Pest Manag Sci **61**:449–457 (2005)
DOI: 10.1002/ps.982

Characterization and PCR-based detection of benzimidazole-resistant isolates of *Monilinia laxa* in California

Zhonghua Ma,[1]* Michael A Yoshimura,[2] Brent A Holtz[3] and Themis J Michailides[1]*

[1]*Department of Plant Pathology, University of California, Davis, Kearney Agricultural Center, 9240 South Riverbend Ave, Parlier, CA 93648, USA*

[2]*Biological Sciences Department, California Polytechnic State University, San Luis Obispo, CA 93407, USA*

[3]*University of California Cooperative Extension, Madera County, Madera, CA 93637, USA*

Abstract: *Monilinia laxa* is a pathogen of brown rot of stone fruit and almond in California, causing blossom blights and fruit rots. In this study, low-level resistance to the benzimidazole fungicides benomyl and thiophanate-methyl was detected in field isolates of *M laxa* collected from stone fruits and almonds in California. Low-resistant (LR) isolates grew in potato dextrose agar (PDA) plates amended with benomyl and thiophanate-methyl at 1 and 5 μg ml^{-1}, respectively, but not in plates amended with benomyl at 5 μg ml^{-1} or thiophanate-methyl at 50 μg ml^{-1}. The benzimidazole LR isolates were characterized by temperature sensitivity and the DNA sequence of the β-tubulin gene. The LR isolates showed high-temperature sensitivity, being sensitive to 1 μg ml^{-1} of benomyl at 28 °C but resistant at 8–24 °C. Analysis of the DNA sequence of the β-tubulin gene showed that the LR isolates had a point mutation at the amino-acid position 240, causing substitution of leucine by phenylalanine. Based on the point mutation, a pair of allele-specific PCR primers was developed for rapid detection of LR isolates of *M laxa*. In addition, a pair of PCR primers specific to *M laxa* was developed on the basis of the differences in the DNA sequence of the intron 6 of β-tubulin gene from *M laxa*, *M fructicola* and other fungal species. The primer pair amplified the expected 376-bp DNA fragment from all *M laxa* isolates tested, but not from 14 other fungal species isolated from stone fruit and almond crops. The restriction endonuclease *BsmA I* recognized the sequence GTCTCC in the PCR products from sensitive (S) isolates only, but not the GTTTCC sequence in the PCR products from LR isolates. The endonuclease digested the 376-bp PCR products from S isolates to produce two bands (111 and 265 bp) on agarose gels. Thus, both allele-specific PCR and the PCR-restriction fragment length polymorphism (PCR-RFLP) methods could be useful for rapidly detecting benzimidazole-resistant isolates of *M laxa* from stone fruit and almond crops in California.

Keywords: allele-specific PCR; brown rot; *Monilinia laxa*; PCR-restriction fragment length polymorphism (PCR-RFLP); *Prunus* spp

1 INTRODUCTION

The benzimidazole fungicides thiophanate-methyl and benomyl have been used widely for the control of brown rot of stone fruit (*Prunus* spp) and almond (*Prunus dulcis* Webb) caused by *Monilinia fructicola* (G Wint) Honey and/or *M laxa* (Aderh & Ruhl) Honey. In California, benomyl and thiophanate-methyl were used extensively for controlling blossom blight caused by the brown rot fungi after the registrations of these fungicides in 1972 and 1979, respectively. By 1977, benomyl-resistant populations of *M fructicola* had become widespread in peach and nectarine orchards throughout California.[1–3] In 1980, benomyl-resistant isolates of *M laxa* were detected for the first time on apricot fruit in California[4] and later on other stone fruit and almond crops.[2] With the withdrawal of benomyl from the market, more and more growers have started using thiophanate-methyl instead. Since positive cross-resistance, which is defined as the resistance of a pathogen to two or more fungicides as a result of the same resistance mechanisms, is very common among benzimidazole fungicides, information on *M laxa* resistance to benomyl will be still useful for the management of thiophanate-methyl resistance in this pathogen.

Resistance to benzimidazole fungicides has been detected in many fungal species. In most cases, resistance is associated with point mutations in

* Correspondence to: Themis J Michailides or Zhonghua Ma, Department of Plant Pathology, University of California Davis, Kearney Agricultural Center, 9240 South Riverbend Ave, Parlier, CA 93648, USA
E-mail: themis@uckac.edu; zhma@uckac.edu

Contract/grant sponsor: USDA/CAR Award; contract/grant number: 2005-5/100-01990
(*Received 2 February 2004; revised version received 17 June 2004; accepted 17 September 2004*)
Published online 10 November 2004

the β-tubulin gene which result in altered amino-acid sequences at the benzimidazole binding site.[5] Previous studies showed that codon changes at positions 198 and 200 conferred benzimidazole resistance in field isolates of most phytopathogenic fungi.[5–8] Mutations at codon 50 and 240 leading to benzimidazole resistance were reported recently in field isolates of *Cladobotryum dendroides* (Bull) W Gams & Hoozemans[9] and *Tapesia yallundae* Wallwork & Spooner,[8] respectively. However, a few exceptions to mutations at these positions (eg 50, 198, 200 and 240) have been reported in benomyl low-resistant (LR) isolates of *Venturia inaequalis* (Cooke) Winter, *Penicillium expansum* Link and *P aurantiogriseum* Dierck,[5] and thiabendazole-resistant isolates of *Gibberella pulicaris* (Fr) Sacc,[10] but the exact molecular mechanisms for their resistance have not yet been determined. For *M laxa*, although resistant isolates have been found in the field since 1980,[2,4] resistance mechanisms have not been documented.

