Phenotype Patterns of Benomyl-Resistant Isolates of *Venturia inaequalis* in Eight Orchards in British Columbia, Canada

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ABSTRACT

Sholberg, P. L., and Yorston, J. M. 1991. Phenotype patterns of benomyl-resistant isolates of *Venturia inaequalis* in eight orchards in British Columbia, Canada. Plant Dis. 75:616-619.

Two hundred and fifty-nine orchard blocks from the interior of British Columbia were screened for benomyl resistance. In 22% of the samples, 85% or more of the conidia tested resistant to 1 μg/ml of benomyl. Eight orchards were selected from this group for further study. Single ascospore isolates were obtained from each orchard, totaling 991 from the eight orchards. Each isolate was tested for benomyl resistance over a range of concentrations from 1 to 500 μg/ml and for sensitivity to 0.5 μg/ml of diethofencarb. The majority of the isolates (74.5%) were resistant to benomyl and came from seven of the eight orchards. Three benomyl-resistant phenotypes were found—low benomyl resistance (LR), high benomyl resistance (HR), and very high benomyl resistance (VHR). Only one LR isolate was found. The HR phenotype was present in 41 isolates from four orchards, although 37 of the 41 isolates came from one orchard. The VHR isolate was found in all seven orchards with benomyl resistance and was present in 671 of the 712 benomyl-resistant isolates that were studied. All of the VHR isolates were sensitive to 0.5 μg/ml of diethofencarb and were negatively correlated cross resistant to *N*-phenylcarbamate. Four orchards had one or more isolates of the HR phenotype in addition to the VHR phenotype. In one orchard, 28.2% of the isolates were the HR phenotype and 67.2% were the VHR phenotype.

An initial survey of 28 orchards in British Columbia, Canada, in 1986 identified resistance of *Venturia inaequalis* (Cooke) G. Wint. to benomyl in 31% of the orchards. A more extensive survey of orchards in 1987 showed that 25.4% were resistant to benomyl at 1 μg/ml (10). Previous studies by Stanis and Jones (11) have identified three benomyl-resistant phenotypes: low resistance (LR), growth of the isolates on media amended with 1 μg/ml but not with 10 μg/ml; medium resistance (MR), growth at 10 μg/ml but not at 25 μg/ml; and high resistance (HR), good growth on media amended with up to 500 μg/ml. This definition was modified in a subsequent publication (3) as follows: high resistance (HR), growth at 5 μg/ml is greater than 50 μg/ml and insensitive to *N*-phenylcarbamate, and very high resistance (VHR), growth rate at 5 μg/ml equals growth rate at 50 μg/ml combined with sensitivity to *N*-phenylcarbamate.

Studies were conducted to determine the phenotypes present in orchards in British Columbia and the extent of benomyl resistance in orchards with a history of benomyl resistance.

Contribution 755, Agriculture Canada Research Station, Summerland, British Columbia.

Accepted for publication 4 December 1990.

MATERIALS AND METHODS

Screening. Twenty apples or 50 leaves with freshly sporulating apple scab lesions were randomly selected from each of 259 orchards in June and July 1988 and brought to our laboratory for benomyl-resistance testing. The method of testing for benomyl resistance was the same as that used in 1986 and 1987 (10).

Sampling. Eight orchards with a high degree of resistance to benomyl were selected for the phenotype screening tests. Leaves with pronounced apple scab lesions during the last week of March 1989 were randomly selected from the

orchard floor and placed in mesh bags and the bags were tied to a fence (at the research orchard) approximately 1 m aboveground. The leaves were allowed to mature for approximately 2 mo when they were brought to the laboratory for examination. Squash mounts were made from pseudothecia from a few leaves in each sample and examined under the microscope for mature ascospores. If the sample did not have mature ascospores, it was allowed to mature for another 2 wk. Leaves from all eight orchards were processed by 15 July 1989.

