evaluating the percentage of fruit infected (Table 3). All fungicide treatments provided adequate protection against these isolates, both at 16 C and after 3 mo of cold storage (0 C). However, vinclozolin and iprodione were less effective than benomyl and CGA 64251 at 8 and 14 days after removal from cold storage.

Significant differences in pathogenicity were evident among the four VR and IR isolates (Table 4). Isolate PIR-5 caused the least decay. The highest mean percentage of infected fruit occurred with PIR-6 at 16 C and with PVR-7 under cold storage conditions. The VR and IR isolates caused more decay in the fruit treated with vinclozolin and iprodione than in the untreated fruit, with the exception of iprodione-treated fruit in cold storage. Averaged over all five observation dates, the percentage of infected fruit in untreated controls was 24 times greater with wild-type than with VR and IR isolates. In untreated fruit showing infections, the diameter of the decayed area averaged 43.5 mm for wild-type and 16.8 mm for VR isolates (Table 5). Neither of the IR isolates caused decay in untreated fruit.

Ten replicates of 12 wounded, untreated, uninoculated fruit were stored at 16 C to determine the incidence of infection caused by inoculum from the field or storage areas. Three of the 120 uninoculated fruit developed decay. Further, 14 of 40 *P. expansum* isolates recovered from decayed fruit that had been inoculated with VR and IR isolates and treated with vinclozolin or iprodione showed no resistance to vinclozolin or iprodione when tested on fungicide-amended agars. Thus, a sizable proportion of the small percentage of decayed fruit in trials with VR and IR isolates may be attributable to natural or contaminating wild-type inoculum, and the actual percentage of fruit infected by VR and IR isolates may be lower than indicated on Tables 3 and 4.

DISCUSSION

Although isolates of *P. expansum* resistant to vinclozolin or iprodione were selected in vitro, we found no evidence of resistance selection in vivo when we tested 228 isolates recovered from decayed fruit that had been treated with vinclozolin or iprodione. Both in this study and in our previous postharvest fungicide trials (7,8), vinclozolin and iprodione were slightly less effective than CGA 64251; they were also less effective than benomyl where benomyl-susceptible isolates of *P. expansum* were used. Our study provides no basis for explaining the differences in decay control provided by the fungicides we tested, but we suspect that fungicides differ in their persistence on fruit under cold storage conditions. The rapid increase in incidence of decay when fruits treated with vinclozolin and iprodione were removed from storage suggests that these compounds lost some of their activity during storage.

In postharvest fungicide tests reported here and elsewhere (7,8), fungicides performed differently at warm temperatures (16–20 C) than in cold storage (0–3 C). Benomyl has consistently provided better control under cold storage conditions than at warmer temperatures, whereas vinclozolin and iprodione sometimes perform better at warm temperatures (8). Because temperature affects the efficacy of postharvest apple fungicides, we suspect that the frequency of fungicide-resistant spores in vitro might also be different if spores were exposed to fungicides at 0–3 C instead of at 20–22 C, as in this study.

The VR and IR isolates selected from fungicide-amended agars and used to inoculate fruit in these experiments were so weakly pathogenic that they probably would not survive in nature unless they were more competitive as saprophytes. But, as reported for *Botrytis cinerea* on grapes (3), more ecologically fit isolates of VR and IR *P. expansum* might develop under the natural selection pressure that would exist if vinclozolin and iprodione were extensively used on a commercial basis.