Mutations in the β-tubulin gene may have pleiotropic effects on fungal growth at high or low temperature. Laboratory induced benomyl-resistant isolates of *Aspergillus nidulans* (Eidam) Winter, *Saccharomyces cerevisiae* Meyer ex Hansen and *Fusarium moniliforme* Sheldon exhibited low or high temperature-sensitive phenotypes.[11,12] Such pleiotropic effects have recently been observed in field-resistant isolates of the plant pathogenic fungus *M fructicola*. In a study on the sensitivity of *M fructicola* to benzimidazoles, we observed that benzimidazole LR isolates showed sensitivity to low temperature, whereas isolates highly resistant to benzimidazoles (HR isolates) exhibited sensitivity to high temperature.[13] Information on the temperature sensitivity of resistant isolates may have a direct impact on strategies for fungicide application. If resistant isolates show sensitivity to high temperature, hot days may be more appropriate times for application of the fungicide in fields where resistant isolates have been detected. Thus, it would be of interest to determine the temperature sensitivity for benzimidazole-resistant isolates of *M laxa*, which is closely related to *M fructicola*.

The current method for detecting benzimidazole resistance in *M laxa* requires the isolation of the pathogen in a pure culture and subsequent plating on a medium containing the fungicide. This procedure is labor intensive and time consuming if large numbers of isolates are to be tested. Advances in molecular biology, particularly the polymerase chain reaction (PCR), have provided new opportunities for rapidly detecting fungicide-resistant isolates of plant pathogens. The techniques of PCR linked with allele-specific probes,[14] PCR-restriction fragment length polymorphism,[15,16] and allele-specific PCR[17,18] have been used successfully to detect fungicide-resistant isolates of several plant pathogens but not *M laxa*.

The objectives of this study were to (1) determine the temperature sensitivity of benzimidazole-resistant isolates of *M laxa*, (2) investigate molecular mechanisms of benzimidazole resistance in field isolates of *M laxa*, and (3) develop rapid molecular techniques to detect benzimidazole-resistant isolates of *M laxa* in California.

2 MATERIALS AND METHODS

2.1 Sensitivity of *Monilinia laxa* to benzimidazole fungicides

Forty-one single-conidial isolates of *M laxa* collected during 1987 to 2002 from nectarine, peach, prune and almond throughout California, as well as one isolate collected from Chile, were used in this study (Table 1). Single conidial isolates were obtained by taking conidia from infected plant tissues, streaking onto acidified (2.5 ml of 25% v/v lactic acid per liter) potato dextrose agar (Microtech Scientific, Orange, CA) (APDA) plates, and 1 day later transferring individual germinating conidia to fresh APDA plates. Isolates were routinely stored on grade 40 silica gels (Davison Chemical, Baltimore, MD) at 4 °C.

To determine the sensitivity of *M laxa* to benzimidazoles, benomyl 500 g kg^{-1} WP (Benlate, Du Pont de Nemours and Company, Wilmington, DE) and thiophanate-methyl 700 g kg^{-1} WP (Elf Atochem North America, Inc, Philadelphia, PA) were dissolved in sterile water and adjusted to 10 mg AI ml^{-1}. Each of the two fungicides was added to PDA medium after sterilization to produce concentrations of 0, 1, 5 or 50 μg AI ml^{-1} medium. Single 5-mm mycelial plugs taken from the edge of a 5-day-old colony of each isolate were placed onto PDA plates amended with each fungicide at each of the above concentrations. Each isolate–fungicide concentration was replicated twice. After the plates had been incubated at 24 °C for 7 days in the dark, mycelial growth on each plate was examined for presence or absence of radial growth. The experiment was performed twice. On the basis of previous studies on benzimidazole sensitivity in *M laxa*, *M fructicola* and other phytopathogenic fungi,[2,4,5] the isolates of *M laxa* were classified into one of the following phenotypes: sensitive (S), with no mycelial growth in PDA plates amended with 1 μg ml^{-1} benomyl; low-resistant (LR), with mycelial growth in plates amended with 1 μg ml^{-1} benomyl but not in those with 5 μg ml^{-1}; and highly resistant (HR), with growth in plates amended with 50 μg ml^{-1} benomyl.

