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Silent Mutation: Characterization of its Potential as a Mechanism for Sterol 14 α -Demethylase Resistance in *Cercospora beticola* Field Isolates from the United States

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Abstract

Sterol demethylation inhibitors (DMIs) are considered among the most effective fungicides used to control Cercospora leaf spot (CLS), caused by *Cercospora beticola* Sacc., in sugar beet. Resistance to DMI fungicides has been reported in the *C. beticola* population from the United States, but the molecular mechanism is not known. It is considered that genetic changes in the *C. beticola* 14α-demethylase (*CbCyp51*) gene may be contributing to DMI resistance. The study investigated a silent mutation (GAG to GAA) at codon 170 as a potential mechanism for *C. beticola* DMI resistance. The *CbCyp51* gene was obtained from DMI-sensitive and -resistant isolates, cloned into a plasmid vector, transformed in an isogenic yeast R-1, and tested for DMI sensitivity. Transformed yeast showed low ED_{50} values (0.02 - 0.09 μg ml^{-1}) as compared to high ED_{50} values from *C. beticola* DMI-resistant isolates (21 - 65 μg ml^{-1}). The finding did not support our hypothesis that a silent mutation in the *CbCyp51* gene may be associated with *C. beticola* DMI resistance. Furthermore, genetic analysis of the *CbCyp51* gene found no mutation in 2 *C. beticola* DMI-resistant isolates from the Central High Plains. Further studies will be required to investigate additional mechanisms which have been associated with DMI resistance in fungi. Thus, we could not develop a molecular-based assay for the rapid detection of *C. beticola* DMI resistance, because no mutation was found in the *CbCyp51* gene. Currently, fungicide sensitivity assay could be the best method screen for *C. beticola* DMI resistance.

Keywords: Cercospora leaf spot; Silent mutation; DMIs; Sugar beet; *Beta vulgaris*

Introduction

Cercospora leaf spot (CLS) of sugar beet (*Beta vulgaris* L.), caused by *Cercospora beticola* Sacc., is the most destructive foliar disease worldwide [1,2]. Severe disease can cause a reduction in extractable sucrose, root yield, and increased concentration of impurities and leading to higher processing losses [3,4]. CLS can be controlled using integrated pest management (IPM) strategies which include planting resistant varieties, crop rotation, deep tillage, control of alternate host plants, or application of foliar fungicides [5-9]. The disease can be controlled by limiting canopy development through the regulation of water use in irrigated areas and nitrogen fertilization [6,9].

Sterol demethylation inhibitors (DMIs) are most widely used for CLS control in sugar beet [9-11]. DMIs protect plants against foliar diseases such as that caused by *C. beticola* [10,11], and members of this group have a medium- risk of resistance due to their single mode of action [12]. Resistance to DMIs has been associated with polygenic-controlled mechanism in which quantitative or additive interaction of several mutant genes can lead to a gradual shift in response to fungicides [9,11,12]. DMI resistance has been reported in fungi, and has been associated with multiple mechanisms [13-27]. DMI resistance in *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*) is due to single nucleotide polymorphic sites (SNPs) on the *Cyp51* gene which led to alterations of the protein [27,28]. In *Penicillium digitatum*, a 126 base pair (bp) insert found on the *Cyp51* gene was associated with DMI resistance [16]. Genetic analysis of the *P. digitatum Cyp51* gene found five tandem repeats on the 126 bp insert. Furthermore, the insert was present in the regulatory region and acted as a transcriptional enhancer, leading to overexpression of the *PdCyp51* gene [16]. DMI resistance has been associated with either uptake or efflux of DMIs which involved an adenosine triphosphate binding cassette (ABC) protein [14,15,29-31].

In *C. beticola,* DMI resistance was reported in field isolates from Greece in which four SNPs were found on the *CbCyp51* gene [32]. Two SNPs led to predicted amino acid changes at positions 297 (E297K) and 330 (I330T) and the mutations were present in *C. beticola* isolates that showed a moderate DMI resistance (MR) phenotype. The third SNP was predicted to lead to an amino acid substitution at position 384 (P384S) and was present in *C. beticola* isolate with a high DMI resistance (HR). The fourth SNP led to predicted synonymous mutation ('silent mutation') at codon 169 and was present on the *CbCyp51* gene in a few Greek *C. beticola* DMI-resistant isolates that showed an overexpression of the *Cyp51* gene. The study hypothesized that the silent mutation may likely be associated with overexpression of the *CbCyp51* gene leading to *C. beticola* DMI resistance [32]. Furthermore, it was indicated that a high ED_{50} value for tetraconazole (>1.0 μg ml^{-1}) was likely associated with overexpression of the *CbCyp51* gene, and was reported in *C. beticola* DMI-resistant isolates from the Red River Valley (RRV) region in the United States [33].

The research objective was to investigate a hypothesis that a silent mutation, on the *CbCyp51* gene, was potentially correlated with *C. beticola* DMI resistance. First, we analyzed the genetic changes on

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the *CbCyp51* gene for DMI-resistant and -sensitive isolates. Second, we investigated whether a silent mutation on the *CbCyp51* gene had a potential role in conferring *C. beticola* DMI resistance. This was achieved by transforming the *CbCyp51* gene from DMI-resistant and -sensitive isolates in an isogenic yeast R-1 (ΔPdr-5), and determined ED_{50} values (effective dose leading to growth inhibition of 50%) for DMI fungicides used for disease control in sugar beet. Transformed yeast R-1 was chosen because it was the fastest method to test the hypothesis as compared to using the *C. beticola,* which required a complex transformation protocol.

