In Situ Detection of Benzimidazole Resistance in Field Isolates of *Venturia inaequalis* in Indiana

Kacie L. Quello, Kimberly S. Chapman, and Janna L. Beckerman, Department of Botany and Plant Pathology, Purdue University, 915 West State St., West Lafayette, IN 47907

ABSTRACT

Quello, K. L., Chapman, K. S., and Beckerman, J. L. 2010. *In situ* detection of benzimidazole resistance in field isolates of *Venturia inaequalis* in Indiana. Plant Dis. 94:744-750.

Venturia inaequalis, the causal agent of apple scab, infects both commercial apples and ornamental crabapples. We found four classes of benzimidazole fungicide sensitivity in the Indiana population: sensitive (S) isolates unable to grow on 0.5 µg active ingredient (a.i.)/ml; low resistant (LR) isolates that grew at 0.5 µg a.i./ml, but not at 5 µg a.i./ml; moderately resistant (MR) isolates that grew at 5 µg a.i./ml, but not at 50 µg a.i./ml; and very highly resistant (VHR) isolates that grew rapidly at 50 µg a.i./ml. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of the β -tubulin gene with two restriction enzymes, *Bst*UI and *Cac*8I, enabled us to rapidly identify benzimidazole resistance among all tested isolates. Sixty-nine percent of the resistant isolates tested possessed the *Bst*UI RFLP at codon 198 that corresponds to VHR, and the remaining LR and MR isolates possessed the *Cac*8I RFLP corresponding to a newly identified resistance allele at codon L240F. Combined, PCR-RFLP correctly identified the resistance status of all isolates tested to date. The preponderance of benzimidazole-resistant isolates from commercial apple orchards and their absence in the landscape on ornamental crabapple suggests that two distinct populations of *V. inaequalis* coexist in Indiana.

Apple scab, caused by the fungus *Venturia inaequalis* (Cooke) G. Winter, is one of the most destructive diseases of apples (16), and is also a significant disease of landscape crabapples in the eastern half of North America (1,12). Fungicides are relied upon to control the disease in the orchard, nursery, and landscape. This frequent use can lead to the selection of resistant populations of the pathogen and loss of efficacy (8,11,30). In commercial apple orchards in the Midwest, up to 12 applications of fungicide may be used for scab control in a single season (13). Scab management of landscape crabapples is less intense, but still involves at least three well-timed, foliar applications of fungicides per season for mature landscape crabapples (1).

Resistance to the benzimidazole class of fungicides has been identified in a number of fungal species, including *V. inaequalis* (20). In most cases, resistance is correlated with point mutations in the β -tubulin gene that result in altered amino acid sequences at the benzimidazole-binding site. Numerous studies have shown that mutations at

codons 6, 50, 167, 198, 200, and 240 in the β -tubulin gene result in benzimidazole resistance in field isolates of phytopathogenic fungi, with different mutations resulting in different levels of resistance to benzimidazoles (20,24). In *V. inaequalis*, the only reported mutations to date occur at codons 198 and 200, and cause moderate and high levels of resistance, respectively (20).

Although the first benzimidazole fungicide, Benlate (active ingredient: benomyl) was voluntarily withdrawn from the market in 2001, it was replaced with another member of the benzimidazole class of fungicides, Topsin M, for commercial apple production, and Cleary's 3336 for ornamental use (active ingredient: thiophanate-methyl). Other benzimidazole-class fungicides (debacarb, carbendazim) are registered and are heavily promoted to arborists and nursery producers for use on landscape trees, including ornamental crabapples. All of these fungicides target the β -tubulin protein. Today, the primary use of benzimidazoles in commercial apple production is for other diseases, but the continued use of this fungicide class in the nursery and landscape industry warrants the continued monitoring of benzimidazole resistance in *V. inaequalis*.

Molecular methods are regularly used to diagnose antifungal and antibiotic resistance in human pathogens (9). For plant pathogens, polymerase chain reaction (PCR) assays, including allele-specific PCR and real-time PCR, allow rapid detection with high specificity and sensitivity

(7,18,19,22,23,26). Restriction fragment length polymorphisms (RFLP) have been used for *Botrytis cinerea*, *Monilinia laxa*, *V. inaequalis*, and *Pyricularia grisea* to test for an association between point mutations in the β -tubulin gene and resistance (10,17,21,25). Of these techniques, PCR-RFLP provides the quickest and least expensive method to rapidly detect fungicide resistance in a diagnostic laboratory.