2.2 Temperature sensitivity of *Monilinia laxa* isolates resistant or sensitive to benzimidazoles

To determine temperature sensitivity, each of the 41 single-conidial isolates of *M laxa* (Table 1) was analyzed for its ability to grow at various temperatures on PDA plates with or without benomyl. A 5-mm mycelial plug taken from the edge of a 5-day-old colony of each isolate was transferred onto a PDA plate amended with benomyl at 0, 1 or 5 μg ml^{-1}. Plates were incubated at 8, 12, 16, 20, 24, 28 or 32 °C for 10 days in the dark, and mycelial growth was

Table 1. Characteristics of *Monilinia laxa*, *M fructicola* and other fungal species collected from stone fruit and almond orchards used in this study

Isolate	Sensitivity to benzimidazoles	Host	Location	Year of isolation	PCR assay with primer pairs MLRF + MLRR/MLF2 + MLR2[a]
Monilinia laxa					
MDA1–MDA11	LR[b]	Almond	Madera, CA	2002	+/+
MDA18	S[b]	Almond	Madera, CA	2002	−/+
MDA37	S	Almond	Madera, CA	2002	−/+
289–292	S	Prune	CA	1987	−/+
293	S	Peach	CA	1987	−/+
487	S	Nectarine	Chile	1992	−/+
515–517	S	Prune	Colusa, CA	1992	−/+
537	S	Prune	Davis, CA	1992	−/+
568–571	LR	Prune	Yolo, CA	1992	+/+
762	LR	Prune	Colusa, CA	1992	+/+
765,766, 768,769	S	Prune	CA	1992	−/+
992	S	Prune	Glenn, CA	1993	−/+
994–997	S	Prune	Glenn, CA	1993	−/+
1001, 1005, 1006, 1043	LR	Prune	Glenn, CA	1993	+/+
M fructicola					
LVN8	S	Nectarine	Merced Co	09/02	−/−
22E5	LR	Peach	Fresno Co	3/94	−/−
MS1	HR[b]	Nectarine	Madera Co	09/02	−/−
F6, C4	ND[c]	Plum	Fresno, CA	2001	−/−
Gf82, Gf104	ND	Prune	Fresno, CA	2001	−/−
2537, 2538	ND	Cherry	Michigan	1998	−/−
532, 533	ND	Cherry	Oregon	1990	−/−
12A, 12B	ND	Peach	Bolivia	2001	−/−
A6	ND	Peach	Australia	1997	−/−
BR6	ND	Nectarine	Australia	1997	−/−
Botrytis cinerea					
1333–1334	ND	Prune	Fresno, CA	1994	−/−
2386	ND	Plum	Fresno, CA	1998	−/−
2367	ND	Nectarine	Fresno, CA	1998	−/−
Alternaria alternata	ND	Peach	Fresno, CA	1999	−/−
Aureobasidium pullulans	ND	Peach	Fresno, CA	1995	−/−
A pullulans	ND	Nectarine	Fresno, CA	1995	−/−
Cladosporium herbarum	ND	Prune	Fresno, CA	1999	−/−
Gilbertella persicaria	ND	Peach	Fresno, CA	1992	−/−
G persicaria	ND	Nectarine	Fresno, CA	1995	−/−
Mucor piriformis	ND	Plum	Fresno, CA	1997	−/−
M piriformis	ND	Peach	Fresno, CA	1993	−/−
M piriformis	ND	Prune	Fresno, CA	1993	−/−
Penicillium digitatum	ND	Plum	Fresno, CA	1996	−/−
P digitatum	ND	Nectarine	Fresno, CA	1996	−/−
Phomopsis sp	ND	Nectarine	Fresno, CA	1999	−/−
Rhizopus stolonifer	ND	Nectarine	Tulare, CA	1991	−/−
R stolonifer	ND	Peach	Kings, CA	1993	−/−
Sclerotinia sclerotiorum	ND	Plum	Fresno, CA	1999	−/−

[a] + and − indicate the presence and absence of the expected 1112-bp and 376-bp PCR fragments by the allele-specific primer pair MLRF + MLRR and species-specific primer pair MLF2 + MLR2, respectively.
[b] S: sensitive, with no mycelial growth in potato dextrose agar (PDA) plates amended with benomyl at 1 μg ml^{-1}. LR: low-resistant, with mycelial growth in PDA plates amended with benomyl at 1 μg ml^{-1} but not in those with benomyl at 5 μg ml^{-1}. HR: highly resistant, with mycelial growth in PDA plates amended with benomyl at 50 μg ml^{-1}.
[c] ND: not determined.

examined for presence or absence of radial growth. Each isolate–fungicide concentration was replicated three times and the experiment was performed twice.

2.3 Isolation of the β-tubulin gene from *Monilinia laxa*

Six isolates (three S isolates: 515, 537 and 992; and three LR isolates: MDA1, MDA2 and 1001) were used for analysis of DNA sequence of the β-tubulin gene. To extract DNA, each isolate was grown in Petri dishes containing 20 ml of potato dextrose broth (DIFCO Laboratories, Detroit, MI) at 24 °C for 7 days. Mycelia were harvested and washed in sterile water, frozen in liquid nitrogen, and lyophilized. DNA from each isolate was extracted by using the FastDNA® Kit (QBIOgene Inc, Carlsbad, CA).

