Detection and Characterization of Benomyl-Resistant *Monilinia laxa* on Apricots

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ABSTRACT

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In 1980, *Monilinia laxa* resistant to benomyl was isolated from decayed fruit and blighted twigs of apricots in California. Resistant isolates grew on Difco potato-dextrose agar (PDA) amended with 1 mg/L benomyl and one isolate grew at 2 mg/L benomyl. Colonies of benomyl-resistant isolates had typical scalloped margins on Difco PDA, except two single-spored benomyl-resistant subcultures that grew very rapidly with no scalloped margins and produced very few conidia. Conidia of three benomyl-resistant isolates germinated normally, but unlike the other benomyl-resistant isolates, were incapable of producing colonies on benomyl-free Difco PDA medium. A greater proportion of conidia of one of these isolates germinated and grew normally on PDA medium prepared with fresh potatoes than on media prepared with Difco PDA. Benomyl-resistant isolates failed to grow out from mycelial plugs when transferred to benomyl-free Difco PDA medium after extended exposure to 3 mg/L benomyl. Benomyl-resistant isolates produced smaller cankers than benomyl-sensitive isolates on inoculated almond shoots.

Additional key words: *Monilinia fructicola*

Benzimidazole fungicides, benomyl, registered in 1972, and thiophanate methyl, registered in 1979, applied at the red-bud stage of bloom (only sepals showing) and a second time near full bloom, provided essentially perfect blossom blight control and eliminated the need for preharvest treatments to control fruit brown rot. In 1967, before registration of benomyl, field isolates of *Monilinia laxa* and *M. fructicola* were sensitive to about 0.1 mg/L benomyl (5,9). The label recommended repeated applications of benomyl with a warning that cover sprays of benomyl would not be effective unless benomyl applications were started at prebloom.

These repeated treatments on peaches resulted in selection of benomyl-resistant isolates of *M. fructicola* by 1975 in both Australia (10) and the United States (Michigan [4]). Two years later, benomyl-resistant *M. fructicola* isolates were detected in California peach orchards (8). In 1977, the manufacturers of benomyl required that benomyl be applied in combination with another fungicide registered for brown rot control in California to prevent or delay development of benomyl-resistant isolates in accordance with a theory published by Delp (2). The 1979 label for stone fruits recommended that thiophanate methyl be used alone or in combination with another fungicide.

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In July 1980, benomyl-resistant isolates of *M. laxa* were detected for the first time on apricot fruit (6) and later on blighted blossoms and twigs of apricots. In this paper, we report the detection of isolates of *M. laxa* with resistance to low levels of benomyl and discuss some characteristics that differ from the benomyl-sensitive isolates of *M. laxa*.

MATERIALS AND METHODS

Isolation from fruit and blighted blossoms. Samples of decaying apricot fruit shipped from San Benito County were collected from a drying yard in Vacaville, CA, during the summer of 1980. That winter, blighted apricot twigs from Contra Costa County orchards were sampled. Isolations were made on potato-dextrose agar (PDA) that contained aqueous extract of 200 g/L fresh potatoes, 15 g/L agar, and 10 g/L dextrose. The medium was adjusted to pH 6.8. Isolates were single-conidial and identified as *M. laxa* on the basis of growth on Difco PDA (3) or by the formation of barrages with *M. fructicola* but not with other *M. laxa* isolates (7).

Determination of benomyl resistance. Benomyl (Benlate 50W) at 1 mg/L a.i. was incorporated in Difco PDA medium before autoclaving and the medium poured into 60-mm petri dishes. Mycelial plugs (4 mm) of each conidial isolate were transferred onto three plates each of benomyl and benomyl-free media and incubated at room temperature (22 ± 1 C). Ten single-conidial subcultures from a benomyl-sensitive isolate (1-77) collected in 1977 were compared with 10 single-conidial subcultures from each of eight benomyl-resistant isolates (2-80–9-80) collected from apricot fruit in 1980. Two benomyl-resistant isolates were collected from apricot twigs in 1981. The diameter of mycelial growth was recorded after 4 and 10 days and the 4-mm mycelial plug size subtracted before statistical analyses. Variability in culture morphology was studied after additional single-conidial transfers.