Isolation. Single ascospore isolates were obtained by cutting 2- to 3-cm squares of leaves and soaking in distilled water for at least 5 min (12). These squares were then placed on the inside of 10-cm-diameter plastic petri plate lids and allowed to discharge their ascospores into 20–30 ml of sterile distilled water for 1–3 hr. A minimum of four petri plates were prepared from each sample, and 10 leaf pieces were used per petri plate. The sterile water was scanned with a dissecting microscope for ascospores of *V. inaequalis*. Ascospores were always found at the bottom of the petri dish and never on the water surface. If several hundred were found in a dish, these were isolated by agitating the contents and pouring 5–10 ml onto 10-cm-diameter plates of 2% water agar. The water agar plates were incubated overnight at 20–22 C. Excess water was removed by passing sterile air over the open plates for 2–3 hr in a laminar flow hood. Germinated ascospores from several plates of each sample were isolated with a sterile needle under a dissecting microscope and individually placed on a 5-cm-diameter plate containing 5 ml of Difco potato-dextrose agar (PDA) acidified with 1.5 ml of 85% lactic acid per liter. Isolates were monitored for contaminants weekly and discarded if contaminated. Uncontaminated isolates grew for 4 wk, at which time they appeared as 1- to 2-cm-diameter olive green colonies. Cultures not used immediately were kept in a 2-C incubator until required.

Testing. Levels of benomyl resistance and sensitivity were determined by exposure to benomyl (1–500 μg/ml). Benomyl (Benlate 50WP) (E. I. du Pont de Nemours & Co., Wilmington, DE) was added to the medium before autoclaving at 121 C for 15 min. The method of Stanis and Jones (11) was modified for the determination of resistance. Bits of mycelium (approximately 1 mm in diameter) were taken from the growing edge of 4-wk-old colonies and inoculated onto benomyl-amended and unamended PDA plates. Growth was evaluated as present or absent after 3 wk of incubation at 22 C.

Similarly, at the time that ascospore colonies were transferred to benomyl-amended media, transfers were made to two plates of PDA amended with 0.5 μg/ml of technical product or diethofencarb 25WP to determine the sensitivity of each isolate. This test was repeated twice for the first 50 isolates from each orchard and a third time for isolates indicating resistance to both diethofencarb and benomyl. The results were the same each time. Diethofencarb (S-32165 25WP and S-32165 technical grade) (Sumitomo Chemical Co., Ltd., Osaka 541, Japan) was dissolved in methanol and added to the PDA when it cooled to 50 C (9). Growth was evaluated as present or absent after 3 wk of incubation at 22 C.

Thirteen double-resistant isolates from three orchards in British Columbia were selected for growth rate studies. Mycelial plugs (5 mm diameter) were taken from cultures that had been growing for 14 days at 22 C and placed on PDA amended with 0.5 μg/ml of diethofencarb or either 5 or 50 μg/ml of benomyl. The plates were incubated at 22 C for 4 wk and the radial growth of each isolate was measured weekly. Each isolate was replicated three times, and the standard deviation of the growth rate was calculated for each isolate.

RESULTS

Screening 259 orchard block samples in July 1988 for benomyl resistance confirmed the 1987 results (10). Benomyl resistance was common in British Columbia, especially in the wetter northern area of the Okanagan Valley, where benomyl has been used since 1973. The resistant orchards occurred over a range of 100 km from Kelowna in the south to Vernon in the north, indicating that resistance was widespread.

Eight orchards with 87% or more of the conidia sampled resistant to 1 μg/ml of benomyl were selected for further study (Table 1). Conidia from these orchards were also highly resistant to dodine because they germinated on PDA amended with 2 μg/ml.

Leaves collected from these orchards the following spring were used to obtain single ascospore cultures. These cultures were tested for benomyl resistance over a wide range of concentrations from 1 to 500 μg/ml. In six of the eight orchards, 85% or more of the isolates were resistant to benomyl, confirming the results of the the previous screening test for these six orchards (Table 2). Two orchards, Kelowna-Glenmore No. 2 and Kelowna-Glenmore No. 3, had a much higher proportion of benomyl-sensitive isolates in the ascospore test than in the previous conidial screening test. Previously, conidia from the two orchards were 100% resistant, but only 24.8 and 0.0%, respectively, were resistant in the ascospore test.

All of the benomyl-resistant isolates except two grew on 100 μg/ml of benomyl, indicating that the majority of isolates had a high level of resistance to benomyl (Table 2). Most of these isolates also grew on 500 μg/ml of benomyl. The two isolates that were susceptible to lower levels of benomyl came from the Oyama area. An isolate from Oyama No. 1 grew on 50 μg/ml of benomyl and an isolate from Oyama No. 2 grew on only 1 μg/ml of benomyl.

