J. Phytopathology **154**, 321–328 (2006)

USDA, Agricultural Research Service, Crops Research Laboratory, Fort Collins, CO, USA

Analysis of β-tubulin Gene Fragments from Benzimidazole-sensitive and -tolerant *Cercospora beticola*

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Received May 24, 2005; accepted August 30, 2005

Keywords: Cercospora leaf spot, fungicide resistance, sugar beet, *Beta vulgaris*

Abstract

Cercospora leaf spot of sugar beet, caused by the fungus *Cercospora beticola*, is a major foliar pathogen on sugar beet. Fungicide sprays have been used extensively to manage Cercospora leaf spot, including the benzimidazole fungicides. Resistance to benzimidazoles has been observed in isolates of *C. beticola*. The precise genetics of this resistance is not known in this fungus. We tested benzimidazole-tolerant and -sensitive isolates and found a single mutation in the β-tubulin gene of benzimidazole-tolerant isolates that corresponds to a mutation known to confer benzimidazole tolerance in other ascomycetes. This mutation is predicted to cause a change from glutamic acid to alanine in the protein product. Isolates containing this mutation further show an increased sensitivity to an *N*-phenylcarbamate, as would be predicted based on the mutant phenotype found in other filamentous fungi. Only a single mutation was found in isolates from different regions of the United States, isolated in different growing seasons.

Introduction

Cercospora leaf spot of sugar beet (*Beta vulgaris* L.), caused by *Cercospora beticola* Sacc., is one of the most important foliar disease of sugar beets (Ruppel, 1986) and causes severe problems worldwide (Holtschulte, 2000). It causes reduced root and sugar yields (Smith and Ruppel, 1973; Shane and Teng, 1992) and reduced viability of roots in storage (Smith and Ruppel, 1971; Franc et al., 2001). The disease is managed by a combination of tillage, rotation, disease resistance and fungicide sprays (Smith and Martin, 1978; Franc et al., 2001). Benzimidazole fungicides have been used extensively in disease management, but tolerance to these fungicides has become a major problem in many beet-growing areas (Georgopoulos and Dovas, 1973; Percich et al., 1987; Campbell et al., 1998; Briere et al., 2001; Weiland and Halloin, 2001). Because early control of the disease is essential for disease management, growers must determine quickly whether, and to what extent, fungicide tolerance is present in the fungal population for effective fungicide use in management programmes.

The mechanism of tolerance to benzimidazole fungicides has been examined in a number of different filamentous fungi. Benzimidazoles act primarily by binding to fungal tubulin and interfering with mitosis and the fungal cytoskeleton (Davidse, 1986; Davidse 1988; Sisler 1988). Most often benzimidazole tolerance is due to mutations in the β-tubulin gene which reduce benzimidazole binding (Davidse and Flach, 1977; Jung et al., 1992; Cooley and Caten, 1993; Reijo et al., 1994). These mutations can be used to rapidly identify tolerant strains with nucleic acid-based methods (Luck and Gillings, 1995). Rapid identification of tolerant strains can aid in determining which fungicides to use in an area, and also can be used to monitor the effect of fungicide resistance management policies.

Some mutations conferring benzimidazole tolerance also confer a sensitivity to *N*-phenylcarbamates (Fujimura et al., 1992, 1994; Davidse and Ishii, 1995; Koenraadt and Jones, 1993). This sensitivity has been used in some areas to manage benzimidazole-tolerant fungal isolates (Yunis et al., 1991; Elad et al., 1995); however, use has been limited because combined resistance to both benzimidazoles and *N*-phenylcarbamates has been found (Elad et al., 1992; Josepovits et al., 1992).

The goals of this study were to isolate the β-tubulin gene from *C. beticola* and compare the sequence of benzimidazole-tolerant and -sensitive isolates to determine whether a mutation in this gene is responsible for tolerance. Only a single mutation conferring resistance was observed in all isolates; therefore we also examined sensitivity to *N*-phenylcarbamates to confirm that the mutation observed caused the phenotype in *C. beticola* that would be expected based on the response of other filamentous fungi.