Materials and Methods

Fungal strains

We analyzed 8 *C. beticola* isolates which included 4 DMI-resistant isolates (RR-08-553, RR-08-760, RR-08-762, and RR-08-940), a DMI-sensitive isolate RR-08-418 from the Red River Valley (RRV) region of the United States; and 2 DMI-resistant isolates (UW11-60 and UW11-81) from the 2010 CLS survey in the Central High Plains (Colorado, Nebraska, Montana and Wyoming) of the United States. One Greek DMI-resistant isolate GR-10-292 [32] was included in the analysis and compared with isolates from the United States. An isogenic yeast R-1 strain (*Ura3*- and *His1*-) was transformed with the *Cyp51* gene from either *C. beticola* DMI-sensitive or *C. beticola* DMI-resistant isolates. The yeast R-1 was a knockout strain lacking a multidrug ABC transporter-Pdr5 [34]. The yeast R-1 strain particularly lacked the protein required for an efflux mechanism which has been associated with a multidrug resistance in fungi [14,15,35].

Fungicides and growth media

DMIs used for fungicide sensitivity assays included tetraconazole (Sipcam Agro USA Inc., Roswell, GA), propiconazole (Syngenta Crop Protection, Greensboro, NC), and difenoconazole (Syngenta Crop Protection). Transformed and non-transformed yeast R-1 strains were cultured on modified selective dropout medium (SM) plates [36]. The modified SM medium included 68 g of yeast nitrogen base (BD Diagnostics, Sparks, MD) and 200 g of ammonium sulfate (Spectrum Quality Products Inc., Gardena, CA). The medium was supplemented with 0.44 µg ml^{-1} of L-histidine (Sigma-Aldrich, St. Louis, MO) and amended with 300 µg ml^{-1} of Geneticin G-418 (Teknova, Hollister, CA). However, control plates were supplemented with 0.44 µg ml^{-1} of L-uracil (Sigma-Aldrich) because non-transformed yeast R-1 strains required L-uracil in order to grow on the modified SM medium [34].

DNA isolation

Mycelia on dry medium, preserved cryogenic vials (Corning Inc., NY) and frozen at -72°C were obtained using a sterile forcep (Fisher Scientific, Pittsburg, PA) to inoculate ~15 ml of nutrient broth (BD Diagnostics). Inoculated cultures were incubated at 27°C by shaking at 200 rpm on an orbital shaker (Lab-line orbital shaker, Romeoville, IL) for 7 days. Mycelia were harvested by straining the liquid medium through sterile cheesecloth, rinsed three times using sterile distilled water (5 ml), lyophilized for 48 h, and pulverized (~20 mg) under liquid nitrogen using a sterile mortar and pestle. We purified DNA using a plant mini kit (DNeasy®, Qiagen Inc., Valencia, CA) and measured absorbance at 260 nm using a spectrophotometer (Nanodrop®-1000, Wilmington, DE).

Sequencing and analysis of *Cyp51* gene from *C. beticola* isolates

Primer pairs (Table 1) were designed based on a consensus sequence of the *Cyp51* gene from *C. beticola* isolates for PCR amplification. PCR reactions (25 µl) included DNA (2 µl), each of the primers (0.4 µM), dNTPs (200 µM each), 1X of a high fidelity buffer with 1.5 mM $MgCl_2$ (Phusion®: ThermoFisher Scientific, Lafayette, CO), 0.4 units $µl^{-1}$ of a high fidelity DNA polymerase (Phusion®: ThermoFisher Scientific), and a final volume was adjusted by the addition of PCR grade water. PCR run parameters included an initial denaturation at 98°C for 30 s, 25 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s with a final extension at 72°C for 10 min. PCR products were separated by gel electrophoresis on a 0.8% agarose gel and 0.5X of Tris borate-EDTA buffer (EMD Chemicals, Gibbstown, NJ), stained with ethidium bromide (IBI Scientific, Peosta, IA), and visualized under UV (570-640nm) illumination (UVP LLC, Upland, CA).

An aliquot (5 µl) was obtained from each PCR product, treated with ExoSAP-IT® reagent (2 µl) (USB Corporation, Cleveland, OH) to remove unincorporated primer pairs and non-specific fragments. The reaction was incubated at 37°C for 15 min and inactivated at 80°C for 15 min. Cleaned PCR products were sequenced at the Sequetech Corporation (Mountain View, CA) using a set of primers that included Cbdm-519F, CBdm906F, and CBdm2284R (Table 1). Three overlapping sequences of the *Cyp51* gene were obtained that included a partial fragment of the promoter region, a complete open reading frame (ORF), and a partial fragment of the 3' untranslated region (3' UTR).

Characterization of *Cyp51* gene from *C. beticola* isolates

Partial sequences were assembled using the web version of CAP3 program and were analyzed using the DNA Baser Software (HeracleSoftware, Germany). The assembled sequences were analyzed for fidelity in which the predicted nucleotides were compared to chromatograms from sequenced data. An alignment was obtained for assembled partial sequences and a sequence from a *C. beticola* DMI-sensitive isolate (GenBank accession # HM778021) and was performed using the *MEGA* ver. 5 software [37]. We determined predicted amino acid residues for the *Cyp51* protein for each *C. beticola* isolate, and compared those residues with predicted amino acid residues for *C. beticola* DMI-sensitive isolate (GenBank accession # ADW54535) using the *MEGA* ver. 5 software [37]. This was performed to identify any potential amino acid changes which could be present on the *C. beticola Cyp51* protein.

