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 ED50 values (0.02 - 0.09 μg ml-1) as compared to high ED50 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 PitCyp51 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 (S384N) 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 ED50 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...
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 \( \Delta D_{\text{R}} \) 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 (UUW1-60 and UUW1-81) 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 His3-) 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 triadimefon (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 forceps (Heracel Scientific, Pittsburg, PA) to inoculate ~15 ml of nutrient broth (BD Diagnostics, Sparks, MD). 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 at 200 rpm on an orbital shaker (Lab-line orbital shaker, Romeoville, IL) for 7 days. Mycelia were harvested by straining the liquid medium at 200 rpm on an orbital shaker (Lab-line orbital shaker, Romeoville, IL) for 7 days. Mycelia were harvested by straining the liquid medium at 200 rpm on an orbital shaker (Lab-line orbital shaker, Romeoville, IL) for 7 days.

**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), 2X Taq buffer (2 µl), dNTPs (200 µM each), 1X of a high fidelity buffer with 1.5 mM Mg\(^{2+}\) (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).

**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 (HeracelSoftware, 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: CbdmBamHI and CbdmNotI (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
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 CbdrmCy5p51F and CbdrmCy5p51R (Table 1). The 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 CbdrmCy5p51F and CbdrmCy5p51R (Table 1). Directional cloning of the Cy5p1 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* Cy5p51 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 at 150 rpm on an orbital shaker (Lab-line orbital shaker) to obtain a cell density of ca. 1 x 10⁶ 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 ml of 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 Cy5p1 gene had a role in DMI resistance. Hormesis model (CRS.5) was used because growth stimulation was observed at a low dose of tetaconazole. The model was a modified non-linear regression based on five parameters using a dose-response curve (**y** = f (**x**)) using the modified procedure [41]. The peak of the dose-response was an estimate of lower limit of the dose-response curve, 'd' estimated the steepness of the curve after the maximal hormetic effect, and 'e' had no straightforward interpretation.

\[
\frac{d - c + f \exp\left(\frac{1}{x}\right)}{1 + \exp\left[H\left[\ln(x) - \ln(e)\right]\right]}
\]  

(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.

### Table 2: Dose-response parameters for *C. beticola* isolates on potato dextrose agar amended with tetaconazole

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ED₅₀</th>
<th>b'</th>
<th>c'</th>
<th>d'</th>
<th>e'</th>
<th>f'</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. beticola isolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMI-sensitive RR-08-418</td>
<td>0.96</td>
<td>0.81</td>
<td>2.34</td>
<td>7.86</td>
<td>0.005</td>
<td>55.53</td>
</tr>
<tr>
<td></td>
<td>(1.2)</td>
<td>(0.2)</td>
<td>(2)</td>
<td>(2.1)</td>
<td>(0.01)</td>
<td>(53.8)</td>
</tr>
<tr>
<td>DMI-resistant RR-08-553</td>
<td>34.4</td>
<td>0.81</td>
<td>-9.41</td>
<td>15.15</td>
<td>10.62</td>
<td>24.07</td>
</tr>
<tr>
<td></td>
<td>(-45.9)</td>
<td>(0.7)</td>
<td>(-1.5)</td>
<td>(-10.7)</td>
<td>(-26.8)</td>
<td></td>
</tr>
<tr>
<td>DMI-resistant RR-08-760</td>
<td>35.9</td>
<td>0.84</td>
<td>-4.28</td>
<td>13.67</td>
<td>11.19</td>
<td>22.49</td>
</tr>
<tr>
<td></td>
<td>(-52.6)</td>
<td>(0.9)</td>
<td>(-1.6)</td>
<td>(-11.5)</td>
<td>(-26.8)</td>
<td></td>
</tr>
<tr>
<td>DMI-resistant RR-08-762</td>
<td>21</td>
<td>1.24</td>
<td>-1.55</td>
<td>14.14</td>
<td>10.77</td>
<td>16.22</td>
</tr>
<tr>
<td></td>
<td>(-41.6)</td>
<td>(2.7)</td>
<td>(-1.6)</td>
<td>(-6.2)</td>
<td>(-21.1)</td>
<td></td>
</tr>
<tr>
<td>DMI-resistant RR-08-940</td>
<td>27.7</td>
<td>0.72</td>
<td>-5.04</td>
<td>13.67</td>
<td>4.24</td>
<td>40.68</td>
</tr>
<tr>
<td></td>
<td>(-91.4)</td>
<td>(-1.3)</td>
<td>(-22.1)</td>
<td>(-1.5)</td>
<td>(-6.4)</td>
<td>(-76.3)</td>
</tr>
<tr>
<td>DMI-resistant GR-10-292</td>
<td>65.6</td>
<td>0.78</td>
<td>-5.39</td>
<td>9.43</td>
<td>9.49</td>
<td>37.82</td>
</tr>
<tr>
<td></td>
<td>(-96.7)</td>
<td>(-0.6)</td>
<td>(-10.9)</td>
<td>(-1.5)</td>
<td>(-9)</td>
<td>(-26.1)</td>
</tr>
</tbody>
</table>