Table 2. PCR primers used in this study

Primer	Sequence (5′-3′)	Base lair location (5′-3′) in β-tubulin gene
TubA (forward)	AAA TGC GTG AGA TTG TA	$-2 \rightarrow 15$
TubR1 (reverse)	TGT ACC AAT GCA AGA AAG CCT T	$1611 \rightarrow 1632$
MfTF	TCG ACA ATA CAA AAT CAC TCC GC	$497 \rightarrow 519$
MLRF (forward)	TGC GTG AGA TTG TAC GTA TAC CTC TC	$4 \rightarrow 29$
MLRR (reverse)	AGT TAA GTT GAC CAG GGA AAC GGA A	$1091 \rightarrow 1115$
MLF2 (forward)	CGA GGC TCT TTA CGA CAT TTG	$985 \rightarrow 1005$
MLR2 (reverse)	TTA TAC TAT GGC CGG GCA GA	$1341 \rightarrow 1360$

The PCR primer pair TubA + TubR1[9] (Table 2) was used to amplify the β-tubulin gene fragment from *M laxa*. The PCR reaction was performed in a 50-µl volume containing 50 ng of fungal DNA, 0.2 µM of each primer, 0.2 mM of each dNTP, 2.5 mM magnesium chloride, 1× Promega Taq Polymerase Buffer (Promega, Madison, WI) and 2 U of Promega *Taq* Polymerase. The PCR amplifications were performed using the following parameters: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 40 s, extension at 72 °C for 1.5 min, and final extension at 72 °C for 10 min. PCR products were examined by electrophoresis in a 1.5% agarose gel in 1× Tris-acetate (TAE) buffer.

The approximately 1.6-kb PCR product from each isolate was purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The purified fragment from each isolate was ligated into the pGEM-T Easy vector (Promega, Madison, WI), and transformed into *Escherichia coli* Cast & Chalm (strain JM109) cells. Recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN) and identified by digestion of plasmids with *EcoR I*. The cloned fragments were sequenced by DBS Sequencing Inc (Division of Biological Sciences, University of California at Davis) using primers T7, SP6 and an internal primer MfTF (Table 2).

The DNA sequences of the β-tubulin gene from the three S and three LR isolates of *M laxa* were translated into amino-acid sequences using standard code by the computer program EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/). The sequence of deduced amino acids was aligned using the computer program Clustal W 1.82 (http://www.ebi.ac.uk/clustalw/, European Bioinformatics Institute, Cambridge, UK). The DNA sequence and deduced amino-acid sequence from the S isolates of *M laxa* were also compared with those from other phytopathogenic fungal species using BLAST of NCBI/GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4 Design of allele-specific PCR primers for LR isolates and PCR primers specific to *Monilinia laxa*

Based on the mutation in the β-tubulin gene from LR isolates of *M laxa*, the allele-specific primer pair MLRF + MLRR (Table 2) was designed for detection of the β-tubulin gene from LR isolates of *M laxa*. The reverse primer MLRR was designed to match the point mutation from C to T at the 3′ end of the primer. In additional, a pair of primers MLF2 + MLR2 specific to *M laxa* was designed based on the sequence of intron 6 of the β-tubulin gene from *M laxa*, which was found to differ from those of other closely related fungal species using BLAST of NCBI/GenBank. The reverse primer MLR2 is located in the intron 6 of the β-tubulin gene and the forward primer MLF2 in the exon 6. Thus, it was expected that the allele-specific primer pair MLRF + MLRR targeted the β-tubulin gene of LR isolates only, but not from sensitive isolates of *M laxa*, and the primer pair MLF2 + MLR2 was specific to *M laxa*.

2.5 Specificity of the PCR primer pair *MLRF + MLRR* and *MLF2 + MLR2* for detection of LR isolates and *Monilinia laxa*, respectively

The 41 isolates of *M laxa*, as well as 34 isolates of other fungal species (Table 1), were used in specificity tests for primer pairs MLRF + MLRR and MLF2 + MLR2. PCR reactions were performed as described above. The PCR amplification parameters were: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 40 s, annealing at 72 °C for 40 s for the primer pair MLRF + MLRR, or 64 °C for the primer pair MLF2 + MLR2, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. A 15-µl aliquot of PCR product of each sample was analyzed in a 1.5% agarose gel in 1× TAE buffer.

2.6 Detection of benzimidazole LR isolates of *Monilinia laxa* using a PCR-restriction fragment length polymorphism (PCR-RFLP) method

The PCR product generated by the species-specific primer pair MLF2 + MLR2 contains the mutation site in the β-tubulin gene. Based on the recognition sequence of *BsmA I* (New England BioLabs Inc, Beverly, MA), this restriction endonuclease is expected to recognize the sequence GTCTCC in the PCR product from S isolates, but not the sequence GTTTCC in the PCR product from LR isolates. A 20-µl aliquot of PCR products generated by the primer pair MLF2 + MLR2 was digested by 1.5 U *BsmA I* at 55 °C for 1 h according to the manufacturer's protocol. Since mixtures of S and LR isolates are present in field

samples, template DNA produced from S and LR isolates mixed in the ratios of 1:1, 10:1 or 1:10 (S:LR) was also used for this PCR-RFLP analysis. Digests were analyzed on 2% agarose gels in 1× TAE buffer.