When the isolates were further tested on 0.5 μg/ml of diethofencarb to determine the phenotypic patterns in the eight orchards, the majority of benomyl-resistant isolates were sensitive to diethofencarb and, thus, were the VHR phenotype (Fig. 1 and Table 3). However, four orchards contained one or more isolates that were resistant to both benomyl and diethofencarb. In the Vernon No. 1 orchard, 37 (28.2%) of its isolates were resistant to both fungicides.

To determine the phenotype of these double-resistant isolates, 13 random samples were tested for growth rate on 5 and 50 μg/ml of benomyl (Table 4). All isolates tested had a lower growth rate at 50 μg/ml of benomyl than at 5, indicating that they belonged to the HR phenotype. The different growth rates on PDA of many of the isolates indicated that they belonged to different clones. We also observed different colony char-

Table 1. Percentage of conidia from eight orchards resistant to 1 μg/ml of benomyl and 2 μg/ml of dodine

Orchard	Benomyl	Dodine
Kelowna No. 1	87	99
Kelowna-Glenmore No. 1	100	100
Kelowna-Glenmore No. 2	100	91
Kelowna-Glenmore No. 3	100	63
Kelowna-East No. 1	100	43
Oyama No. 1	100	100
Oyama No. 2	100	42
Vernon No. 1	100	86

Table 2. Percentage of ascospore isolates from eight orchards resistant to benomyl

Orchard	Isolates tested	Benomyl (μg/ml) 1	10	25	50	100	500
Kelowna No. 1	91	100.0	100.0	100.0	100.0	100.0	97.8
Kelowna-Glenmore No. 1	89	89.9	89.9	89.9	89.9	89.9	87.6
Kelowna-Glenmore No. 2	137	24.8	24.8	24.8	24.8	24.8	24.8
Kelowna-Glenmore No. 3	141	0.0	0.0	0.0	0.0	0.0	0.0
Kelowna-East No. 1	118	100.0	100.0	100.0	100.0	100.0	98.3
Oyama No. 1	139	85.6	85.6	85.6	85.6	84.9	84.9
Oyama No. 2	145	100.0	99.3	99.3	99.3	99.3	93.1
Vernon No. 1	131	95.4	95.4	95.4	95.4	95.4	95.4

acteristics among the various isolates such as color and sporulation. The Vernon No. 1 orchard had a high number of the HR phenotypes in addition to the VHR phenotype (Fig. 2).

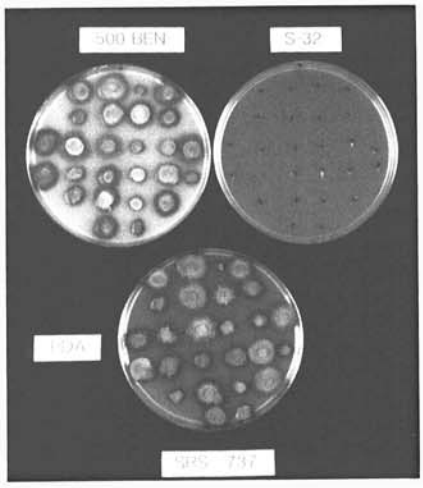

Fig. 1. Benomyl VHR isolates of *Venturia inaequalis* from Kelowna-East No. 1 on potato-dextrose agar (PDA), PDA amended with 500 μg/ml of benomyl (500 BEN), and PDA amended with 0.5 μg/ml of diethofencarb (S-32). Twenty-six isolates were evaluated simultaneously with bits of mycelium from single-ascospore colonies. Examples show growth 4 wk after inoculation.

DISCUSSION

Initial screening of conidia from Kelowna-Glenmore No. 2 and No. 3 indicated that all conidia were resistant to 1 μg/ml of benomyl, but when ascospore isolates from leaves collected the following spring were tested, only 24.8 and 0.0%, respectively, were resistant.

The reason for this difference could be the lack of fitness of the progeny or a sampling error. Lalancette et al (7) showed that although fitness was not related to benomyl resistance, it could be linked. They hypothesized that if benomyl was not used in one of the orchards they examined, the orchard would revert back to sensitivity because the resistant isolates were not as fit as the sensitive isolates. Alternately, the sample size may have been too small to detect the level of resistance found previously. Although leaves were randomly sampled from throughout the orchards, only 10–20 were actually used to obtain the cultures used for the benomyl test.