The objectives of this study were (i) to develop a rapid molecular method to detect benzimidazole resistance of *V. inaequalis* directly from leaf lesions, and (ii) to test the populations of *V. inaequalis* derived from the commonly grown apple cultivars in the commercial orchard and the commonly grown crabapples in the landscape. To accomplish this task, we needed to determine whether the mycelium growth assay agreed with results from PCR-RFLP and could be used to detect benzimidazole resistance directly from leaf lesions.

MATERIALS AND METHODS

Fungal isolates. Sixty-seven single-spore *V. inaequalis* isolates were collected from three orchards in Indiana during the 2007 season, and 35 isolates were collected from crabapples from five other sites with a history of chemical management, including isolates from two crabapples that were injected with a combination of debacarb and carbendazim but developed scab later in the season, in 2009. Single-spore isolates were obtained by streaking excised apple scab leaf lesions onto 2% water agar (Difco, Sparks, MD). After incubation at 19°C for 24 h, germinated conidia were picked with a sterilized needle and placed on potato dextrose agar (PDA) (Difco). The germinated spores were grown in the dark at 23°C until sufficient growth was present to transfer the colony onto a new PDA plate, and only one colony per lesion was used for this study.

Assessment of *V. inaequalis* isolates sensitivity to benzimidazoles. Twenty-five isolates of *V. inaequalis* were tested for benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, 95%, Sigma-Aldrich cat. no. 381586-5G) and thiophanate-methyl (Topsin M 70WP, United Phosphorus Inc.) sensitivity using conventional mycelium assays as described by Koenraadt et al. (20). Briefly, a 4-mm section of agar with mycelium was placed on both PDA amended with 0.5 µg active

Corresponding author: J. L. Beckerman
E-mail: jbeckerm@purdue.edu

K. L. Quello and K. S. Chapman contributed equally to this work.

Accepted for publication 25 February 2010.

doi:10.1094/PDIS-94-6-0744

© 2010 The American Phytopathological Society

ingredient (a.i.)/ml of benomyl, and PDA amended with 0.5 µg a.i./ml of thiophanate-methyl, and on a PDA unamended control. Three replicates of each isolate were tested. The plates were incubated at 19°C for 4 weeks. Two measurements of radial growth of each colony were made at right angles to each other. The relative growth (RG) was calculated by taking the average radial growth of each isolate on the amended media and dividing it by the average radial growth of the isolate on the nonamended media and multiplying it by 100. Resistance levels were identified based upon previously established baselines (14,15,20). Regression analyses of the RG of isolates on benomyl and on thiophanate-methyl were compared to controls with SAS version 9.1.3.

DNA extraction and PCR amplification. DNA was extracted from lyophilized mycelia using a Qiagen DNeasy Blood and Tissue kit (cat. no. 69504) according to the manufacturer's recommendations. Primer BtubF14 (5'-AACCAAATTGGTGCTGCC TTCTG-3') was developed using the β-tubulin sequence published by Koenraadt et al. (20). Primer BtubR (5'-TGGAGG ACATCTTAAGACCACG-3') was identical to primer B described by Koenraadt et al. (20). PCR amplification was performed in a 50-µl volume containing 2-µl DNA (~50 ng), 200 µM dNTPs, 0.2 µM primer BtubR, 0.2 µM primer BtubF14, 1.5 mM MgCl₂, 5× buffer provided by the manufacturer, and 1 unit of GoTaq Flexi (Promega, Madison WI). Amplifications were completed in a thermal cycler (Applied Biosystems, Carlsbad, CA) programmed for 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C.

RFLP analysis of β-tubulin. Approximately 1 µg of amplified β-tubulin PCR product that was amplified by BtubF14 and BtubR was digested with 1 unit of *Bst*UI restriction enzyme (New England BioLabs, cat. no. R0518S) or 1 unit of *Cac*8I restriction enzyme (New England BioLabs cat. no. R0579S) in 1× buffer 4, provided by the manufacturer, at 37°C for 3 h as per the manufacturer's suggestion. PCR products (both undigested and digested) were resolved using a 1.2% agarose gel (UltraPure, Invitrogen, cat. no. 15510-019) at 60 volts in 1× TBE buffer for approximately 2 h. All gels were stained with ethidium bromide and were photographed with a Gel Logic 200 Imaging System (Kodak, Carestream Health, Rochester, NY).

Sequence analysis of novel isolates lacking the RFLP. Sequence analysis was performed on the β-tubulin gene for 14 of the 15 isolates that were found to be resistant by mycelial growth assay at the 5.0 µg a.i./ml discriminatory dose of fungicide but did not have the expected benzimidazole-resistance RFLP found in previously identified resistant field isolates (15). The

amplified PCR products were sequenced at Purdue University's low throughput lab with an ABI 3700 instrument. Bioedit sequence alignment editor (www.mbio.ncsu.edu/BioEdit/BioEdit.html) was used to compare the forward and reverse sequences. The nucleotide sequences were converted to their corresponding amino acid sequences and aligned using Clustal W 1.8 in the Bioedit program to the previously published *V. inaequalis* β-tubulin coding sequence (GenBank M97951.1; 20).