2.7 Rapid DNA extraction for detection of benzimidazole LR isolates of *Monilinia laxa*

To test application of the allele-specific PCR or PCR-RFLP method for rapid detection of benzimidazole resistance, 37 isolates of *M laxa* (not included in Table 1) whose sensitivities to benzimidazoles were unknown, were collected from naturally infected plums and almonds in California in 2002. DNA from mycelia and spores of each isolate was extracted using a boiling mycelial method.[19] Briefly, for each isolate, approximately 2 mg (fresh weight) of mycelia were collected by gently scraping the surface of a culture on APDA with a sterilized loop, placed into a 1.5-ml microcentrifuge tube containing 30 µl of 1 M Tris-HCl (pH 8.0), and overlaid with two drops of mineral oil (Sigma, St Louis, MO). Samples were boiled at 98–100 °C for 20 min and immediately placed on ice for 5 min. After centrifuging at 10 000 *g* for 2 min, a 2-µl aliquot of supernatant below the oil was used as template DNA for PCR amplification with the primer pair MLRF + MLRR or MLF2 + MLR2. In addition, the internal transcribed spacer primer pair ITS1 + ITS4[20] was used to test the quality of the template DNA. PCR reactions were performed under the same amplification conditions as described above for the allele-specific primer pair MLRF + MLRR, and the species-specific primer pair MLF2 + MLR2. PCR amplifications with the primer pair ITS1 + ITS4 were performed using the same conditions as described previously for the primers MLF2 + MLR2 except that the annealing temperature of 56 °C was used. After PCR amplification, a 20-µl aliquot of PCR product generated by the primer pair MLF2 + MLR2 from each isolate was digested by the endonuclease *BsmA I* under the conditions described above.

The same 37 isolates were also used to determine their sensitivities to benomyl in PDA plates amended with benomyl as described above. The results from this conventional bioassay method were compared with those from the allele-specific PCR and PCR-RFLP assays for detecting benzimidazole-resistant isolates of *M laxa*.

3 RESULTS

3.1 Sensitivity of *Monilinia laxa* to benomyl and thiophanate-methyl

After incubation at 24 °C for 7 days, 20 out of 41 isolates were growing on PDA amended with benomyl at 1 µg ml^{-1}, and these isolates were also growing on the plates with thiophanate-methyl at either 1 or 5 µg ml^{-1} but not at 50 µg ml^{-1}. None of these 20 isolates grew on PDA amended with benomyl at 5 or 50 µg ml^{-1}. Therefore, these 20 isolates were considered LR isolates (Table 1). Highly resistant isolates were not detected among the samples surveyed. The remaining 21 did not grow on PDA with 1 µg ml^{-1} benomyl, but 16 of them grew slowly (1–6 mm colony diameter) on PDA amended with 1 µg ml^{-1} thiophanate-methyl after 7 days of incubation.

3.2 Sensitivity of *Monilinia laxa* isolates to low and high temperatures

After incubation at various temperatures for 10 days, the 20 LR isolates grew poorly on PDA amended with 1 µg ml^{-1} benomyl at 28 °C, but they grew well at 8–24 °C (Fig 1). The remaining 21 S isolates did not grow on PDA amended with 1 µg ml^{-1} benomyl at any temperature. None of the 41 isolates tested grew on PDA with or without benomyl at 32 °C.

3.3 Partial sequences of the β-tubulin gene from *Monilinia laxa*

A single DNA fragment (1634-bp) was amplified with the primer pair TubA + TubR1 (Table 2) from each of the six (three S and three LR) isolates sequenced. The partial β-tubulin gene amplified with the primer pair TubA + TubR1 from *M laxa* contained six introns and seven exons. The sequence was submitted to GenBank (accession number AY349149). Deduced amino-acid sequences from S isolates 515, 537 and 992 were 398 residues in length and identical in sequence. The sequenced section of the β-tubulin gene included all positions known to affect the sensitivity to benzimidazoles in other fungi.[5,8,13] The deduced amino-acid sequence of the β-tubulin gene from S isolates of *M laxa* showed similarities of 99% to that of *M fructicola* (GenBank accession Number AAP40256) and *Botryotinia fuckeliana* (P53373), and 98% similarity to *Erysiphe pisi* (CAA57491). However, the DNA sequence of intron 6 of the β-tubulin gene of *M laxa*, in which the species-specific reverse primer