Plasmid construction and yeast transformation

Partial fragments of the *C. beticola Cyp51* gene were obtained using a pair of primers: Cbdm-519F and Cbdm2284R and a nested PCR was performed using a second pair of primers: CbdmBamHIF and CbdmNotIR (Table 1), to obtain PCR products with an open reading frame (ORF) of the *C. beticola Cyp51* gene (~1625 bp). Standard cloning was performed and was followed by sequencing to confirm

Primer	Sequences (5' to 3')	References
Cbdm-519F	gttgtatgccgctttggagt	This work
Cbdm208F	gcatcgacccgtacaagttc	This work
Cbdm906F	agaggtggcacacatgatga	This work
Cbdm2284F	ttgcttcaatactggatgctt	This work
CbdmBamHIF	cgatggatccgttgtatgccgctttg	This work
CbdmNotIR	tttgcggccgcagtgtgtccaagg	This work
CbdmCyp51F	tgccacgcgacgagacattcaagatgagc	This work
CbdmCyp51R	cagctcctttgctgaccagaccgtagc	This work

Table 1: Primers used for PCR amplification of *Cyp*51 gene.

	Modified log-logistic model[1]					
C. beticola isolate	**ED_{50}**	**b**[2]	**c**[3]	**d**[4]	**e**[5]	**f**[6]
DMI-sensitive RR-08-418	0.96	0.81	2.34	7.86	0.005	55.53
	(1.2)[7]	(-0.2)	(-2)	(-2.1)	(-0.01)	(-53.8)
DMI-resistant RR-08-553	34.4	0.81	–4.91	15.15	10.62	24.07
	(-45.9)	(-0.7)	(-10.6)	(-1.5)	(-10.7)	(-26.8)
DMI-resistant RR-08-760	35.9	0.84	–4.28	13.67	11.19	22.49
	(-52.6)	(-0.9)	(-10.7)	(-1.6)	(-11.5)	(-26.8)
DMI-resistant RR-08-762	21	1.24	–1.55	14.14	10.77	16.22
	(-41.6)	(-2.7)	(-11.3)	(-1.6)	(-6.2)	(-21.1)
DMI-resistant RR-08-940	27.7	0.72	–5.04	13.67	4.24	40.68
	(-91.4)	(-1.3)	(-22.1)	(-1.5)	(-6.4)	(-76.3)
DMI-resistant GR-10-292	65.6	0.78	–5.39	9.43	9.49	37.82
	(-96.7)	(-0.6)	(-10.9)	(-1.5)	(-9)	(-26.1)

Table 2: Dose-response parameters for C. *beticola* isolates on potato dextrose agar amended with tetraconazole. Dose-response parameters were obtained using a four-parameter log-logistic model [45] and shown are model parameters and ED50 (medium effective dose reducing growth by 50%) for each C. *beticola* isolate on PDA amended with tetraconazole and compared with non-amended control.

[1]Modified log-logistic model (CRS.5) with fixed α = 0.25
[2] Steepness of the curve after the maximal hormetic effect
[3] Lower limit of the dose-response curve
[4] Upper limit of the dose-response curve
[5] Lower bound of the dose in which growth was reduced by 50%
[6] Denotes the theoretical upper bound of the hormetic effect
[7] Numbers in parentheses are standard errors

whether our insert which included the plasmid vector was present and determined its orientation [38]. Partial PCR products of the *Cyp51* gene were inserted between BamHI and NotI restriction sites of the pCM189-URA3 vector [39]. Plasmid constructs included a pCM189-URA3::mut*Cyp51* (*CbCyp51* from RRV DMI-resistant isolate), pCM189-URA3::mut*Cyp51* (*CbCyp51* from Greek DMI-resistant isolate), and pCM189-URA3::wt*Cyp51* (*CbCyp51* from DMI-sensitive isolate). Each construct was transformed in *E. coli* competent cells using a standard heat-shock procedure as described in a QIAgene expression kit (Qiagen, Inc.). Transformed *E. coli* competent cells were cultured on a Lauria-Bertani (LB) agar plates, supplemented with 100 µg ml^{-1} of filter-sterilized ampicillin sodium salt (Sigma-Aldrich, St. Louis, MO), and incubated at 30°C overnight. LB broth (5 µl) was inoculated using a single colony of *E. coli* competent cells and incubated at 30°C overnight by shaking at 200 rpm on an orbital shaker (Lab-line orbital shaker, Romeoville, IL). Plasmid was purified from overnight cultures using a Qiaprep® miniprep kit (Qiagen Inc.), and transformed in isogenic yeast R-1 strain [*MATα PDR1-3pdr5::KANMX4 ura3 his1 yor1 pdr10 pdr11 ycf1 pdr3*] [34] using a *S. cerevisiae* direct transformation kit (Wako Chemicals USA, Inc., Richmond, VA).

Detection of *C. beticola Cyp51* messenger RNA

Total RNA was extracted from overnight cultures from either transformed or non-transformed yeast R-1 strain using a yeast RNA kit (E.Z.N.A®: Omega Bio-Tek Inc., Norcross, GA). Total RNA concentration was determined by measuring the absorbance at 280 nm using a spectrophotometer (NanoDrop-1000®: Wilmington, DE). Purified total RNA (5 µl) was separated by gel electrophoresis in 1% agarose gel, 1X Tris-borate-EDTA buffer (EMD Chemicals) and visualized as described above. The total RNA was reverse-transcribed to cDNA using a qScript® cDNA synthesis kit (Quanta Biosciences Inc., Gathersburg, MD), which included random oligonucleotide primers. The cDNA was PCR-amplified using a pair of primers such as Cbdm*Cyp51*F and Cbdm*Cyp51*R (Table 1). These primer pairs spanned a region of ~425 base pairs that included targeted partial sequence of the *Cyp51* gene and a partial fragment of the plasmid vector. PCR products were cleaned using the ExoSAP-IT® reagent (2 µl) and sequenced at the University of Wyoming-Nucleic Acid Exploration Facility (UW-NAEF) in both directions using Cbdm*Cyp51*F and Cbdm*Cyp51*R (Table 1). Directional cloning of the *Cyp51* gene insert within the plasmid vector was confirmed by PCR amplification. The sequences were subjected to nucleotide-nucleotide BLAST search [40] and compared with other sequences in the NCBI/GenBank® database.