Materials and Methods

Isolates used in this study

Of the 27 isolates (Table 1) primarily used in this study, 12 were obtained from USDA-ARS Sugarbeet Research Unit's *Cercospora* collection in Fort Collins, CO. These isolates were collected from sugar beet leaf lesions during the 1970s from various states in the USA, including AZ, CO, MD and TX; and one isolate, GR-1, was obtained from Greece. An additional isolate, CAH-90, was collected in CA in 1990. Isolates were single-spored to obtain pure cultures and maintained on potato dextrose agar (PDA; Becton and Dickinson Company, Franklin Lakes, NJ, USA) plates at 25°C. For long-term storage, cultures were maintained on PDA slants under sterile mineral oil at 4°C. The remaining 15 isolates were collected in 2003 from various states in the USA including CO, MT, NE and WY as described by Briere et al. (2001). Leaf collections were made during visits to productive sugar beet fields that were exhibiting Cercospora leaf spot symptoms. Although benzimidazoles fungicides were used in the past in the region, and may occasionally still be used, no effort was made to bias the collections towards fields with previous or current exposure to benzimidazoles.

Sensitivity of *C. beticola* to benzimidazole fungicides

Sterile molten PDA was cooled to approximately 48°C. A stock suspension 50 μg/ml of methyl benzimidazolecarbamate (MB; Benlate®, du Pont de Nemours and Company, Wilmington, DE, USA) was prepared in sterile distilled water. Appropriate volumes were added to achieve the concentrations listed below. About 15 ml of cooled, amended medium was dispensed into each Petri dish with the aid of an automatic dispensing unit. The poured plates were left to dry in a laminar flow hood for 24 h before use. The concentration of amended medium prepared was MB 5 μg/ml.

Each isolate tested on fungicide-amended media was subcultured from the stored source plate onto PDA, and incubated for 12–14 days at 23°C with a 12-h photoperiod. Conidial suspensions from each isolate were prepared by pipetting 1 ml of sterile distilled water on a 1×2 cm section of the colony. This area of the colony was lightly rubbed with a sterile glass rod to loosen conidia from the mycelia. For each isolate, non-amended PDA and MB-amended PDA plates were inoculated with three equally spaced 1.0 μl aliquots of the conidial suspension. *Cercospora beticola* strains with known insensitivity to MB (provided by J. J. Weiland, USDA, Fargo, ND) were used as insensitive or tolerant controls. Inoculated plates were incubated at 22°C with a 12-h photoperiod. The diameter of each colony was measured after 7 days with a digital caliper.

The percentage inhibition of each test isolate grown on each amended medium was determined by

Table 1
Cercospora beticola isolates used in this study

Isolate name	State of origin	Year isolated	Collector[a]	MB ED_{50}[b]	DFC ED_{50}[c]
13a	NE	2003	Franc	100	< 1
14b	WY	2003	Franc	> 150	< 1
16a	WY	2003	Franc	> 100	< 1
17b	MT	2003	Franc	100	< 1
1a	CO	2003	Franc	100	< 1
20c	CO	2003	Franc	100	< 1
5a	CO	2003	Franc	> 100	< 1
6b	NE	2003	Franc	100	< 1
AD-76	AZ	1976	SBRU	> 100	< 1
CA-90	CA	1990	SBRU	> 100	< 1
H1-12	TX	1973	SBRU	> 100	< 1
H5-12	TX	1973	SBRU	85	< 1
Td-5	TX	1976	SBRU	60	< 1
WY4.4	WY	2003	Franc	100	< 1
10c	NE	2003	Franc	< 0.1	> 100
27d	CO	2003	Franc	< 0.1	> 100
31d	CO	2003	Franc	< 0.1	> 100
4b	CO	2003	Franc	< 0.1	> 100
6c	NE	2003	Franc	< 0.1	> 100
C-3	CO	1976	SBRU	< 0.1	> 100
GR-1	Greece	1973	Georgopoulos	< 0.1	> 100
HC-10	TX	1973	SBRU	< 0.1	> 100
HC-13	TX	1973	SBRU	< 0.1	> 100
MD-1	MD	1970	SBRU	< 0.1	> 100
MD-2	MD	1970	SBRU	< 0.1	> 100
Tb-17	TX	1976	SBRU	4	70
WY2.2	WY	2003	Franc	< 0.1	> 100