Conidial germination and colony growth. Colonies were grown from single conidia on benomyl-free oatmeal agar under constant light at 22 ± 1 C. Conidia were removed by touching the colony with a wire loop of sterile distilled water that was then placed in 10 ml of water. The conidial suspension was streaked onto plates containing Difco PDA. Plates were held at room temperature (22 ± 1 C), and single conidia were obtained after 18 hr. Germinated conidia that produced colonies were recorded for each of 12 single-conidial transfers.

Pathogenicity test. Pathogenicity on Drake almond shoots was determined for benomyl-sensitive isolate 1-77 and two benomyl-resistant subcultures, 7-80E and 7-80L. A 2-mm-diameter mycelial plug from a 2-day-old mycelial culture was placed onto a portion of an actively growing shoot where the bark had been removed with a 4-mm-diameter cork borer. The inoculated shoot portion was wrapped with a paraffin strip and masking tape. Inoculations were performed in the University of California, Davis, campus orchard on September 1982 and the extent of canker development was measured after 18 days. Ten replicates were made per isolate or subculture.

RESULTS

Determination of benomyl resistance. Isolates producing mycelial growth in 4–10 days on Difco PDA medium amended with 1 mg/L benomyl were scored as resistant to benomyl. All 10 subcultures of 1-77 failed to grow on Difco PDA medium amended with 1 mg/L benomyl, whereas all 80 subcultures, 10 each from 8 isolates from apricot fruit, grew on benomyl-amended medium (Table 1). Also, two of 16 isolates from apricot twigs were benomyl resistant. None of the isolates grew on Difco PDA amended with 4 mg/L benomyl. Benomyl-resistant isolates on 1 mg/L benomyl medium grew slower than on benomyl-free medium, indicating some sensitivity to 1 mg/L benomyl.

Significant differences occurred in mycelial growth among subcultures of

benomyl-sensitive (1-77) and benomyl-resistant (2-80) isolates (Table 2). After 4 days, the slowest-growing benomyl-sensitive subculture grew 45% less than the fastest-growing benomyl-sensitive subculture (range 14.4–26.4 mm), and after 10 days, the difference was only 23% (range 48.6–63 mm).

Significant differences occurred in growth rates among sensitive isolates. Similar differences in mycelial growth rates were observed among colonies of benomyl-resistant subcultures of 2-80 on benomyl-free Difco PDA (Table 2). On 1 mg/L benomyl medium, nine of 10 benomyl-resistant subcultures produced colonies during the 4-day incubation period. Ranking in colony diameter among the isolates at 4 days was not consistent with the ranking at 10 days.

On Difco PDA, two (subcultures D and E) of the 10 subcultures from isolate 7-80 were morphologically different from the eight subcultures that were morphologically similar to the parent. These morphologically different benomyl-resistant subcultures were less sensitive to benomyl, showed no apparent lobing on 1 mg/L benomyl medium (Fig. 1), and grew twice as fast as the eight remaining subcultures or isolate 7-80 on both unamended or benomyl-amended media. Benomyl resistance remained stable after three serial transfers onto Difco PDA and subsequent storage at 0 C.

Mycelial death occurred with prolonged exposure of benomyl-resistant isolates to concentrations of benomyl greater than 1 mg/L. Mycelial plugs of eight apricot benomyl-resistant isolates were placed on 1, 3, 5, and 10 mg/L benomyl for a 14-day period at 22 ± 1 C. These plugs were transferred onto benomyl-free Difco PDA medium and incubated 8 days. Mycelia grew out from all plugs transferred from 1 mg/L, whereas 75, 25, and 25% grew from those on 3, 5, and 10 mg/L, respectively.