Temperature sensitivity of novel isolates lacking the RFLP. Mycelium assays

with fungicide-amended PDA (0.5 µg/ml thiophanate-methyl) and unamended PDA were completed as stated above for the novel resistant isolates lacking the *Bst*UI RFLP, two sensitive isolates, and one resistant isolate with the *Bst*UI-RFLP at 10, 23, and 27°C, to test for temperature sensitivity and to further characterize these isolates.

Detection of benzimidazole-resistant isolates of *V. inaequalis* from scab lesions by PCR. To test the application of the PCR-RFLP method for rapid detection of benzimidazole resistance, apple or crabapple leaves were collected from the field

Fig. 1. Relationship of fungicide sensitivity with 0.5 µg active ingredient (a.i.)/ml of thiophanate-methyl and 0.5 µg a.i./ml of benomyl by mycelia growth assay for *Venturia inaequalis*. After subtracting diameter of original plug, relative colony diameters were calculated by dividing diameter on fungicide-amended medium by diameter on nonamended medium and multiplying by 100 to determine percent relative growth.

Fig. 2. Sensitivity of isolates of *Venturia inaequalis* collected in 2007 from Indiana apple orchards to the fungicide thiophanate-methyl. Sensitive (S) isolates did not grow on 0.5 µg active ingredient (a.i.)/ml. Low-resistant (LR) isolates grew at 0.5 µg a.i./ml, but not at 5 µg a.i./ml. Moderately resistant (MR) isolates grew at 5 µg a.i./ml, but not at 50 µg a.i./ml. Very highly resistant (VHR) isolates grew rapidly at 50 µg a.i./ml.

and either stored in the refrigerator for up to 9 months or lyophilized directly after collection. Leaf lesions were excised with a sterile scalpel and placed in a 1.5-ml Eppendorf tube. Tissue was then lyophilized in a Freezone Six Liter Bench Top Freeze Dryer (Labconco, Kansas City, MO). Genomic DNA from the leaf and leaf

lesion was extracted using a micro mortar and pestle, followed by a Plant DNeasy Qiagen kit (cat no. 69104). In order to amplify the small amount of DNA, primer KQ18 (5'-CACCGTCAACATGCGTGA AATTG-3') was created upstream of BtubF14 using the β -tubulin sequence. PCR amplification was performed in a 25-

μ l volume containing 4 μ l of DNA (~100 ng/ μ l), 400 μ M dNTPs, 0.4 μ M primer BtubR, 0.4 μ M primer KQ18, 1.5 mM MgCl₂, 5 $\times$ buffer provided by the manufacturer, and 1 unit of GoTaq Flexi (Promega). PCR amplification was performed again in a 25- μ l volume containing 2 μ l of the amplified region obtained from the reaction using primer KQ18 and BtubR (~100 ng/ μ l), 400 μ M dNTPs, 0.4 μ M primer BtubR, 0.4 μ M primer BtubF14, 1.5 mM MgCl₂, 5 $\times$ buffer provided by the manufacturer, and 1 unit of GoTaq Flexi (Promega). The PCR amplifications, restriction digests, and gel resolution and photography were performed as described above.

RESULTS

Sensitivity of *V. inaequalis* isolates to benzimidazoles. Twenty-five of the 67 apple orchard isolates were tested to compare sensitivity to both benzimidazole and thiophanate-methyl. After incubation at 19°C for 30 days, the subpopulation grown on both thiophanate-methyl and benomyl at 0.5 μ g a.i./ml had relative growth that ranged from 0 to 147%. Regression analysis of the relative growth on benomyl and thiophanate-methyl showed a strong correlation ($R = 0.8882$, $P < 0.0001$) between the effects of two fungicides on *V. inaequalis* (Fig. 1). Subsequent radial mycelial growth assays using only thiophanate-methyl at increasing concentrations identified several categories of fungicide resistance in orchard isolates: 6 sensitive isolates (S), 5 low-resistance isolates (LR), 10 moderately resistant isolates (MR), and 46 very highly resistant isolates (VHR) (Fig. 2). Any growth reported above 0% was regarded as resistant (20,28). Of the 67 apple orchard isolates tested, 61 isolates tested resistant at 0.5 μ g/ml thiophanate-methyl. The relative mycelial growth of this in vitro assay ranged from 0 to 151%. Of the 61 resistant isolates, 56 tested resistant on amended medium with 5 μ g a.i./ml with relative growth ranging from 0 to 155%. Of these, 46 isolates tested resistant on medium amended with 50 μ g/ml thiophanate-methyl, with the relative growth ranging from 0 to 102%. Although 91% of the orchard isolates were resistant to benzimidazole fungicides, none of the isolates from landscape crabapples were resistant to these fungicides (Table 1).