[a]Collectors are the Sugar Beet Research Unit (SBRU) collection from E. G. Ruppel and the University of Wyoming collection from G. D. Franc (Franc). The isolate from Greece was provided to the SBRU collection by S. G. Georgopoulos.
[b]The effective dose of methyl benzimidazolecarbamate (MB) for 50% reduction in growth of *C. beticola*. All doses are as μg of compound per ml of media. Doses were calculated from the dose–response curve for each isolate.
[c]The effective dose of diethofencarb (DFC) for 50% reduction in growth of *C. beticola*. All values are as μg of compound per ml of media.

comparing its growth on non-amended PDA. The mean value for all three colonies for each treatment was calculated. The diameter of the initial inoculum drop on the plate was approximately 3 mm and this diameter was subtracted from the mean colony diameter for both the amended and non-amended control for each isolate before computing percentage inhibition. The percentage inhibition for each isolate was then calculated according to Bugbee (1995) with the following equation, [(Non-amended control – Amended/Non-amended control) × 100]. Colonies with diameters > 3 mm were considered insensitive or tolerant. All isolates were tested at least two times.

DNA extraction

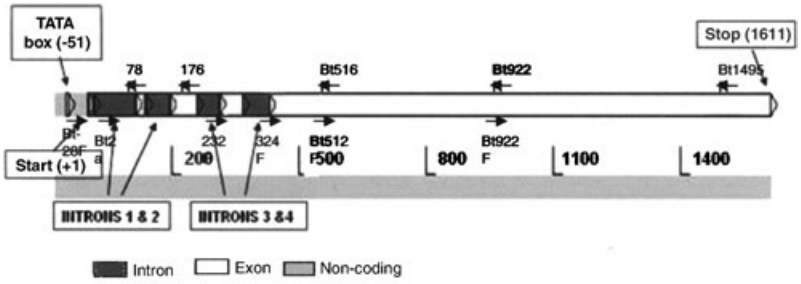
Cercospora beticola isolates were grown in 50 ml glucose-yeast extract-casein medium (GYEC; 15 g glucose, 3 g yeast extract and 5 g casein hydrolysate per l) at 29°C on a shaking incubator at 200 rpm for 4–5 days. Mycelia were rinsed with sterile distilled water over sterile cheesecloth and lyophilized for 48–72 h. The dried tissue was ground using a mixer mill (Retsch, Haan, Germany) and nucleic acids were extracted using the Easy DNA kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The nucleic acid pellet was suspended in 100 μl Tris-EDTA buffer (TE; Invitrogen Life Technologies) and digested with 40 μg/ml RNase (Invitrogen Life Technologies). Genomic DNA samples were subjected to gel electrophoresis on 0.9% agarose gels in 0.5X Tris-borate-EDTA buffer (TBE; Sambrook et al., 1989), and visualized using a UV transilluminator. DNA quantification was obtained by comparison of H33258 dye incorporation detected with a Hoefer DyNA Quant® 200 fluorometer (Amersham Biotech, Milano, Italy).

Polymerase chain reaction

All polymerase chain reactions (PCR) were performed in thermal cycler (Eppendorf AG, Hamburg, Germany) in either 50 μl or 25 μl volumes using 50 ng or 25 ng fungal genomic template and 0.15 μM or 0.3 μM of appropriate primers, respectively. A 0.2 mM concentration of each deoxynucleoside triphosphate, 3 mM of $MgCl_2$, 1X MBI reaction buffer (Fermentas, Inc., Hanover, MD, USA) and 1.25 U of *Taq* polymerase (Fermentas, Inc.) were used for each reaction. The following PCR run parameters were used; an initial preheating for 1 min at 94°C, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 62°C for 30 s and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. PCR products were separated by gel electrophoresis as described above. All primers used for DNA amplification are illustrated in Fig. 1.