Determination of dose-response

To determine dose-response for *C. beticola* isolates, a fungicide sensitivity assay was performed on a potato dextrose agar (BD Diagnostics, Sparks, MD) using the modified procedure [41]. PDA was amended with each DMI fungicide prepared to a final concentration of 0, 0.001, 0.01, 0.1, 1, 10, and 100 µg ml^{-1} of the active ingredient and inoculated with *C. beticola* isolates prepared from sugar beet leaf extract agar (SBLEA) [42] plates. We grew yeast R-1 strain transformed with the *C. beticola Cyp51* gene overnight cultures (5 ml) in a yeast extract-peptone-dextrose (YPD) broth (BD Diagnostics). The YPD broth [36] was amended with 300 µg ml^{-1} of Geneticin G-418 as described above. The overnight cultures were incubated at 30°C by shaking on a 150 rpm orbital shaker (Lab-line orbital shaker) to obtain a cell density of ca. 1 x 10^6 cells ml^{-1}, which is equivalent to ~0.1 optical density (OD) at 600 nm [43]. Sterile tubes with YPD (5 ml) were either amended with DMI fungicide or non-amended controls, and each was inoculated with 0.5 x 10^5 ml^{-1} cells [43]. Additionally, each culture (5 ml) was amended with a DMI fungicide prepared to a final concentration such as 0, 0.01, 0.02, 0.04, 0.08, 0.1, 0.12, 0.16, and 0.32 µg ml^{-1}. Each amended YPD broth (5 ml) was inoculated either with transformed or non-transformed yeast R-1, and incubated at 30°C by shaking at 150 rpm on an orbital shaker (Lab-line orbital shaker) for 24 h. We measured optical density (OD) at 600 nm for cell cultures (200 µl) in 96-well plate.

Statistical analysis

Data analysis was performed using the language of R statistical computing environment [44] and estimated dose-response using a Cedergreen-Ritz-Streibig modified model [45]. Our null hypothesis was that a silent mutation at codon 170 on the *Cyp51* gene had a potential role in *C. beticola* DMI resistance. Hormesis model (CRS.5) was used because growth stimulation was observed at a low dose of tetraconazole. The model was a modified non-linear regression based on five parameters using a dose-response curve (*drc*) statistical add-on package [46,47]. Below was the expression that defined the relationship between response (y) and dose (x)

$$y = c + \frac{d - c + f\exp\left(\frac{1}{x^{\alpha}}\right)}{1 + \exp\{b[\ln(x) - \ln(e)]\}} \quad \text{(CRS.5)}$$

Parameters from the CRS.5 model were estimated at fixed alpha value (α=0.25). The dose-response estimated '***y***' which was the maximal response at zero dose, '***c***' was an estimate of lower limit of the dose-response curve, '***b***' estimated the steepness of the curve after the maximal hormetic effect, and '***e***' had no straightforward interpretation

in this model, but it estimated a lower bound on the ED_{50} value [45]. Whereas ('***d*** + ***f***') estimated upper bound on growth stimulation ('***f***'>0) whereby a larger value of '***f***' was correlated with increased growth stimulation (hormesis) as long as its value was positive. For instance, statistical analysis for hormesis was the same as testing for '***f***' = 0 based on fixed α value (0.25). ED_{50} value for each *C. beticola* isolate was estimated based on the modified log-logistic model [45,47] and results were classified as either low (<0.01 μg ml^{-1}), medium (0.01 to 1.0 μg ml^{-1}), or high (>1.0 μg ml^{-1}) [33]. Dose response-curves were fitted based on radial growth of *C. beticola* or optical density (OD) at 600 nm of yeast measurements.

Prediction of *C. beticola Cyp51* mRNA model structure

Putative secondary structures of the *Cyp51* mRNA of *C. beticola* were predicted based on the DNA sequences of a DMI-sensitive isolate CB6-80 (GenBank accession # HM778021) and a DMI-resistant isolate RR-08-940 (GenBank accession # HM778022) using the *RNAfold* program of the Vienna RNA Websuite (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) [48]. Determination of secondary RNA structure was performed using the *RNAfold* and was based on a comparative sequence analysis [23]. The algorithm constrains prediction of minimum free energy (MFE), and provides the best thermodynamic folding by taking into consideration factors such as base-pairing and unpaired regions of the sequence [49]. Additionally, the algorithm computes MFE on the assumption of a 'nearest neighbor model' achieved through the application of empirical estimates of thermodynamic parameters on neighboring interactions and loop entropies, and used as a mechanism for scoring folding structures [23,49].

Results

Fungicide sensitivity

Growth inhibition was determined for each *C. beticola* isolate from the Red River Valley (RRV) and Central High Plains of the United States and compared with that for a DMI-resistant isolate from Greece. Radial growth was measured from PDA plates amended with DMIs, inoculated with each *C. beticola* isolate, and compared with corresponding measurements obtained from a non-amended PDA control (Figure 1).

Results revealed that *C. beticola* DMI-sensitive isolate RR-08-418 had low ED_{50} on tetraconazole of 0.96 μg ml^{-1} compared to DMI-resistant isolates with ED_{50} values included 21.0 and 65.6 μg ml^{-1} (Table 3). In addition, all *C. beticola* isolates showed hormesis [46,50] at low concentrations of tetraconazole (Figure 2). No ED_{50} value was obtained for propiconazole and difenoconazole, because these DMIs were too effective against the tested *C. beticola* isolates at doses below 0.01 μg ml^{-1}.