After studying 991 isolates from eight orchards in British Columbia, it appears that three phenotypes are present. Only one LR isolate was found, possibly because the rates of application of benomyl or benomyl cross-resistance products (thiophanate-methyl) produce an effective residue higher than 1 μg/ml and, thus, have eliminated most of these isolates from the population. The HR phenotype was common in one orchard and occurred in three other orchards. The VHR phenoytpe was by far the most common, occurring in all seven orchards with benomyl resistance. Of the 712 isolates resistant to benomyl, 671 (94.2%) were the VHR phenotype.

The VHR phenotype is negatively correlated cross-resistant to *N*-phenylcarbamates and is effectively controlled with 0.5 μg/ml of diethofencarb, an *N*-phenylcarbamate. Diethofencarb controlled all isolates of *V. inaequalis* from two orchards, Kelowna No. 1 and Kelowna-East No. 1, where resistance to benomyl was 100% (Table 3 and Fig. 1). Kato (6) found that field applications of diethofencarb mixed with a benzimidazole fungicide were very effective for controlling gray mold of vegetables and grapes where benomyl resistance had been a problem. On the other hand, when diethofencarb was used in commercial cucumber greenhouses, it failed in one out of four sites because a new phenotype which was resistant to benzimidazole as well as *N*-phenylcarbamates appeared and was pathogenic on cucumber seedlings (4). We already know that the HR phenotype with both benomyl and diethofencarb resistance exists in our orchards. The value of diethofencarb may lie in using it to eliminate the VHR phenotype and leave only the HR phenotype.

There is indirect evidence that the HR phenotype may be less fit than the VHR

Table 3. Number of ascospore isolates resistant to benomyl, diethofencarb, or both from eight orchards

Orchard	Isolates tested	Benomyl (100 μg/ml)	Diethofencarb (0.5 μg/ml)	Both
Kelowna No. 1	91	91	0	0
Kelowna-Glenmore No. 1	89	80	9	0
Kelowna-Glenmore No. 2	137	34	104	1
Kelowna-Glenmore No. 3	141	0	141	0
Kelowna-East No. 1	118	118	0	0
Oyama No. 1	139	119	22	2
Oyama No. 2	145	144	2[a]	1
Vernon No. 1	131	125	43	37

[a]One of these isolates was sensitive to 10 μg/ml of benomyl.

Table 4. Growth rate (mm/day) of *Venturia inaequalis* isolates with double resistance to diethofencarb and benomyl

Orchard and isolate	Growth rate on PDA	Diethofencarb (0.5 μg/ml)	Benomyl (5.0 μg/ml)	Benomyl (50 μg/ml)
Vernon No. 1				
1	0.25 ± 0.05	0.21 ± 0.03	0.18 ± 0.03	0.05 ± 0.01
2	0.31 ± 0.02	0.33 ± 0.04	0.25 ± 0.03	0.04 ± 0.00
3	0.35 ± 0.02	0.32 ± 0.03	0.27 ± 0.01	0.11 ± 0.00
4	0.38 ± 0.01	0.38 ± 0.01	0.35 ± 0.02	0.09 ± 0.03
5	0.42 ± 0.03	0.39 ± 0.04	0.36 ± 0.03	0.06 ± 0.01
6	0.45 ± 0.01	0.50 ± 0.03	0.49 ± 0.04	0.15 ± 0.02
7	0.57 ± 0.00	0.56 ± 0.01	0.53 ± 0.02	0.18 ± 0.03
8	0.63 ± 0.01	0.68 ± 0.04	0.61 ± 0.08	0.30 ± 0.12
9	0.63 ± 0.01	0.65 ± 0.02	0.57 ± 0.03	0.14 ± 0.15
10	0.75 ± 0.10	0.87 ± 0.01	0.87 ± 0.04	0.54 ± 0.16
11	0.88 ± 0.04	0.92 ± 0.02	0.77 ± 0.04	0.28 ± 0.00
Kelowna Glenmore No. 2				
1	0.23 ± 0.04	0.22 ± 0.04	0.19 ± 0.04	0.05 ± 0.00
Oyama No. 1				
1	0.67 ± 0.04	0.41 ± 0.16	0.60 ± 0.09	0.25 ± 0.12