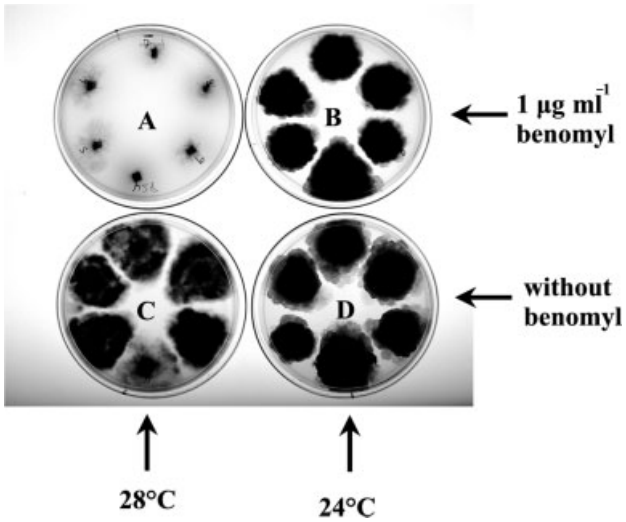

Figure 1. Temperature effects on mycelial growth of six benomyl low-resistant isolates of *Monilinia laxa* on potato dextrose agar plates amended with benomyl at 1 µg ml^{-1} (A and B), and without benomyl (C and D) at 28 °C (A and C) and 24 °C (B and D).

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                                    3'-AGACGGGCCGGTATCATATT-5':MLR2
                                    <-------------------
ML:  5'-TAAGTTTACTCTACTGTCTGCCCGGCCATAGTATAACTCGCATATACTGAC-TTTTTTTTACAGC
MF:     TAAGTTTACTCTACTGCCTTCTCGGTCATCATACAGCCCGCATATACTAATACTTTTTCTGCAGC
BC:     TAAGTTTGCCCTGTAATCAA-TCTGCCAAAAT----CTTGTAGAAACTAAC----TTTCTGTAGC
        ******* * **     *    * * **   *    *  * * * *** *     *** *  ***
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Figure 2. Alignment of DNA sequences of the intron 6 of β-tubulin genes from *Monilinia laxa* (*ML*, GenBank accession number AY349149), *Monilinia fructicola* (*MF*, AY283676), and *Botrytis cinerea* (*BC*, U27198). * Indicates identity of the nucleotide in the compared species. The arrow indicates the position of the primer MLR2 and the DNA sequence of this primer is present above the arrow.

MLR2 is located, had only 75 and 48% similarity to that of *M fructicola* and *B fuckeliana* (Fig 2), respectively. All three S isolates of *M laxa* had the sequence of CTC (leucine) at codon position 240 in the β-tubulin gene. All three LR isolates were identified as having codon 240 converted from CTC to TTC (phenylalanine). Apart from this mutation, the deduced amino-acid sequence of the partial β-tubulin gene was identical for the six isolates sequenced.

3.4 Detection of LR isolates of *Monilinia laxa* with allele-specific PCR and PCR-RFLP methods

The allele-specific primer pair MLRF + MLRR consistently amplified the expected 1112-bp DNA fragment from each of the 20 LR isolates tested, but not from any of the 21 S isolates and 34 isolates of other fungal species, including benzimidazole low- and high-resistant isolates of *M fructicola* (Table 1, Fig 3).

The species-specific primer pair MLF2 + MLR2 amplified a 376-bp DNA fragment from each of the 41 isolates of *M laxa* tested, but not from any of the 34 isolates of other fungal species (Table 1, Fig 4) obtained from stone fruit and almond.

The endonuclease *BsmA I* digested the 376-bp PCR product generated by the species-specific primer pair MLF2 + MLR2 from each of the three S isolates into two fragments 111 and 265 bp in length on agarose gels (Fig 5). In contrast, the enzyme did not digest the PCR products from three LR isolates (Fig 5). The same results were also observed for the remaining 18 S and 17 LR isolates listed in Table 1 (data not shown). When using DNA produced from the S and

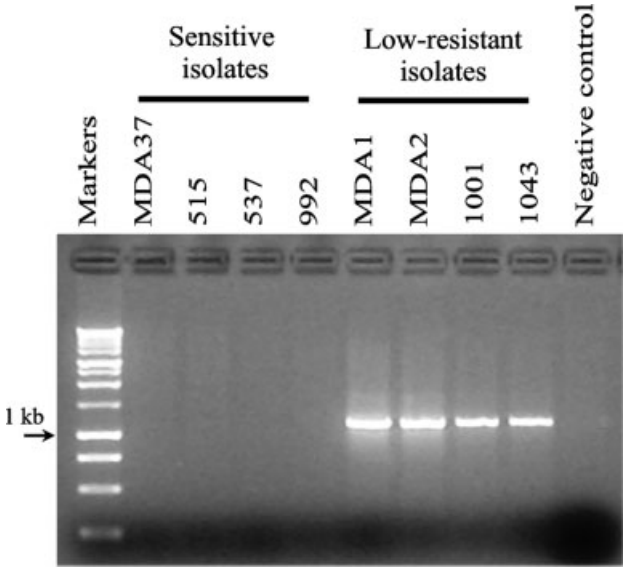

Figure 3. Specificity of the allele-specific PCR primer pair MLRF + MLRR for detection of benzimidazole low-resistant isolates of *Monilinia laxa*. Negative control is without template DNA. Markers are the 1-kb DNA ladders (Promega, Madison, WI).