Detection of thiophanate-methyl resistance by PCR-based RFLP assay. A single 1,244-bp fragment of the β -tubulin gene was amplified from field isolate by primer pair BtubF14 and BtubR regardless of resistance status in all isolates, and served as a positive control for the PCR reaction. The sequence of this product was compared to the GenBank submission (accession number: M97951.1) and contained the portion of the β -tubulin gene that included all positions known to affect

Table 1. Deduced amino acid changes in the β -tubulin gene of *Venturia inaequalis* and phenotypic response to benzimidazole-class fungicides

Amino acid changes	Growth at 0.5 μ g/ml	Growth at 5 μ g/ml	Growth at 50 μ g/ml	Resistance phenotype ^a	No. of isolates orchard/landscape
None	—	—	—	S	6/35
None	+	—	—	LR	0/0
F200Y	+	+	—	MR	0/0
E198K	+	+	+	HR	0/0
E198A	+	+	+	VHR	46/0
L240F	+	+ or —	—	LR or MR	15/0

^a Fungicide sensitivity (S), low resistance (LR), moderate resistance (MR), high resistance (HR), and very high resistance (VHR), were previously defined based upon growth at listed benzimidazole concentrations (20,26).

Fig. 3. A, Expected digestion sites for *Bst*UI of the amplified β -tubulin product. Black arrows denote location of the restriction site 5'...CGCG...3'. Asterisk indicates additional cleavage site with the mutation E198A. **B,** Gel electrophoresis analysis of *Bst*UI digest of β -tubulin gene amplified from isolates of *Venturia inaequalis*. Lane 1, 1-kb DNA ladder (Invitrogen); lanes 2 to 4, digested polymerase chain reaction (PCR) product of sensitive isolates; lanes 5 to 7, digested PCR product of resistant isolates.

the sensitivity to benzimidazoles in *V. inaequalis* and other fungi (24). *Bst*UI digestion of the PCR product was expected to result in the production of four fragments of 125, 243, 339, and 537 bp if resistant, or three products with fragments of 243, 339, and 662 bp if sensitive (Fig. 3). The six isolates previously identified as sensitive to thiophanate-methyl did not contain the *Bst*UI-RFLP. However, only 46 of the 67 isolates (69%) identified as resistant to thiophanate-methyl by the relative growth assay possessed the *Bst*UI-RFLP that resulted in the 662-bp fragment being cut into fragments of 125 and 537 bp. Thus, 15 of the isolates scored as sensitive to thiophanate-methyl based upon PCR-RFLP were actually either low resistant (could grow at 0.5 µg a.i./ml, but not at 5 µg a.i./ml) or moderately resistant (could grow at 5 µg a.i./ml, but not at 50 µg a.i./ml). Sequence analysis of the 1,244-bp DNA fragments amplified from 14 LR isolates lacking the expected *Bst*UI-RFLP identified a point mutation corresponding to codon 240 that would result in an amino acid change from leucine to phenylalanine, as was previously identified as conferring LR to MR to benzimidazoles in *M. laxa* (25). The predicted amino acid differences between benzimidazole-resistant isolates with the described RFLP, fungicide-resistant isolates lacking described RFLP, and fungicide-sensitive isolates are shown in Figure 4.

This mutation at codon 240 corresponds to a loss of a *Cac*8I restriction site, which we used to identify *V. inaequalis* isolates with LR or MR, and to discriminate from the previously described HR and VHR isolates. After amplification, *Cac*8I-digested PCR products resulted in (i) five fragments of 84, 230, 246, 273, and 411 bp or (ii) four fragments of 230, 246, 276, and 495 bp (Fig. 5). All 15 LR and MR isolates that were resistant to thiophanate-methyl possessed the RFLP produced by *Cac*8I; the remaining 52 isolates, both S and VHR, did not possess this RFLP corresponding to LR or MR.

Temperature sensitivity of novel isolates lacking the RFLP. After incubation at various temperatures for 4 weeks, the relative growth of the novel isolates with LR and MR did not exhibit any notable temperature sensitivities distinct from wild type at the listed temperatures (Fig. 6), unlike those reported in *M. laxa* (25).