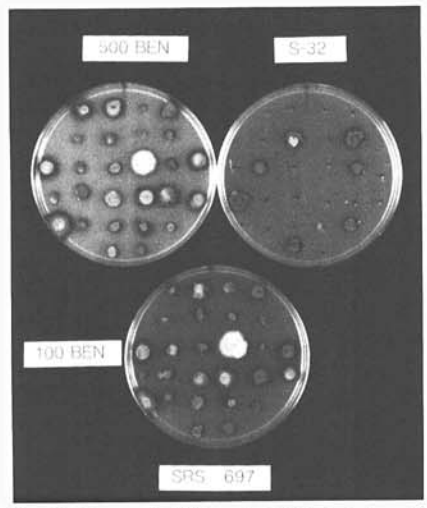

Fig. 2. Benomyl HR and VHR isolates of *Venturia inaequalis* from Vernon No. 1 on potato-dextrose agar (PDA) amended with 100 and 500 μg/ml of benomyl (100 BEN and 500 BEN, respectively) and PDA amended with 0.5 μg/ml of diethofencarb (S-32). The HR isolates are those that are growing on diethofencarb. Twenty-seven isolates were evaluated simultaneously with bits of mycelium from single-ascospore colonies. Examples show growth 4 wk after inoculation.

phenotype. First, it was common in only one orchard we studied, indicating that it may not compete well with the VHR phenotype. In their study of ascospore progenies from crosses between HR and sensitive (S) phenotypes, Jones et al (3) found that in two crosses, the ratio of S to HR was not 1:1 but 2:1. This could mean that some of the ascospores of the benomyl-resistant progeny lost the ability to germinate. It appears to happen quite often, considering that it occurred in two of the seven crosses that were made. We have recorded a similar ratio of sensitive to resistant progeny in some of the crosses we have made between S and HR isolates (P. L. Sholberg, *unpublished*). Research on the HR phenotype could have practical importance if it is discovered that this phenotype disappears at a faster rate from the *V. inaequalis* population than previously thought for benomyl-resistant isolates.

Resistance in *V. inaequalis* to benomyl is attributed to a single gene with different alleles of this gene conferring different levels of resistance (5,8,11). Our failure to detect alleles other than the VHR allele sensitive to diethofencarb supports the finding of Jones et al (3) that very high resistance to benomyl is genetically linked to sensitivity to diethofencarb and that the mechanism of benomyl resistance in such strains is probably different from the mechanism in the HR strains. Identification of genes for the HR and VHR phenotypes by molecular biological techniques could confirm or refute this hypothesis.

Benzimidazoles are specific inhibitors of microtubule assembly that act by binding to their heterodimeric subunit, the tubulin molecule (1). Binding of antimicrotubular drugs to tubulin results in inhibition of microtubule assembly (2). Diethofencarb is structurally related to the benzimidazoles possessing a benzene ring instead of a benzimidazole ring. Takahashi et al (13) showed that substituted *N*-phenylcarbamates show high fungicidal activity against benomyl-resistant fungi on agar medium. The *N*-phenylcarbamates interfere with microtubule functioning in *Saccharomyces pombe* (Lindner) Jörgensen , which seems to involve α-tubulin (1). Negative cross resistance of benzimidazole-resistant isolates of *Botrytis cinerea* Pers.:Fr., *Cercospora beticola* Sacc., *Fusarium nivale* Ces. ex Berl. & Voglino, *Mycosphaerella melonis* (Pass.) Chiu & J. C. Walker, *Pseudocercosporella herpotrichoides* (Fron) Deighton, and *V. nashicola* Tanaka & Yamamoto to *N*-phenylcarbamates could be attributed to altered microtubule stability (2). On the other hand, the mechanism of benomyl-resistance in the VHR phenotype may involve β-tubulin and altered affinity of tubulin to benomyl because mutations are usually found in the β-tubulin gene (2). Research on the biochemistry and the molecular genetics of tubulin from these two phenotypes would clarify this problem.

ACKNOWLEDGMENTS

We thank Tanya Jones of the British Columbia Ministry of Agriculture and Fisheries for assistance in the screening tests, Eric Demaere and Paula Haag of Agriculture Canada for assistance in the laboratory tests, and Bill Fleming for the photographs.

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