Isolation of putative β-tubulin gene from *C. beticola*

PCR amplification of β-tubulin gene fragments were performed using generic primers for filamentous ascomycetes, Bt2a and Bt2b (Glass and Donaldson, 1995). PCR products were cloned into plasmid vectors using the TOPO TA Cloning Kit (Invitrogen Life Technologies). The primers, M13F and M13R (Integrated DNA Technologies, Coralville, IA, USA), were used in PCR analysis of recombinant plasmids. The fragments were sequenced on an automated sequencer (LI-COR Biotechnology, Lincoln, NE, USA) using labelled primers according to the manufacturer's protocol.



Primer	Sequence (5'-3')
Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC
Bt2b	ACC CTC CGT GTA GTG ACC CTT GGC
78R	CTG CTC CAC TTA TCC ATT GCG ATA TTC T
176R	TGG TGC TCT GCT CAA TGC GGG CGT ACC T
232F	TCC ACA GGC TTC CGG TAA CAA GTA TGT C
324F	GCC AGC TTT TCC GCC CAG ACA ACT TCG T
Bt-28F	ACA AAC AAC ACC GCC AAA ATT CC
Bt512F	**CCA GCT TTT CCG CCC AGA CAA C**
Bt516R	ACG AAG TTG TCT GGG CGG AAA AG
Bt922F	GCT TGC CGT GAA CAT GGT GCC GT
Bt922R	**ACG GCA CCA TGT TCA CGG CAA GC**
Bt1495R	TCG TTC ATG TTG GAC TCA GCC T

Fig. 1 *Cercospora beticola* β-tubulin gene sequences (AD-762, GenBank accession number AY856374 and C-3, GenBank accession number AY856373) with polymerase chain reaction primers and their location in the gene. The primers used for amplification of the 500 bp fragment for the majority of the isolates are in bold text

On the basis of the gene fragment sequence, new primers, 78R, 176R, 232F and 324F (Fig. 1), were designed to amplify the flanking sequences of the β-tubulin gene fragment by genome walking. Using the Clonetech Universal Genome Walker Kit (BD Biosciences, Palo Alto, CA, USA), a 2500 bp upstream fragment and 1800 and 1200 bp downstream fragments were amplified from two isolates, AD-762 (benomyl-tolerant, Ben^T) and C-3 (benomyl-sensitive, Ben^S). These fragments were purified using a gel extraction kit (Qiagen, Inc., Valencia, CA, USA), cloned into plasmid vectors (above), and PCR analysis of recombinants was performed as described above. The fragments were sequenced by Macrogen, Inc. (Seoul, Korea) using the primers, M13F and M13R. Sequences were edited and contigs assembled using SEQUENCHER 4.1 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Based on the entire putative gene sequence, the primers Bt-28F, Bt512F, Bt922F, Bt516R, Bt922R and Bt1495R (Fig. 1) were designed to amplify the gene and/or gene fragments from the remaining studied isolates in either two overlapping 1 kb fragments or in three 500 bp fragments for subsequent sequencing using the automated sequencing method described above. Sequence alignments were performed using the CLUSTALW algorithm.

Sensitivity of *C. beticola* to fungicides

In other filamentous fungi, amino acid substitutions at codon 198 have been shown to confer sensitivity to *N*-phenylcarbamate fungicides (Fujimura et al., 1992, 1994; Koenraadt and Jones, 1993). To confirm the phenotype from the observed genetic difference in the *C. beticola* β-tubulin, the 27 isolates for which DNA sequence information had been obtained (Table 1) were tested for sensitivity to the *N*-phenylcarbamate fungicide, diethofencarb (DFC). Fungicide sensitivity was determined in a radial growth assay, as described for MB sensitivity, except that a range of fungicide concentrations was used. Both MB and DFC were added separately to molten PDA at concentrations of 0.1, 1, 10 and 100 μg/ml. Conidial suspensions were placed on the plates and colony diameter determined after 7 days with digital calipers. In 2004, an additional 96 *C. beticola* isolates were collected from leaves with Cercospora leaf spot symptoms in CO, MT, NE and WY and tested for fungicide sensitivity on PDA amended with MB at 1 and 5 μg/ml or DFC at 5 and 50 μg/ml. Isolates were collected from fields with no regard to fungicide use.