Dose-response curve for a DMI-sensitive isolate RR-08-418 was compared with that for five DMI-resistant isolate RR-08-553, DMI-resistant isolate RR-08-760, DMI-resistant isolate RR-08-762, DMI-resistant isolate RR-08-940, and DMI-resistant isolate GR-10-292. Points included are radial growth measurements at a given dose of tetraconazole. The lines were fitted using the dose-response curve [47].

Sequence analysis of *Cyp51* gene from *C. beticola* isolates

Approximately 2.4 kb of the *C. beticola Cyp51* gene was obtained that included a partial sequence of the promoter as well as an entire coding region for each isolate using a set of four primers (Table 1). Mutations were identified from 4 *C. beticola* field isolates when compared to a baseline sequence of a DMI-sensitive isolate. First, 2 RRV *C. beticola* isolates RR-08-553 and RR-08-940 contained an identical SNP (silent mutation) and was predicted not to lead an amino acid change at codon 170 (Figure 3). Second, we identified two SNPs (silent mutations), predicted not to lead to an amino change at codon 355 for a DMI-resistant isolate RR-08-553 and codon 211 for a DMI-resistant isolate RR-08-760 (Table 3). Two non-identical SNPs were identified from the *C. beticola Cyp51* gene, in which one SNP was predicted to lead to amino acid substitution at position 12 (D12N) for a DMI-resistant isolate RR-08-762. A second SNP was predicted to lead to amino acid substitution at position 195 (P195A) for a DMI-resistant isolate RR-08-760. However, 2 *C. beticola* DMI-resistant isolates (UW11-60 and UW11-81) were identical to the baseline sequence of the *CbCyp51* gene obtained from a DMI-sensitive isolate RR-08-418 (Table 3).

Predicted *CbCyp51* Messenger RNA Structure of *C. beticola* Isolate

Predicted structure of the messenger RNA (mRNA) was determined using *RNAfold* based on the *C. beticola Cyp51* sequence of a DMI-sensitive isolate CB6-80 and a DMI-resistant isolate RR-08-940 (Figure 3). The putative mRNA structure for DMI-resistant isolate RR-08-940 with a silent mutation which did not lead to predicted amino acid substitution at position 170 had a slightly different folding pattern on the lower region as compared to that from DMI-sensitive isolate CB6-80 (Figure 4).

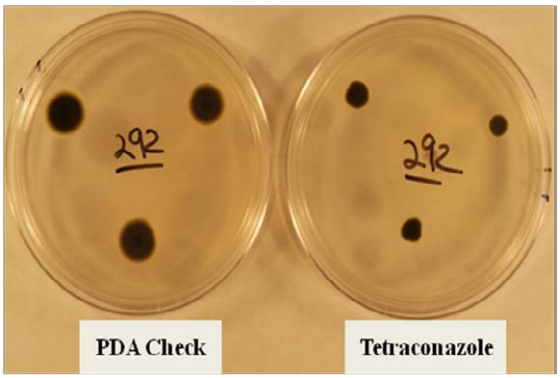

Figure 1: Radial growth obtained from a *C. beticola* DMI-resistant isolate GR-10-292. Radial growth from a non-amended potato dextrose agar (PDA) was compared with PDA amended with tetraconazole (1 μg ml^{-1}).

	C. beticola isolates	Origin of isolate	DMI Phenotype	Mutation(s)
1	RR-08-418	Red River Valley	Sensitive	baseline sequence
2	UW11-60	Central High Plains	Resistant	baseline sequence
3	UW11-81	Central High Plains	Resistant	baseline sequence
4	RR-08-553	Red River Valley	Resistant	GAG to GAA at 170 and AAG to AAA at 355 (silent mutations)
5	RR-08-760	Red River Valley	Resistant	CCT to GCT (P195A)
6	RR-08-762	Red River Valley	Resistant	GGC to GGG at 211 (silent mutation) and GAC to AAC (D12N)
7	RR-08-940	Red River Valley	Resistant	GAG to GAA at 170 (silent mutation)

Table 3: Single nucleotide polymorphic sites (SNPs) from partial sequences of the *Cyp51* gene for *C. beticola* isolates.

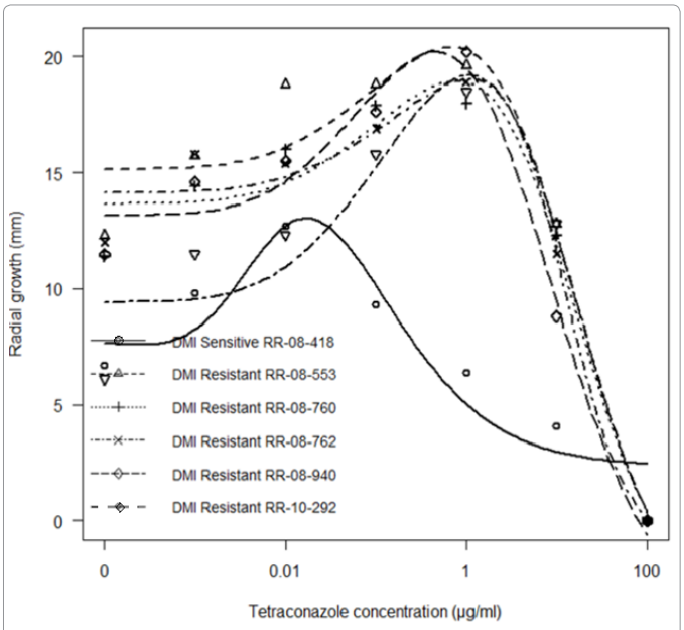

Figure 2: Dose-response curves for *C. beticola* DMI-sensitive and -resistant isolates on potato dextrose agar amended with tetraconazole.