The large variation in the incidence of resistant spores among the four isolates we tested in vitro suggests that a larger number of isolates should be tested to provide an accurate estimate of the incidence of VR and IR spores. However, trends in the incidence of resistant spores as affected by fungicide and fungicide concentration were similar for all four isolates. Extrapolating from the incidence of resistant spores detected in vitro, we suspect that a problem with fungicide resistance in *P. expansum* would develop more rapidly following commercial use of vinclozolin than of iprodione. The exception might be the use of vinclozolin at the concentration of 1,200 μg/ml, because the frequency of resistant spores was very low at this concentration. However, even if vinclozolin were recommended at 1,200 μg/ml, many growers would either purposefully or inadvertently (through daily addition of water to tanks) use lower concentrations and thus increase the selection pressure

Table 4. Effects of seven postharvest treatments and of two vinclozolin-resistant and two iprodione-resistant isolates of *Penicillium expansum* on percentage of decayed McIntosh fruits following incubation at 16 or 0 C

Treatment[w] or isolate	Rate (μg/ml)	Decayed fruit (%)				
		16 C[x]		Cold storage (0 C)[y]		
		14 days	20 days	0 days	8 days	14 days
Water check	···	2.3 b[z]	5.3 b	0.4 ab	1.6 ab	5.5 b
Benomyl	300	0.0 a	0.5 a	0.0 a	0.0 a	0.1 a
CGA 64251	45	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Vinclozolin	600	29.1 d	38.6 d	1.2 bc	9.4 bc	27.8 c
Vinclozolin	1,200	29.2 d	39.4 d	3.3 c	5.4 bc	25.6 c
Iprodione	600	11.6 c	18.3 c	0.2 b	1.1 ab	22.0 c
Iprodione	1,200	12.1 c	18.2 c	0.1 ab	0.9 ab	19.4 c
PVR-4	···	6.8 b[z]	8.3 ab	0.8 ab	3.6 b	14.4 bc
PVR-7	···	10.8 bc	15.1 b	1.5 b	3.3 b	22.2 c
PIR-5	···	1.4 a	2.5 a	0.0 a	0.1 a	3.4 a
PIR-6	···	16.8 c	32.5 c	0.2 ab	0.8 ab	5.9 ab

[w] Treatments were applied 27 September to wounded, inoculated fruit by dipping in fungicide or water check for 20 sec.
[x] Fruits held at 16 C were evaluated 14 days and again 20 days after inoculation.
[y] Fruits were kept in cold storage from 28 September 1979 to 3 January 1980 and were evaluated upon removal (0 day) and again after an additional 8 and 14 days at 16 C.
[z] Numbers are grand means of percentage of decayed fruit for treatments and isolates from 4 × 7 factorial experiments with five replicates of 12 apples in each treatment-isolate cell. The arc sin transformation was used for statistical analysis. Means within columns for treatments or for isolates followed by the same letter are not significantly different (LSD, $P = 0.05$).

Table 5. Comparison of mean diameters of decayed tissue in untreated McIntosh apple fruits inoculated with four isolates of *Penicillium expansum*

Isolate	Mean diameter (mm) when stored at		
	16 C	Cold storage[y]	
	14 days	0 days	8 days
P-34	33.1 b[z]	42.2 b	58.9 b
P-39	29.4 ab	41.0 b	56.2 b
PVR-4	16.0 a	10.0 a	8.2 a
PVR-7	12.0 a	27.3 a	29.9 a

[y] Fruits were stored for 97 days at 0 C and evaluated the day of removal from cold storage (0 day) and after 8 days at 16 C.
[z] Numbers are means for all decayed fruit in five replicates of 12 apples each. A completely randomized design was used for statistical analysis. Means in each column followed by the same letter are not significantly different (Waller-Duncan's Exact Bayesian K-ratio LSD rule, $P \leq 0.05$).

for pathogenic resistant isolates. With either of the dicarboximide fungicides, the relatively common occurrence of cross-resistance to the related dicarboximide and to benomyl eliminates the possibility of managing the resistance problem by alternating or combining these fungicides.

Further research on the genetics of resistant isolates is needed to determine why the incidence of VR isolates is greater than that of IR isolates, why IR isolates are cross-resistant to vinclozolin whereas not all VR isolates are resistant to iprodione, and why susceptibility to benomyl sometimes changes when isolates become resistant to iprodione but does not change when they become resistant to vinclozolin.

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