Results

Sensitivity to benzimidazole in isolates of *C. beticola* from sugar beet leaves

All isolates examined grew well in the absence of fungicides. Isolates used in this study varied in their sensitivity to MB. Isolates classified as tolerant showed 80% or more growth on PDA with 5 μg/ml MB, compared with the growth on unamended PDA. When tested on a variety of MB concentrations, the effective dose for 50% reduction in growth (ED_{50}) calculated for the MB-tolerant isolates used in this study was 60 μg/ml or higher (Table 1).

Eleven of the 12 isolates classified as sensitive to MB (Table 1) showed $< 30\%$ of the colony diameter on amended media compared with unamended PDA. These isolates all had ED_{50} values of < 0.1 μg/ml MB. One isolate, Tb-17, while classified as MB-sensitive, showed measurably more growth on MB than the other sensitive isolates. This isolate had an ED_{50} of more than 1 μg/ml of MB, and complete growth inhibition at 10 μg/ml.

Isolation of putative β-tubulin gene from *C. beticola*

PCR amplification of genomic DNA from the Ben^S *C. beticola* isolate, C-3, using primers Bt2a and Bt2b generated two products (approximately 460 and 344 bp). Sequence analysis confirmed that the larger insert was a 460 bp putative β-tubulin gene fragment, which exhibited 99% DNA identity to the corresponding fragment in *C. beticola* strain Cb. (GenBank accession number AF146116) and 97% identity to the corresponding fragment in *Cercospora piaropi* strain 10 (accession number AF146097, Tessmann et al., 2001). The smaller 344 bp insert showed 98% identity to a partial β-tubulin sequence from *Fusarium oxysporum* (GenBank accession number AF433263.1), but was not pursued any further in this study.

After amplification and sequencing of genomic regions flanking the putative β-tubulin gene fragment, contig assembly resulted in 1890 bp and 2506 bp DNA sequences for AD-762 (GenBank accession number AY856374) and C-3 (GenBank accession number AY856373), respectively. An alignment of the two sequences showed 99.4% nucleotide identity between the two *C. beticola* isolates. Analysis of the consensus sequence by homology searches and previously described fungal splice site motifs (Shi and Perlin, 2001) revealed a 1611 bp putative β-tubulin gene sequence including a TATA box at -51, four introns, and five exons encoding a putative protein of 448 amino acids. The putative nucleotide consensus sequence showed high homology to β-tubulin genes from other ascomycetous fungi, including 78.9% identity to *Mycospharella pini* (accession number AF044975) and 73.2% identity to *M. graminicola* (accession number A4547264.1). The entire putative exon 5 or the last 1187 bp of the consensus sequence showed 100% identity to β-tubulin gene fragments from many ascomycetous fungi, including *M. pini*, *M. graminicola*, *Neurospora crassa* (accession number XM_323372.1), *Magnaporthe grisea* (accession number XM_368640) and *Gibberella zeae* (accession number A4303689). The predicted protein showed 91.7% identity to *M. pini* (accession number AAC02112.1) and 91.3% identity to *M. graminicola* (accession number AA555060.1).

Of the base pair differences between AD-762 and C-3, a point mutation was discovered in putative

exon 5 which is predicted to result in an amino acid substitution at predicted codon 198; GCG (alanine) in Ben^T AD-762 substituted for GAG (glutamic acid) in Ben^S C-3. Using the primers Bt-28F, Bt922R, Bt512F and Bt1495R, verification of 1448 bp within the whole gene sequence was achieved by amplifying and sequencing two overlapping 1 kb fragments in two additional *C. beticola* isolates, Ben^S HC-10 and Ben^T HI-12. An alignment of these two sequences showed 99.7% sequence identity. Sequence alignment showed high similarity between these two isolates and C-3 and AD-762, with 99.2% identity to the previously described consensus sequence generated from C-3 to AD-762. The consistently observed exception was at predicted codon 198, for which a GCG was observed in the Ben^T isolates (HI-12 and AD762) and GAG in the Ben^S isolates (Fig. 2).

Sequence analysis of β-tubulin gene fragments containing potential amino acid substitution

For the remaining 23 isolates in the primary study, only a 500 bp fragment was amplified and sequenced using primers Bt512F and Bt922R, in order to analyse the region in exon 5 containing predicted codon 198. All Ben^S isolates used in this study had the sequence GAG (Glu) at codon position 198, while all Ben^T isolates contained the amino acid substitution of GCG (Ala) at this position (Fig. 2).