RFLP analysis to detect benzimidazole resistance in field isolates of *V. inaequalis* in situ. DNA was extracted from the lesions of fresh and stored leaf tissue, or lyophilized tissue. We successfully amplified the 1,244-bp fragment of the β-tubulin gene from all tissue. The healthy (lesion-free) leaf tissue did not produce an amplicon. When applied on landscape crabapple, PCR-RFLP with *Bst*UI failed to identify any mutation at codon 198; PCR-RFLP with *Cac*8I did not identify a muta-

tion at codon 240 (Fig. 7). To date, no resistance, or any corresponding mutations, have been found at codon 198 and 240 in the *V. inaequalis* population that infects crabapple (Fig. 8). This demonstrates that the PCR-RFLP assay can be successfully applied directly to lesions, bypassing the need for culture of the pathogen.

DISCUSSION

In this study, we found that thiophanate-methyl resistance in *V. inaequalis* was widely distributed throughout Indiana apple orchards, despite a current lack of use for scab control. Interestingly, we did not find any benzimidazole resistance in the *V. inaequalis* population derived from landscape crabapples, suggesting that there are two distinct field populations of *V. inaequalis*, the orchard and the landscape, and limited gene flow between these populations. Fungicide resistance in *V. inaequalis* infecting landscape crabapples has not been reported, even though the use of fungicides, including the benzimidazole

class, is common in both nursery production and the landscape. From commercial orchards, we identified *V. inaequalis* isolates that represented low, moderate, and very high levels of benzimidazole resistance. After characterizing the resistance phenotype of isolates with mycelium assays, sequence analysis of the β-tubulin gene identified two RFLPs that could be used to identify the presence of benzimidazole resistance in 100% of the Indiana isolates, and in so doing, detected a previously unreported benzimidazole resistance allele in the *V. inaequalis* population. The identified point mutation would result in a change at codon 240 from the observed TTG (leucine) in sensitive isolates to TTT (phenylalanine) in low and moderately resistant isolates. Benzimidazole resistance associated with this point mutation was also reported in *M. laxa* (25). This assay was successfully applied directly to scab lesions on both commercial and ornamental *Malus* species, and was used to confirm the occurrence of fungicide resistance in the commercial orchards, and in the *V.*

M97951.1 (S)	ASGNKFVPRAVLVLDLEPGTMDAVRAGPFGQLFRPDNFVFGQSGAGNNWAKGHYTEGAE
B5-4 (LR)	*****
MYD3-2 (LR)	*****
B8-5 (MR)	*****
B10-5 (MR)	*****
B10-6 (MR)	*****
MYD4-2 (MR)	*****
MYD4-5 (VHR)	*****
198	
M97951.1 (S)	LVDQVLDVVRREAGCCLQGFGQITHSLGGGTGAGMGTLLISKIREFPDRMMATFSV
B5-4 (LR)	*****
MYD3-2 (LR)	*****
B8-5 (MR)	*****
B10-5 (MR)	*****
B10-6 (MR)	*****
MYD4-2 (MR)	*****
MYD4-5 (VHR)	*****
240	
M97951.1 (S)	VPSPKVSDTVVEPYNATLSVHQLVENSDEFICIDNEALYDICMRTLKLNPSYGDNLH
B5-4 (LR)	*****
MYD3-2 (LR)	*****
B8-5 (MR)	*****
B10-5 (MR)	*****
B10-6 (MR)	*****
MYD4-2 (MR)	*****
MYD4-5 (VHR)	***** *A*****
240	
M97951.1 (S)	LVSVMMSGVTTCLRFPGQLNSDLRKLAVNMVFPRLHFFM
B5-4 (LR)	***** *F*****
MYD3-2 (LR)	***** *F*****
B8-5 (MR)	***** *F*****
B10-5 (MR)	***** *F*****
B10-6 (MR)	***** *F*****
MYD4-2 (MR)	***** *F*****
MYD4-5 (VHR)	*****

Fig. 4. Comparison of putative amino acid sequences from codon 54 to 267 of the β-tubulin gene of *Venturia inaequalis*. M97951.1 is the sequenced sensitive isolate deposited in GenBank (20). The β-tubulin gene sequence of isolates with low resistance (LR) to thiophanate-methyl, moderate resistance (MR) to thiophanate-methyl, and very high resistance (VHR) to thiophanate-methyl is given. LR is characterized by growth at 0.5 µg/ml benomyl, but not at 5 µg/ml. MR is characterized by growth at 5 µg/ml benomyl, but not at 50 µg/ml. VHR is characterized by rapid growth at 50 µg/ml benomyl. Asterisk indicates the same amino acid. All MR and LR isolates contain a point mutation at codon 240 that changes leucine to phenylalanine. The VHR isolate contained a point mutation at codon 198 that changed glutamic acid to alanine as described by Koenraadt et al. (20).