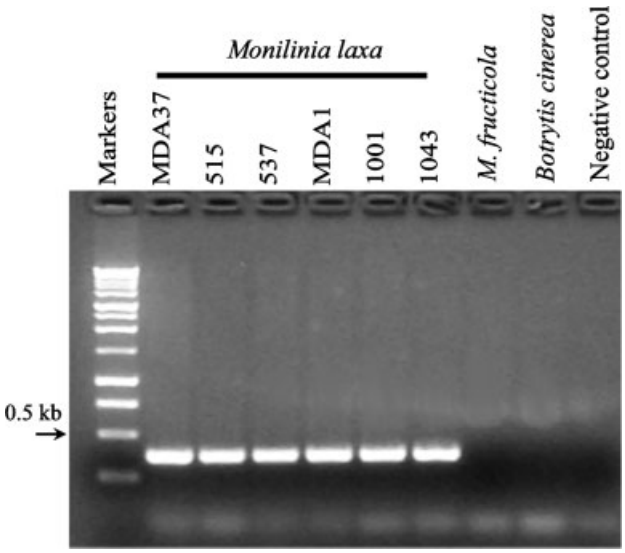

Figure 4. Specificity test for the PCR primer pair MLF2 + MLR2 in detecting *Monilinia laxa*. Negative control is without template DNA. Markers are the 1-kb DNA ladders (Promega, Madison, WI).

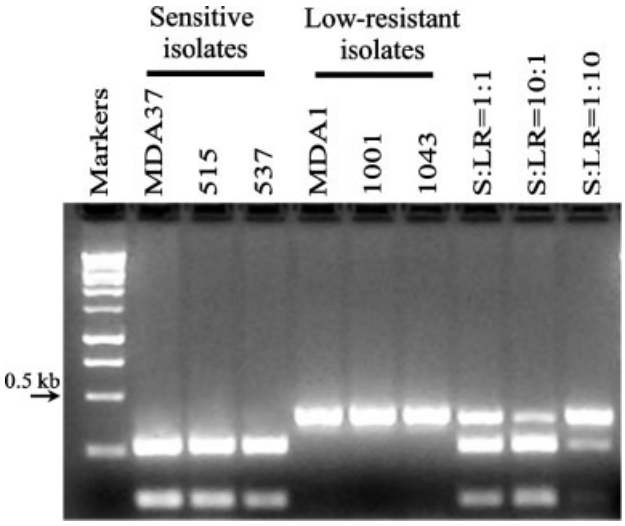

Figure 5. Restriction endonuclease *BsmA I* digestion of the 376-bp PCR products of the β-tubulin gene generated by the species-specific PCR primer pair MLF2 + MLR2 from benomyl-sensitive (S), low-resistant (LR), or from the mixtures of S and LR isolates of *Monilinia laxa* at various rates. Markers are the 1-kb DNA ladders (Promega, Madison, WI).

LR isolates mixed in the ratios of 1:1, 10:1 or 1:10, the digests showed different intensities in agarose gels (Fig 5).

The boiling-mycelial method generated amplifiable template DNA for PCR amplification. Using a 2-µl aliquot of supernatant obtained by boiling approximately 2 mg mycelia in 30 µl of 1 M Tris-HCl, the primer pair ITS1 + ITS4 and the species-specific primer MLF2 + MLR2 consistently amplified approximately 480-bp and 376-bp fragments, respectively, from each of the 37 *M laxa* isolates (not included in Table 1). Digestion of the PCR products generated by the primer pair MLF2 + MLR2 with the endonuclease *BsmA I* showed that 29 out of the 37 isolates were LR isolates. The allele-specific primer pair MLRF + MLRR also amplified the expected fragment (1112-bp) from these 29 isolates, but not from the remaining eight isolates. Bioassay results showed that these 29 LR isolates grew in PDA plates amended with benomyl at 1 µg ml^{-1} but not in those with 5 µg ml^{-1} benomyl after incubation at 24 °C for 1 week. None of remaining eight isolates grew in plates amended with benomyl at either 1 or 5 µg ml^{-1}.

4 DISCUSSION

In this study, we observed only one resistance level (low resistance) to benzimidazoles in *M laxa* isolates collected in California. However, both low and high-resistant isolates of *M fructicola*, which is closely related to *M laxa*, were observed in field isolates in California, and high-resistant (HR) isolates of *M fructicola* were able to grow in PDA plates amended with benomyl at 500 µg ml^{-1}[13,21] Results from inoculation tests showed that the full rate of thiophanate-methyl effectively controlled the disease caused by S isolates, but not the disease caused by either LR or HR isolates of *M fructicola*.[21] Analysis of the sequence of the β-tubulin gene showed that a single base pair mutation at codon position 240 was correlated to the low resistance level to benzimidazoles in *M laxa*. The mutation resulted in a change at codon 240 from CTC (leucine) in sensitive isolates to TTC (phenylalanine) in low-resistant isolates. This point mutation leading to benzimidazole resistance was also recently reported from three field benzimidazole-resistant isolates of *Tapesia yallundae*.[8] The LR isolates of *M laxa* in this study had different DNA fingerprinting profiles generated by microsatellite primers (data not shown), and these isolates were obtained in different years (from 1987 to 2002) at diverse locations in California, in some cases separated by more than 300 km. The results indicate this point mutation had occurred independently in *M laxa*, which is similar to the mutations in the β-tubulin genes occurring independently among different isolates of *M fructicola*.[13]