The predicted mRNA folding structure from *C. beticola* DMI-resistant isolate RR-08-940 (GenBank accession # HM778022) showed a lower minimum free energy (mfe) of –565.92 kcal mol^{-1} as compared to a similar mfe of –567.82 kcal mol^{-1} from DMI-sensitive isolate CB6-80 (GenBank accession # HM778021).

Heterologous transcription of the *C. beticola Cyp51* messenger RNA in yeast

Results showed *C. beticola Cyp51* messenger RNA was produced in transformed yeast R-1 strain and was not found in control (Figure 5). PCR products were sequenced, aligned using ClustalW2 [22] and partial sequences showed a 100% identity (e-value = 0) to partial sequence of *C. beticola* DMI-sensitive isolate CB6-80 (GenBank accession # HM778021) or DMI-resistant isolate RR-08-940 (GenBank accession # HM778022).

Dose-response curves were estimated by measuring an optical density (OD) at 600 nm. Yeast R-1 strains included untransformed, transformed with plasmid, and transformed with *Cercospora beticola*: DMI-sensitive isolate RR-08-418 [Transformed Sensitive (418)], a DMI-resistant isolate RR-08-940 [Transformed Resistant (940)], and a DMI-resistant isolate GR-10-292 [Transformed Resistant (292)].

Non-transformed yeast R-1 strain, transformed with plasmid, and transformed with constructs with the *C. beticola Cyp51* gene from a DMI-sensitive isolate RR-08-418, a DMI-resistant RR-08-940, or DMI-resistant GR-10-292 showed medium ED_{50} values of between 0.02 and 0.09 µg ml^{-1} in culture amended with tetraconazole (Table 4). Furthermore, all transformed yeast R-1 strains showed hormesis (growth stimulation) [46,50] at low concentrations of tetraconazole (Figure 6). Growth stimulation was significant especially at low tetraconazole dose (one-sided t-test, $p<0.05$) for 3 yeast strains (Table 4). However, no response curve was obtained for propiconazole and difenoconazole because the two DMI fungicides were effective at all rates used.

Discussion

DMIs are used for CLS control in sugar beet [18] and resistance has been reported in *C. beticola* field isolates [11,18,33,51,52]. However, the mechanism of *C. beticola* DMI resistance is not known. DMI resistance

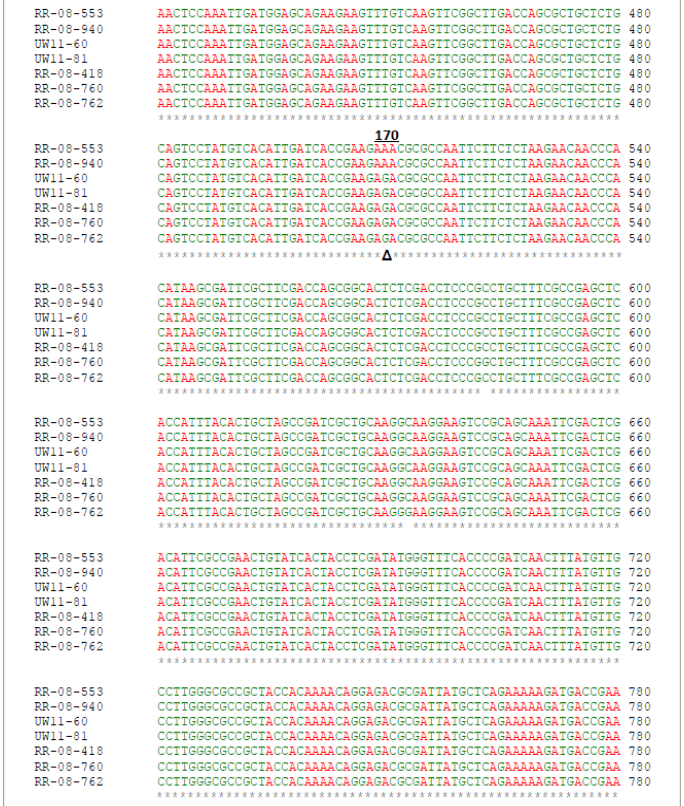

Figure 3: Alignment of partial sequences of the *Cercospora beticola Cyp51* gene. Shown are partial sequences from DMI-resistant (RR-08-553; RR-08-760; RR-08-762, RR-08-940, UW11-60; UW11-81); a point mutation (GAG to GAA) at codon 170 (**Δ**).

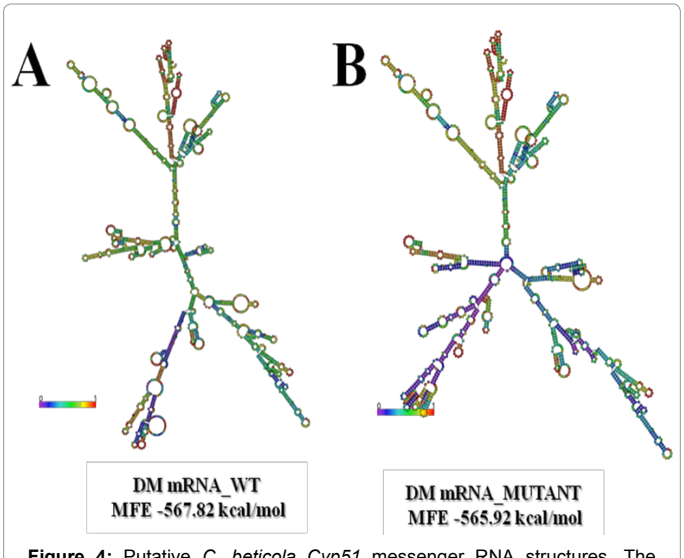

Figure 4: Putative *C. beticola Cyp51* messenger RNA structures. The putative mRNA structures were obtained for (**A**) DMI-sensitive isolate CB6-80 (WT) and (**B**) DMI-resistant isolate RR-08-940 (mutant).