Fig. 5. A, *Cac8I* expected digestion sites of the amplified β -tubulin product. Black arrows indicate sequence 5'...GCNNGC...3'. Asterisk indicates loss of a cleavage site with the point mutation corresponding to codon change L240F. **B,** Gel electrophoresis analysis of *Cac8I* digestion of β -tubulin amplified from isolates of *Venturia inaequalis*. Lane 1, EZ Load Molecular Rulers (BioRad); lanes 2 and 3, polymerase chain reaction (PCR) product of very highly resistant (VHR) and sensitive (S) isolates, respectively; lanes 4 and 5, PCR product of moderately resistant (MR) and low-resistant (LR) isolates, respectively; lanes 6 and 7, digested PCR product of VHR and S isolates, respectively; lanes 8 and 9, digested PCR products of MR and LR isolates, respectively.

Fig. 6. Relative growth of moderately and low-resistance isolates of *Venturia inaequalis* lacking the *BstUI*-restriction fragment length polymorphism (RFLP), and of resistant isolate B11-2 and sensitive isolate LC2-3 at varying temperatures. NA indicates that isolates did not grow on unamended or amended media.

inaequalis population infecting crabapples treated with an injection of debacarb and carbendazim, or with a history of chemical management. The ability to quickly and conclusively identify fungicide resistance *in situ* is important because the viability of *V. inaequalis* conidia recovered from commercial orchards treated with pesticides is usually low. These data suggest a similar approach can be used to screen for fungicide resistance to other classes of fungicides where a single gene is associated with resistance.

This study relied upon an amplified gene fragment that included the resistant allele E198A in such a way as to use the resulting *BstUI*-RFLP to discriminate sensitive isolates from those VHR isolates possessing the RFLP. By using PCR-RFLP, as opposed to the allele-specific PCR reaction, a built-in control (the PCR product) is included. However, this assay is not without flaws, and would not detect resistant isolates that arose from other

Fig. 7. Gel electrophoresis of β -tubulin amplified polymerase chain reaction (PCR) amplicon and PCR-restriction fragment length polymorphism (RFLP) to identify thiophanate-methyl resistance in *Venturia inaequalis* isolates. Lane 1, 1-kb DNA ladder (Invitrogen); lane 2, polymerase chain reaction (PCR) product from a pure culture isolate; lanes 3 and 4, PCR product from apple scab leaf lesions; lane 5, PCR product of leaf DNA; lane 6, *BstUI*-digested PCR product from lane 2; lanes 7 and 8, *BstUI*-digested PCR product from lanes 3 and 4, respectively (sensitive).

Fig. 8. Gel electrophoresis of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) products from *Venturia inaequalis* lesions *in situ* from crabapples. DNA was directly isolated from lesions, the 1,244-bp fragment of β -tubulin amplified, and then digested with either *BstUI* or *Cac8I*. Lane 1, EZ Load Molecular Rulers (BioRad); lanes 2, 5, 8, 11, and 14, β -tubulin amplified crabapple isolates. Lanes 3, 6, 9, 12, and 15, *BstUI*-digested β -tubulin amplified crabapple isolates. Lanes 4, 7, 10, 13, and 16, *Cac8I*-digested β -tubulin amplified crabapple isolates. All isolates indicated no selected mutation at codons 198 and 240.

resistance mutations unless they too resulted in the development of a *Bst*UI-RFLP. The mutation at codon L240F was found after sequencing 6 of the 15 PCR products from isolates that did not contain a point mutation at codon 198 but were identified as resistant using relative growth assays. This PCR-RFLP screen, using restriction enzymes *Bst*UI and *Cac*8I, correctly identified fungicide resistance in 100% of the isolates tested from Indiana.

The benzimidazole class of fungicides contains four different active ingredients (benomyl, thiophanate-methyl, debacarb, and carbendazim) with the same site-specific mode of action. It is important to note that each benzimidazole fungicide varies in efficacy for scab control, and that benzimidazole and its metabolized by-product carbendazim have similar toxicity, whereas intact thiophanate-methyl is less toxic than either carbendazim or debacarb (6). However, any isolate (regardless of fungal population) that has a mutation within the β -tubulin gene at any of the previously reported sites (6, 50, 198, 200, 240) will have some resistance to this class of fungicides (20,24). Continued application of these fungicides, even for the control of other pathogens in the orchard or landscape, will result in continued selection for resistance in *V. inaequalis*. Furthermore, benzimidazole resistance has been found to persist in other fungal populations, even in the absence of benzimidazole use for periods in excess of 5 years (18).