Sensitivity to *N*-phenyl carbamate in isolates of *C. beticola*

All isolates tested that were tolerant to the benzimidazoles according to the criteria used showed an increased sensitivity to DFC (Table 1). All isolates classified as MB-tolerant had ED_{50} values of <1 μg/ml of DFC and had complete growth inhibition at 10 μg/ml (Fig. 3). MB-sensitive isolates had calculated ED_{50} values of 70 μg/ml or more. Eleven of the 12 MB-sensitive isolates had ED_{50} values of over 100 μg/ml, the highest concentration tested. Isolate Tb-17, which had a higher tolerance to MB than the other sensitive isolates, had an estimated ED_{50} of 70 μg/ml. With the 96 isolates collected from the field and tested in 2004, there was a strong negative correlation ($r = -0.971$) between growth on MB and growth on DFC (Fig. 4).

Discussion

Only a single mutation in the β-tubulin gene was consistently found in all MB-tolerant isolates examined, from different regions and different years. This mutation was observed at predicted codon 198. Mutations at codon 198 in the β-tubulin gene of a number of ascomycetes have been demonstrated to confer

Fungus	Type	No.	196197198199200201	Origin	Year	X
C. beticola	R	13a	TCCGACGCGACCTTCTGT	NE	2003	Ala
C. beticola	R	14b	TCCGACGCGACCTTCTGT	WY	2003	Ala
C. beticola	R	16a	TCCGACGCGACCTTCTGT	WY	2003	Ala
C. beticola	R	17b	TCCGACGCGACCTTCTGT	MT	2003	Ala
C. beticola	R	1a	TCCGACGCGACCTTCTGT	CO	2003	Ala
C. beticola	R	20c	TCCGACGCGACCTTCTGT	CO	2003	Ala
C. beticola	R	5a	TCCGACGCGACCTTCTGT	CO	2003	Ala
C. beticola	R	6b	TCCGACGCGACCTTCTGT	NE	2003	Ala
C. beticola	R	AD-76	TCCGACGCGACCTTCTGT	AZ	1976	Ala
C. beticola	R	CA-90	TCCGACGCGACCTTCTGT	CA	1990	Ala
C. beticola	R	H1-12	TCCGACGCGACCTTCTGT	TX	1973	Ala
C. beticola	R	H5-12	TCCGACGCGACCTTCTGT	TX	1973	Ala
C. beticola	R	Td-5	TCCGACGCGACCTTCTGT	TX	1976	Ala
C. beticola	R	WY4.4	TCCGACGCGACCTTCTGT	WY	2003	Ala
C. beticola	S	10c	TCCGACGAGACCTTCTGT	NE	2003	Glu
C. beticola	S	27d	TCCGACGAGACCTTCTGT	CO	2003	Glu
C. beticola	S	31d	TCCGACGAGACCTTCTGT	CO	2003	Glu
C. beticola	S	4b	TCCGACGAGACCTTCTGT	CO	2003	Glu
C. beticola	S	6c	TCCGACGAGACCTTCTGT	NE	2003	Glu
C. beticola	S	C-3	TCCGACGAGACCTTCTGT	CO	1976	Glu
C. beticola	S	GR-1	TCCGACGAGACCTTCTGT	Greece	1973	Glu
C. beticola	S	HC-10	TCCGACGAGACCTTCTGT	TX	1973	Glu
C. beticola	S	HC-13	TCCGACGAGACCTTCTGT	TX	1973	Glu
C. beticola	S	MD-1	TCCGACGAGACCTTCTGT	MD	1970	Glu
C. beticola	S	MD-2	TCCGACGAGACCTTCTGT	MD	1970	Glu
C. beticola	S	Tb-17	TCCGACGAGACCTTCTGT	TX	1976	Glu
C. beticola	S	WY2.2	TCCGACGAGACCTTCTGT	WY	2003	Glu
P. italicum	R		TCCGACAAGACTTCTGT			Lys
P. italicum	S		TCCGACGAGACTTCTGT			Glu
V. inaequalis	R		TCTGACAAGACATTCTGC			Lys
V. inaequalis	R		TCTGACGCGACATTCTGC			Ala
V. inaequalis	S		TCTGACGAGACATTCTGC			Glu
Predicted Amino acid			SerAsp X ThrPheCys			