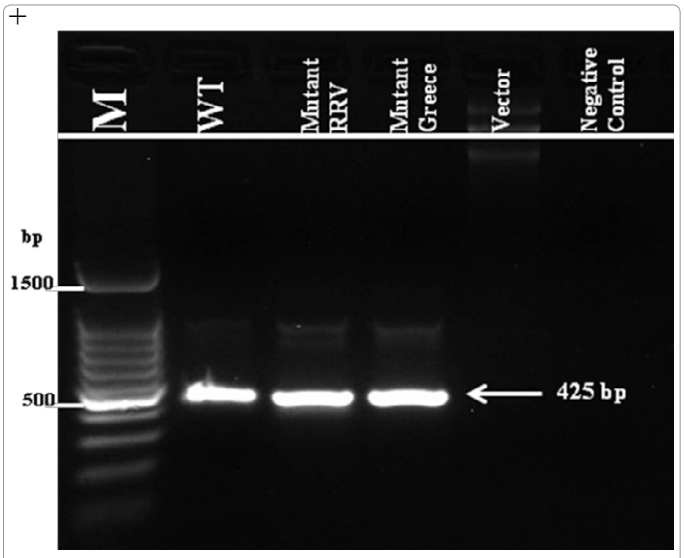

Figure 5: Transcripts from an isogenic yeast R-1 transformed with the *C. beticola Cyp51* gene. Shown are transcripts produced in yeast R-1 strain as well as PCR products (~425 bp each). Constructs included pCM189-URA3:*Cyp51* (RRV DMI-sensitive RR-08-418), pCM189-URA3:mut *Cyp51* (RRV DMI-resistant RR-08-940), and pCM189-URA3:mut*Cyp51* (Greek DMI-resistant GR-10-292). **M**: 100 bp DNA Marker (Promega).

has been associated with mutations predicted to lead to amino acid substitution on the *Cyp51* gene from other fungi [13-15,27,35]. Partial sequences of the *CbCyp51* gene were obtained from *C. beticola* isolates and analyzed for genetic changes. Genetic analysis found a few single nucleotide polymorphic sites (SNPs) within the coding region of the *CbCyp51* gene of five DMI-resistant isolates (Table 3). However, the genetic analysis found no single insertion sequence within the promoter region of *C beticola* DMI-resistant or -sensitive isolates as was reported for either *P. digitatum* DMI-resistant strains [16] or *Monilinia fruticola* DMI-resistant isolates [53]. Results indicated the SNPs identified from 5 DMI-resistant isolates were unlikely associated with *C. beticola* DMI resistance. This is because the genetic analysis found no mutations on the *CbCyp51* gene from 2 Central High Plains DMI-resistant isolates (Table 3). Similarly, a high degree of sequence variation was reported in the coding and the flanking regions for *C. beticola* isolates from the Red River Valley region in the United States, but none of those mutations could be associated with DMI-resistance because SNPs were present in isolates which showed both low and high ED_{50} values [33].

It was noted that among the genetic changes include a silent mutation in the *C. beticola* 14α-demethylase (*CbCyp51*) gene which may be associated with *C. beticola* DMI resistance. Our genetic analysis found a silent mutation (GAG to GAA) at codon 170 (Figure 3). An identical SNP was reported in a few DMI-resistant isolates from Greece [32] as well as two RRV DMI-resistant isolates. Hence, our study investigated whether the silent mutation at codon 170 had a potential role in conferring *C. beticola* DMI resistance. Putative mRNA structures of *C. beticola* DMI-sensitive isolate CB6-80 and *C. beticola* DMI-resistant isolate RR-08-940 were obtained using the *RNAfold*, (Figure 4). This was to determine whether the silent mutation at codon 170 may lead to changes in the *CbCyp51* mRNA structure which could be associated with *C. beticola* DMI resistance. Genetic mutations could potentially change mRNA structures leading to alteration in the amount of protein produced in cells. The *RNAfold* is considered to be a reliable program for finding and comparing hairpins free from pseudoknots (non-nested structural elements), in which, "the analysis determines similarities between centroid and minimum free energy structure as well as using base-pair distance ensemble between two predicted structures" [48]. The *RNAfold* program computes the energy contributions of elementary substructures (which support parts of a structure) leading to prediction of secondary structure associated with the total minimum free energy for each mRNA structure and it is based on the nearest-neighbor thermodynamic method [23,49]. The silent mutation at codon 170 on the *Cyp51* messenger RNA for *C. beticola* DMI-resistant isolate RR-08-940 led to a putative mRNA structure with a total minimum free energy of –565.92 kcal mol^{-1} as compared to –567.82 kcal mol^{-1}, which was obtained for a *C. beticola* DMI-

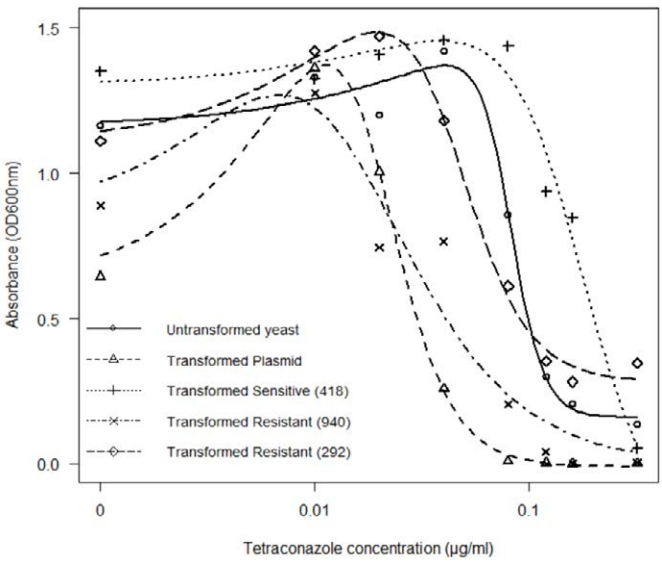

Figure 6: Dose-response curves for yeast R-1 strains in yeast extract-peptone-dextrose (YPD) culture treated with tetraconazole.