Positive cross-resistance is very common among benzimidazole fungicides. In this study, we found that all benomyl S and LR isolates were sensitive and resistant to thiophanate-methyl, respectively. However, the minimum inhibitory concentrations of *M laxa* to benomyl and to thiophanate-methyl were different. The S isolates of *M laxa* did not grow in PDA plates with 1 µg ml^{-1} benomyl; 76% of S isolates, however, grew slowly in plates with 1 µg ml^{-1} thiophanate-methyl. The 50% effective concentrations (ie the fungicide concentration at which fungal growth is reduced by 50%) of S and LR isolates of *M laxa* to benomyl were more than 10 times those to thiophanate-methyl (data not shown). The inhibitory differences of these two benzimidazole fungicides have also been found in other pathogens including *M fructicola*[13,21] and *Helminthosporium solani* Durieu & Mont,[22] perhaps because activity of thiophanate-methyl at a given concentration is less than that of benomyl.

In a previous study on resistance of *M fructicola* to benzimidazoles, we observed that the benzimidazole high-resistant isolates, having a mutation at codon 198 in the β-tubulin gene, were heat-sensitive and did not show high resistance to benomyl at 31 °C, whereas the LR isolates of *M fructicola*, having a mutation at codon 6, were cold-sensitive and did not show low resistance to benomyl at temperatures below 15 °C.[13] A laboratory-induced benomyl-resistant isolate of *F moniliforme* with a point mutation at codon position 50 also showed cold sensitivity. It is interesting that the LR isolates of *M laxa*, having the mutation at the codon 240, showed heat sensitivity, which is similar to that of HR isolates of *M fructicola*. The results suggest that resistant fungal isolates with mutations in the front part of β-tubulin gene (eg from codon 6 to 50) and in the middle part (eg from codon 198 to 240) might have cold and heat sensitivity, respectively. This hypothesis needs to be further confirmed with benzimidazole-resistant isolates of other fungal species.

Identification of *M laxa* and *M fructicola* generally relies on morphological and cultural characteristics. The identification method based on fungal morphology requires considerable expertise because some morphological criteria are overlapping among *Monilinia* spp.[23] Therefore, PCR-based diagnostic assays were developed for identification of *M fructicola* and *M laxa*. To date, several PCR primer pairs[24–28] are available to detect *M fructicola*, whereas only one primer pair, ITS1Mlx + ITS4Mlx, which was designed based on the DNA sequence of the nuclear ribosomal DNA internal transcribed spacers (ITS), has been developed for identification of *M laxa*.[23] In this study, based on the unique DNA sequence of the intron 6 of the β-tubulin gene from *M laxa*, we developed the species-specific PCR primers, which could be used not only for the detection of *M laxa* in California but also for the detection of benzimidazole low-resistant *M laxa* after PCR products are digested with *BsmA I*.

The current bioassay method takes at least one week to confirm benzimidazole resistance in *M laxa*. This study showed that the results from both the allele-specific PCR and PCR-RFLP assays were in agreement with those from the conventional bioassay

method in the detection of benzimidazole resistance in *M laxa*. Once as few as 2–5 mg fungal materials are available, the rapid allele-specific PCR method combined with the boiling-mycelial DNA extraction method can provide definitive diagnosis of LR isolates of *M laxa* within a few hours. The simple, cheap and rapid boiling-mycelial method, however, did not work with the detection of *M laxa* in infected plant tissues directly (data not shown), which may result from PCR inhibitors derived from plant tissues of stone fruits and almonds.[28,29] In several other studies, we found that the Qbiogene FastDNA kit (Carlsbad, CA) could be used to extract amplifiable template DNA from plant tissues for PCR diagnosis of phytopathogenic fungi or fungicide-resistant genotypes.[30–32] When a large number of samples need to be tested, however, commercial kits are expensive. In order to use the simple and cheap boiling-mycelial method for extracting DNA of *M laxa* from plant tissues, infected plant tissues need to be incubated for 2 to 3 days. Mycelia and spores growing on infected tissues can be harvested and used for the detection of benzimidazole resistance using the allele-PCR or PCR-RFLP method. Therefore, the rapid and reliable detection techniques would allow for the rational selection of fungicides to control brown rot and also permit a rapid method for evaluating the effectiveness of resistance management programs in stone fruit and almond orchards.

ACKNOWLEDGEMENT

This study was part of a research project funded by USDA/CAR Award No 2005-5/100-01990. The authors thank David P Morgan for technical assistance.

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