Fig. 2 Sequence alignment for *Cercospora beticola* β-tubulin predicted codons 196–201 compared with sequence of other ascomycetous fungi. Other fungi are *Penicillium italicum* and *Venturia inaequalis*. All benzimidazole-tolerant isolates examined had the same single base pair change. Such a mutation in other fungi confers benzimidazole tolerance and diethofencarb sensitivity

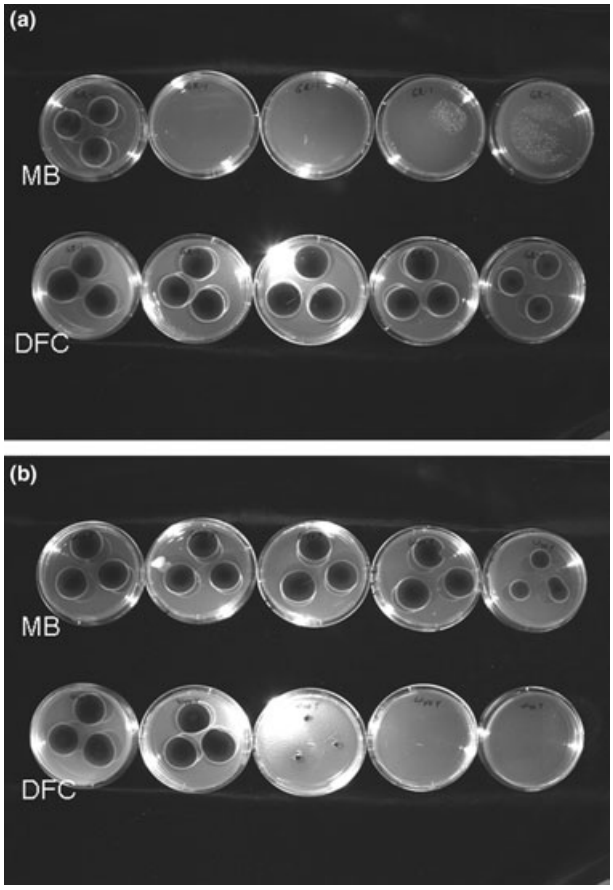


Fig. 3 Response of different *Cercospora beticola* isolates to fungicides. Plates shown have a range of fungicide concentrations (left to right: 0, 0.1, 1.0, 10 and 100 μg/ml), with methyl benzimidazolecarbamate (MB) on the top and diethofencarb (DFC) in the lower row. (a) Benzimidazole-sensitive isolate GR-1; (b) benzimidazole-tolerant isolate WY4.4

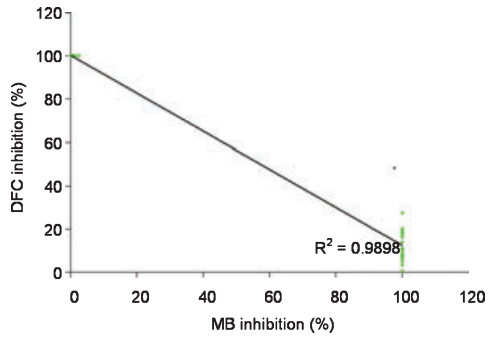


Fig. 4 Relative inhibition of growth of *Cercospora beticola* isolates on methyl benzimidazolecarbamate (MB) vs. diethofencarb (DFC). A strong negative correlation was observed

resistance or tolerance to benzimidazole fungicides (Fujimura et al., 1992; Luck and Gillings, 1995; Butters and Holloman, 1999). This is one of the codons most frequently reported altered in field isolates of ascomycetes with benzimidazole resistance, with changes at codon 200 also being reported (Koenraadt et al., 1992; Koenraadt and Jones, 1993; Butters and Holloman, 1999). Several different amino acid substitutions, including glycine, lysine, alaninine, and valine, have been reported at codon 198 in field isolates of various fungi (Koenraadt et al., 1992; Butters and Holloman, 1999). Only one of the several possible codon changes was found in all of the *C. beticola* isolates examined in this study.