	Modified log-logistic model[1]					
Yeast strain	**ED_{50}**	**b[2]**	**c[3]**	**d[4]**	**e[5]**	**f[6]**
Untransformed yeast	0.04	5.76	0.15	1.17	0.08	2.07
	(0.004)[7]	(-1.8)	(-0.06)	(-0.06)	(-0.004)	(-0.96)
Transformed Plasmid	0.07	3.21	–0.01	0.64	0.02	20.66*
	(-0.007)	(-0.6)	(-0.04)	(-0.07)	(-0.002)	(4.06)
Transformed Sensitive (418)	0.05	2.51	–0.30	1.31	0.17	1.78
	(-0.007)	(-0.8)	(-0.36)	(-0.06)	(-0.029)	(-1.08)
Transformed Resistant (940)	0.02	1.65	–0.00	0.89	0.01	25.87*
	(-0.043)	(-0.2)	(-0.06)	(-0.07)	(0.006)	(-17)
Transformed Resistant (292)	0.09	2.89	0.28	1.12	0.04	6.93*
	(-0.005)	(-0.6)	(-0.06)	(-0.07)	(-0.005)	(-2.15)

Table 4: Dose-response parameters and ED50 (medium effective dose reducing growth by 50%) for non-transformed and transformed yeast R-1 strain in yeast extract-peptone-dextrose (YPD) broth amended with tetraconazole

The parameters were determined using a Cedergreen-Ritz-Streibig modified log-logistic model [45].

[1]Modified log-logistic model (CRS.5) with fixed α = 0.25 [45]

[2]Steepness of the curve after the maximal hormetic effect

[3]Lower limit of the dose-response curve

[4]Upper limit of the dose-response curve

[5]Lower bound on the dose at *d-c* reduced by 50%

[6]Denotes the theoretical upper bound of the hormetic effect

[7]Numbers in parentheses are standard errors

*Significantly different from zero ($p<0.05$)

sensitive isolate CB6-80 (Figure 4). Low folding free energy has been associated with low translation rates in addition to the occurrence of high transcript turnovers in *Saccharomyces cerevisiae* [54]. Our results found two different stable structures and were predicted to have similar free energies, although a minimum shift of –2.1 kcal mol^{-1} of predicted minimum free energy was observed between these two putative mRNA structures. The shift, however, was not significant to support our hypothesis that the silent mutation at codon 170 in the *CbCyp51* gene may likely be associated with *C. beticola* DMI resistance.

The genetic analysis of two RRV *C. beticola* DMI-resistant isolates found a few silent mutations on the coding region of the *Cyp51* gene (Table 3). This was determined using a partial fragment of the *CbCyp51* gene obtained from *C. beticola* DMI-sensitive RR-08-418 and DMI-resistant isolates (RR-08-940 and GR-10-292), cloned in a plasmid vector, and transformed into an isogenic yeast R-1 strain. Our results indicated that low concentrations of tetraconazole led to growth stimulation (hormetic response) of *C. beticola* isolates (Figure 2). Hormesis was first reported in yeast [55], and was later reported in fungi such as *Pythium aphanidermatum* mefonoxam-resistant strain, the causal organism of damping off disease in sugar beet, in which 10% growth stimulation was observed in culture amended with sub-lethal doses of mefonoxam [56]. A similar effect was reported for *Sclerotina homoeocarpa*, the causal organism of Dollar leaf spot in turf grass, in which growth stimulation was observed in culture amended with sub-lethal concentrations of trinexapac- ethyl [57]. However, we do not believe that hormesis may be associated with DMI resistance in *C. beticola* isolates because growth stimulation was mainly observed for low concentration of tetraconazole.

Results from heterologous transcription did not support our hypothesis that the silent mutation at codon 170 could be associated with *C. beticola* DMI resistance, because transformed yeast R-1 strains showed low ED_{50} values between 0.01 and 0.17 μg ml^{-1} (Table 2 and Figure 6). However, we determined that the messenger RNA was produced (Figure 5), which proved that the insert was being transcribed in the yeast R-1 strain. A similar silent mutation was reported from a few *C. beticola* DMI-resistant isolates from the Red River Valley (RRV) region in the United States, but it was noted that the silent mutation was unlikely associated with *C. beticola* DMI resistance [33]. The silent mutation, however, was found from three variants of *C. beticola* isolates that showed either low or high EC_{50} values, and it was suggested that the mutation was likely not associated with an overexpression in the *CbCyp51* gene [33]. The first incidence of DMI resistance was reported for two *C. beticola* isolates (UW11-60 and UW11-81) from the Central High Plains region, but the genetic analysis of the *CbCyp51* gene revealed that the isolates showed identical sequence to that of a DMI-sensitive isolate (RR-08-418). Hence, results did not support the hypothesis that the silent mutation at codon 170 was likely conferring *C. beticola* DMI resistance. Although propiconazole and difenoconazole were included in the study, no response curves were obtained because the fungicides were effective at low doses (<0.01 μg ml^{-1}) as compared to tetraconazole.

We could not develop a diagnostic assay for the rapid detection of *C. beticola* DMI resistance because the mechanism is not known. Hence, further studies will be required to investigate additional mechanisms which have been associated with DMI resistance in other fungi [14,15,35]. Currently, screening for *C. beticola* for DMI resistance could effectively be performed using fungicide sensitivity.

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