Benzimidazole resistance has been detected in many fungal species (3,14,15,30). Traditionally, benzimidazole resistance is detected by isolating a single spore of the pathogen in pure culture, and subculturing that isolate onto fungicide-amended medium and on unamended medium, and comparing the growth of the fungus between both plates. This procedure is labor intensive and time-consuming, requiring up to several weeks for adequate growth of *V. inaequalis* (2). Use of a PCR-RFLP assay applied directly to DNA amplified from fungal lesions can quickly discriminate fungicide resistant isolates from sensitive isolates. McKay and Cooke (27) used a PCR-based RFLP to identify thiabendazole resistance in *Helminthosporium solani*. In 2003, Cunha and Rizzo (5) used this method to identify thiophanate-methyl and benomyl resistance in *H. solani* populations in California. A PCR-based RFLP method to determine benomyl resistance has been performed with *V. inaequalis*, but the PCR-based RFLP was not correlated with the phenotype, nor was it applied to field isolates *in situ* (29). As we found in this study, had we used only one RFLP or had we not screened for phenotype prior to the PCR-RFLP screen, we would have under-reported MBC resistance in 31% of the isolates. However, there still remains the possibility of new resistance alleles

developing that would remain undetected, which may lead to under-reporting of benzimidazole-resistant isolates.

Nevertheless, using a PCR-RFLP based assay may be the best option to screen for fungicide resistance from late-season scab lesions. *V. inaequalis* cannot be reliably cultured in the Midwest after early to mid-June due to the competing leaf microflora. When leaves are collected from the field and streaked onto water agar to isolate single *V. inaequalis* spores, saprophytes regularly outgrow the *V. inaequalis* conidia, and pure cultures cannot be obtained. Therefore, conventional mycelium assays or biochemically based assays (4) cannot be used to test for fungicide resistance on mid- to late-season isolates. PCR and/or other molecular procedures may be essential to test for fungicide resistance in *V. inaequalis* in the late season.

This study demonstrates the persistence of benzimidazole resistance in orchard populations of *V. inaequalis*, as most commercial apple growers in Indiana no longer rely on thiophanate-methyl for scab control, but utilize it for control other diseases of apple. One surprising finding from this study was the fact that we failed to detect benzimidazole resistance in the *V. inaequalis* population infecting crabapples. This finding supports the continued use of this fungicide to control scab in this setting, and also suggests barriers to gene flow within these two populations of *V. inaequalis*.

Molecular detection of benzimidazole resistance is more convenient and less costly than mycelial growth assays. Using the *in situ* PCR-based method, LR, MR, and VHR to benzimidazole can be detected within 2 days compared to 4 to 6 weeks by conventional assay procedures. However, its use is tempered by the recognition that it does not identify all possible alleles conferring resistance, and may under-report resistance. To date, 100% of isolates surveyed were accurately identified, and the combination of faster results and lack of expense may justify its use in screening for resistance in nursery and landscape populations. This will allow nursery growers and arborists to quickly modify spray programs to exclude or tank-mix reduced efficacy fungicides in order to better control the disease in ornamental crabapples.

ACKNOWLEDGMENTS

We thank Corie Shaner for her technical assistance, and S. Goodwin and G. Shaner for editorial and scientific comments on the manuscript. We thank Adam Leonberger for his photography assistance and image preparation. This work was supported by startup funds from Purdue University.

LITERATURE CITED

1. Beckerman, J., Chatfield, J., and Draper, E. 2009. A 33-year evaluation of resistance and pathogenicity in the apple scab-crabapples pathosystem. *HortScience* 44:599-608.
2. Braun, P. G., and McRae, K. B. 1992. Compo-

sition of population of *Venturia inaequalis* resistant to myclobutanil. *Can. J. Plant Pathol.* 14:215-220.