Conidial germination and colony growth. Germination of conidia from benomyl-sensitive and benomyl-resistant isolates was 100% on benomyl-amended Difco PDA during an 18-hr incubation period at 22 ± 1 C. Germ tubes of benomyl-sensitive isolates failed to elongate and produce colonies. Some benomyl-resistant isolates continued elongating and developed into colonies but others did not (Table 1). On benomyl-free medium, conidia of benomyl-sensitive isolates germinated and produced colonies, whereas some conidia of benomyl-resistant isolates failed to produce colonies. The percentage of conidia from benomyl-resistant isolates producing colonies ranged from 1 to 67 in the first experiment and from 0 to 50 in the second. Colonies were most difficult to obtain from germinated single conidia of isolates 9-80 and 8-80 on Difco PDA; however, on PDA prepared from fresh potatoes, colonies were produced more consistently from single conidia.

Isolate 8-80 was used to determine if the type of PDA used influenced conidial germination and colony development. Conidial suspensions were plated on Difco PDA or on PDA prepared from fresh potatoes. After a 20-hr incubation at room temperature, percentage germination was determined. On Difco PDA, 69% of the conidia did not germinate, 23% germinated and grew normally, and 8% germinated but had reduced germ-tube length (ie, length of germ tube less than or equal to length of conidium). On PDA made from fresh potatoes, 15% of the conidia did not germinate and 85% germinated and grew normally. Mycelial plugs from several single-conidial isolates of 8-80 growing on PDA prepared from fresh potatoes were transferred to Difco PDA containing 1 mg/L benomyl. All grew and formed colonies, indicating

Table 1. Mycelial growth rates of *Monilinia laxa* from apricot fruit on Difco PDA with and without benomyl

Monilinia laxa isolate[a]	Average diameter of mycelial growth (mm)[b]				Percent germinated conidia forming mycelial colonies[c]	
	After 4 days		After 10 days			
	1 mg/L benomyl	No benomyl	1 mg/L benomyl	No benomyl	Exp. 1	Exp. 2
1-77	0.0 z[d]	19.0 xy	0.0 y	54.4 vw	100	100
2-80	17.3 vw	23.2 vwx	46.3 vw	55.1 vw	58	42
3-80	17.0 vw	20.7 wxy	44.8 vw	47.2 wx	17	25
4-80	16.7 vw	25.9 v	41.6 wx	54.1 vw	17	33
5-80	13.0 x	21.1 wxy	36.2 x	43.6 x	25	33
6-80	18.5 v	26.5 v	52.4 v	63.2 v	42	50
7-80	13.6 x	21.6 wxy	43.6 wx	51.0 wx	67	25
8-80	8.5 y	17.6 y	43.4 wx	47.4 wx	8	8
9-80	15.2 wx	23.7 vw	44.9 vw	54.4 vw	17	0

[a] Benomyl-sensitive isolate (1-77) was isolated in 1977 and benomyl-resistant isolates (2 to 9-80) were isolated in 1980.
[b] Measurements minus plug diameter of 4 mm. Incubated at 22 ± 1 C.
[c] Percentage of germinated conidia that formed mycelial colonies after 36-hr incubation at 22 ± 1 C from 12 single-conidial tranfers onto Difco PDA.
[d] Values in a column followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

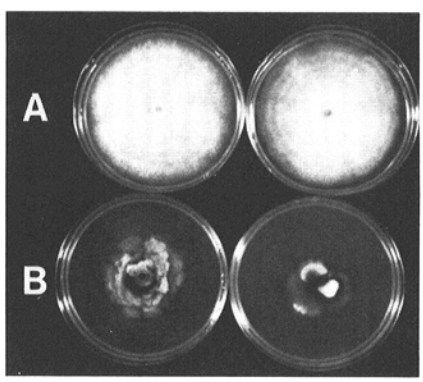

Fig. 1. Comparison of morphologically different *M. laxa* isolates on Difco PDA amended with 1 mg/L benomyl. **(A)** White, mycelial isolates 7-80D and 7-80E. **(B)** Sister isolates 7-80K and 7-80L, typical *M. laxa*.