As predicted based on the detected sequence for the MB-tolerant isolates, all isolates showed an increased sensitivity to the *N*-phenylcarbamate DFC. Because the mutation observed is predicted to confer such sensitivity (Fujimura et al., 1992, 1994; Koenraadt and Jones, 1993), and these results are consistent with the conclusion that the mutation described in this study confers the expected phenotype upon the *C. beticola* isolates examined; support for the role of the observed mutation in conferring the MB tolerance is strengthened. The observation that all isolates obtained from the field in 2004 showed this negative cross-resistance indicates that this cross-resistance can be used to look for changes in the benzimidazole-tolerant *C. beticola* population.

The sensitivity of MB-tolerant isolates to DFC might allow use of this or related compounds in managing MB-tolerant pathogens in the field. However, efforts to use *N*-phenylcarbamates to manage benzimidazole-tolerant fungi generally have not worked long term (Elad et al., 1992; Josepovits et al., 1992). Other mutations in the β-tubulin can arise that confer benzimidazole tolerance without *N*-phenylcarbamate sensitivity (Koenraadt and Jones, 1993; Fujimura et al., 1994). If *N*-phenylcarbamates are used, they should be used in combination with other materials attempt to manage for fungicide resistance and should not be used alone.

The differences between the response of one isolate classified as MB-sensitive, isolate Tb-17 and the other MB-sensitive isolates is of interest. The slightly elevated level of tolerance to MB determined in this isolate would be unlikely to have an obvious effect in the field, as the level of MB used to treat plants is higher than this (manufacturer's label). Such variability may indicate the presence of other isolates with varying tolerance levels. This isolate showed the same predicted amino acid sequence as all of the other MB-sensitive isolates over the area sequenced, an area that would be expected to cover several of the previously reported sites for mutations conferring benzimidazole tolerance (Fujimura et al., 1992; Jung et al., 1992; Koenraadt et al., 1992; Cooley and Caten, 1993). It is possible that this isolate might have a mutation at a different site in the β-tubulin gene, outside of the region sequenced. Alternatively, *C. beticola* may have a second β-tubulin gene. This is unlikely, because most filamentous fungi have only a single β-tubulin gene (reviewed in Ayliffe et al., 2001), and only one β-tubulin gene has been reported from the closely related *Mycosphaerella* spp. However, two β-tubulin genes have been reported in a few fungi (May et al., 1987; Panaccione and Hanau, 1990; Buhr and Dickman, 1994). In the initial tests, a second fragment, with high

identity to a putative β-tubulin gene sequence was found. This was not pursued further in the present study, but would be of interest to investigate further in the future. It is also possible that some other factor is causing this altered sensitivity to MB. The level of tolerance here is much lower than is usually reported in field isolates (Georgopoulos and Dovas, 1973; Campbell et al., 1998; Briere et al., 2001).

The high level of identity between the sequence from *C. beticola* and *Mycospharella* species is not unexpected. Other researchers have demonstrated that *Mycospharella* and *Cercospora* species are closely related and may be a single genus (Stewart et al., 1999; Goodwin et al., 2001). Our results showing the high identity in the β-tubulin gene between *C. beticola* and *M. pini* and *M. graminicola* agrees with the results of previous researchers observed for rDNA (Stewart et al., 1999) and ITS region (Goodwin et al., 2001) sequences.

Finding only a single mutation in MB-tolerant isolates from different years and geographic regions raises the possibility of developing a rapid screen to differentiate between MB-sensitive and MB-tolerant isolates in the field. Such screens have been examined for other fungi (Luck and Gillings, 1995). A rapid MB tolerance screen could be of use to growers in making informed management decisions, and to researchers examining the potential of different fungicide resistance management practices.

Acknowledgements

The authors would like to thank A. L. Hill, P. Reeves, R. M. Spence and W. L. Stump for valuable technical assistance. Partial funding for the collection and fungicide sensitivity testing of isolates was provided by Western Sugar Joint Research Committee. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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