3. Chandler, W. A., Daniell, J. W., and Littrell, R. H. 1978. Control of peach disease in an orchard having benomyl-tolerant *Cladosporium carpophilum*. *Plant Dis. Rep.* 62:783-786.
4. Cox, K. D., Quello, K., Deford, R. J., and Beckerman, J. L. 2009. A rapid method to quantify fungicide sensitivity in the brown rot pathogen *Monilinia fructicola*. *Plant Dis.* 93:328-331.
5. Cunha, M. G., and Rizzo, D. M. 2003. Development of fungicide cross resistance in *Helminthosporium solani* populations from California. *Plant Dis.* 87:798-803.
6. Delp, C. J. 1995. Benzimidazole and related fungicides. Pages 291-303 in: *Modern Selective Fungicides—Properties, Applications, Mechanisms of Action*. H. Lyr, ed. Gustav Fischer Verlag, New York.
7. Delye, C., Bousset, L., and Corio-Costet, M.-F. 1998. PCR cloning and detection of point mutations in the eburiole 14a-demethylase (CYP51) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. *Curr. Genet.* 34:399-403.
8. Eckert, S. W. 1988. Historical development of fungicide resistance in plant pathogens. Pages 1-3 in: *Fungicide Resistance in North America*. American Phytopathological Society, St. Paul, MN.
9. Fluit, A. C., Visser, M. R., and Schmitz, F. J. 2001. Molecular detection of antimicrobial resistance. *Clin. Microbiol. Rev.* 14:836-871.
10. Fontaine, A., Remuson, F., Fraissinet-Techet, L., Micoud, A., Marmeisse, R., and Melayah, D. 2008. Monitoring of *Venturia inaequalis* harboring the QoI resistance G143A mutation in French orchards as revealed by PCR assays. *Pest Manag. Sci.* DOI 10.1002/ps1649.
11. Hewitt, H. G. 1998. *Fungicides in crop protection*. CAB International, New York.
12. Iles, J. K., and Vold, A. M. 2003. Landscape tree cultivar preferences in Iowa. *J. Arboricult.* 29:331-336.
13. Jones, A. L., and Aldwinckle, H. S. 1991. *Compendium of Apple and Pear Diseases*. American Phytopathological Society, St. Paul, MN.
14. Jones, A. L., and Ehret, G. R. 1980. Resistance of *Coccomyces hiemalis* to benzimidazole fungicides. *Plant Dis.* 64:767-769.
15. Jones, A. L., and Walker, R. J. 1976. Tolerance of *Venturia inaequalis* to dodine and benzimidazole fungicides in Michigan. *Plant Dis. Rep.* 60:40-44.
16. Keitt, G. W., and Palmiter, D. H. 1938. Heterothallism and variability in *Venturia inaequalis*. *Am. J. Bot.* 25:338-344.
17. Kim, Y.-S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. *Phytopathology* 93:891-900.
18. Koch, P. L., Grau, C. R., Jo, Y.-K., and Jung, G. 2009. Thiophanate-methyl and propiconazole sensitivity in *Sclerotinia homoeocarpa* populations from golf courses in Wisconsin and Massachusetts. *Plant Dis.* 93:100-105.
19. Koenraad, H., and Jones, A. L. 1992. The use of allele-specific oligonucleotide probes to characterize resistance to benomyl in field strains of *Venturia inaequalis*. *Phytopathology* 82:1354-1358.
20. Koenraad, H., Somerville, S. C., and Jones, A. L. 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology* 82:1348-1354.
21. Luck, J. E., and Gillings, M. R. 1995. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using polymerase chain reac-

- tion. Mycol. Res. 99:1483-1488.
22. Luo, Y., Ma, Z., and Michailides, T. 2007. Quantification of allele E198A in beta-tubulin conferring benzimidazole resistance in *Monilinia fructicola* using real-time PCR. Pest Manag. Sci. 63:1178-1184.
 23. Ma, Z., and Michailides, T. 2004. A real-time PCR assay for the detection of azoxystrobin-resistant *Alternaria* populations from pistachio orchards in California. Crop Prot. 23:1259-1263.
 24. Ma, Z., and Michailides, T. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotype in phytopathogenic fungi. Crop Prot. 24:853-863.
 25. Ma, Z., Yoshimura, M. A., Holtz, B., and Michailides, T. 2005. Characterization and PCR-based detection of benzimidazole-resistant isolates on *Monilinia laxa* in California. Pest Manag. Sci. 61:449-457.
 26. Ma, Z., Yoshimura, M. A., and Michailides, T. J. 2003. Identification and characterization of benzimidazoles resistance in *Monilinia fructicola* from stone fruit orchards in California. Appl. Environ. Microbiol. 69:7145-7152.
 27. McKay, G. J., and Cooke, L. R. 1997. A PCR-based method to characterize and identify benzimidazole resistance in *Helminthosporium solani*. FEMS Microbiol. Lett. 152:371-378.
 28. Shabi, E., Katan, T., and Marton, K. 1983. Inheritance of resistance to benomyl in isolates of *Venturia inaequalis* from Israel. Plant Pathol. 32:207-211.
 29. Svircev, A. M., Gill, J. J., Zhou, T., Castle, A., and Chiba, M. 2000. A comparison of techniques for assessing benzimidazole resistance in *Venturia inaequalis*. J. Phytopathol. 148:205-210.
 30. Wicks, T. 1974. Persistence of benomyl tolerance in *Venturia inaequalis*. Plant Dis. Rep. 60:818-819.