Table 2. Differences in mycelial growth of single-conidial subcultures of benomyl-sensitive and benomyl-resistant *Monilinia laxa*

Time of reading	Benomyl	Subcultures (mm diameter)[a]										
		A	B	C	D	E	F	G	H	I	J	$\bar{X}$
		Benomyl-sensitive isolate 1-77[b]										
4 Days	−	26.4 w[c]	23.0 wx	21.0 xy	19.4 xy	19.2 xy	19.0 xy	16.8 xy	16.6 xy	15.6 y	14.4 y	19.0
10 Days	−	62.6 w	63.0 w	53.8 x	53.4 x	54.4 x	53.2 x	48.6 x	49.0 x	53.6 x	52.6 x	54.4
		Benomyl-resistant isolate 2-80[b]										
4 Days	−	30.4 w	28.0 wx	27.8 wx	26.0 x	25.2 x	25.0 x	24.6 x	20.0 y	19.6 yz	15.8 z	24.2
10 Days	−	52.8 x	57.6 w	59.4 w	59.6 w	58.6 w	57.4 w	56.4 w	53.8 w	53.6 w	41.4 x	57.1
4 Days	+	21.0 w	17.0 xy	13.8 y	13.8 y	19.4 wx	17.4 x	17.8 wx	17.8 wx	17.8 wx	...[d]	17.3
10 Days	+	39.6 z	45.6 xyz	48.8 wxy	41.4 w	54.4 w	46.6 wxyz	40.6 yz	46.2 wxyz	53.0 wx	...[d]	46.2

[a] Five 4-mm mycelial plugs for each replicate were placed in the center of a 90-mm petri dish containing Difco PDA medium without benomyl (−) or with 1 mg/L benomyl (+) and incubated at 22 ± 1 C.
[b] Single-conidial isolates from culture originating from the initial isolation from host tissue.
[c] Values in a row followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[d] Not determined.

these cultures were still benomyl resistant.

Pathogenicity test. Both benomyl-resistant subcultures (7-80E and 7-80L) were less pathogenic on Drake almond shoots than the benomyl-sensitive isolate (1-77). Canker lengths produced by benomyl-resistant isolates 7-80E and 7-80L averaged 3 and 13 mm, respectively, whereas the average canker length for benomyl-sensitive isolate 1-77 was 55 mm. Cankers produced by benomyl-resistant isolates generally moved acropetally, whereas cankers produced by the benomyl-sensitive isolate extended in both directions from the inoculation site.

DISCUSSION

Development of benomyl-resistant *M. laxa* was expected because of the extensive use of benzimidazole fungicides alone or in combination with other fungicides. The delay in detection of benomyl-resistant *M. laxa* on apricots compared with *M. fructicola* on peaches may be related to fewer applications made each year on apricots. Only one or two applications are made on apricots, whereas on peaches, one or two applications are made during bloom and two or more before harvest. The delay in the development of resistance appears to be related to reduced selection pressure. All benomyl-resistant *M. laxa* isolates found in our survey were sensitive to 4 mg/L benomyl. Two isolates were less pathogenic in producing twig cankers and produced fewer conidia in culture. Some isolates produced conidia that germinated but did not produce colonies on Difco PDA. The reason for the difference in conidial germination of certain benomyl-resistant isolates between Difco PDA and fresh PDA is under investigation.

These findings, together with data by Cañez and Ogawa (1) showing reduced ability of some benomyl-resistant *M. laxa* isolates to infect almond and prune blossoms, indicate that *M. laxa* with low level benomyl resistance (1 mg/L) is less likely to survive than benomyl-sensitive *M. laxa*. Further field studies are needed using a larger sample size to determine how competitive these benomyl-resistant isolates are in the absence of benomyl. With continued use of benzimidazole fungicides, alone or in combination with protectant fungicides on apricots, populations of benomyl-resistant isolates are expected to increase as shown with *M. fructicola*. Also, resistant isolates more competitive with the wild type may develop. In the meantime, it appears appropriate to alternate fungicides with modes of action different from that of benzimidazole to reduce selection pressure for higher levels of benzimidazole resistance in *M. laxa*.

Studies are in progress to determine the competitiveness of benomyl-resistant *M. laxa* and to determine if different levels of benomyl resistance occur in apricot orchards. Also being studied is the mechanism by which conidia of benomyl-resistant isolates germinate but fail to produce colonies on benomyl-free media.

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