Modified log-logistic model

1. Modified log-logistic (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

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whether our insert which included the plasmid vector was present and determined its orientation [38]. Partial PCR products of the Cy5p1 gene were inserted between BamHI and NotI restriction sites of the pCM189-URA3 vector [39]. Plasmid constructs included pCM189-URA3::mutCy5p1 (CbCy5p1 from RVM DMI-resistant isolate), pCM189-URA3::mutCy5p1 (CbCy5p1 from Greek DMI-resistant isolate), and pCM189-URA3::wtCy5p1 (CbCy5p1 from DMI-sensitive isolate). Each construct was transformed in *E. coli* competent cells using a standard heat-shock procedure as described in a QIAexpression 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 ml) 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 Qiagen mini prep kit (Qiagen Inc.) and transformed in isogenic yeast R-1 strain [MATa PDR1 3Δpdr5::KANMX4 ura3 his1 yor1 pdr10 pdr11 ycf1 pdr3] [34] using a *S. cerevisiae* direct transformation kit (Wako Chemicals USA, Inc., Richmond, VA).
in this model, but it estimated a lower bound on the ED₅₀ value [45]. Whereas \((\bar{d} + f)\) 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 a fixed value \((0.25)\). ED₅₀ value for each C. beticola isolate was estimated based on the modified log-logistic model [45,47] and results were classified as either low \((<0.01 \mu g ml^{-1})\), medium \((0.01 to 1.0 \mu g ml^{-1})\), or high \((>1.0 \mu g ml^{-1})\) [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 Web suite (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 DMIIs, 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 ED₅₀ on tetracozole of 0.96 µg ml⁻¹ compared to DMI-resistant isolates with ED₅₀ 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 tetracozole (Figure 2). No ED₅₀ value was obtained for propiconazole and difenoconazole, because these DMIIs 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. Radial growth from a non-amended potato dextrose agar (PDA) was compared with PDA amended with tetracozole (1 µg ml⁻¹).

**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 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 acid 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).

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

Table 3: Single nucleotide polymorphic sites (SNPs) from partial sequences of the Cyp51 gene for C. beticola isolates.
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 ED₅₀ 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
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 ED₅₀ 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-resistant GR-10-292.

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

\[ \text{Yield of protein} = \frac{\text{ED}_{50}}{1 + \left( \frac{\text{dose}}{\text{ED}_{50}} \right)^{b}} \]

where \( a \) and \( b \) are parameters estimated from the data. The ED₅₀ was determined as the dose at which the yield of protein is 50% of the maximum yield. The parameters \( a \) and \( b \) were estimated using the modified log-logistic model, and the results are shown in Table 4. The model provides a good fit to the data, with an R² value of 0.98.

**Table 4:** Dose-response parameters and ED₅₀ for yeast R-1 strains in yeast extract-peptone-dextrose culture treated with tetracyanazole

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Modified log-logistic model¹</th>
<th>Yeast strain</th>
<th>Modified log-logistic model¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed</td>
<td>( ED_{50} \times 10^{-3} )</td>
<td>Untransformed</td>
<td>( ED_{50} \times 10^{-3} )</td>
</tr>
<tr>
<td>Transformed Plasmid</td>
<td>( ED_{50} \times 10^{-3} )</td>
<td>Transformed Plasmid</td>
<td>( ED_{50} \times 10^{-3} )</td>
</tr>
<tr>
<td>Transformed Sensitive</td>
<td>( ED_{50} \times 10^{-3} )</td>
<td>Transformed Sensitive</td>
<td>( ED_{50} \times 10^{-3} )</td>
</tr>
<tr>
<td>Transformed Resistant</td>
<td>( ED_{50} \times 10^{-3} )</td>
<td>Transformed Resistant</td>
<td>( ED_{50} \times 10^{-3} )</td>
</tr>
</tbody>
</table>

¹ Modified log-logistic model (CRS.5) with fixed \( a = 0.25 \) [45]
² Sleepiness 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
⁸ 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 \textit{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 \textit{CbCyp51} gene may likely be associated with \textit{C. beticola} DMI resistance.

The genetic analysis of two RRV \textit{C. beticola} DMI-resistant isolates found a few silent mutations on the coding region of the \textit{Cyp51} gene (Table 3). This was determined using a partial fragment of the \textit{CbCyp51} gene obtained from \textit{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 tetracanozol led to growth stimulation (hormetic response) of \textit{C. beticola} isolates (Figure 2). Hormesis was first reported in yeast [55], and was later reported in fungi such as \textit{Pythium aphanidermatum} methoxanox-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 \	extit{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 \textit{C. beticola} isolates because growth stimulation was mainly observed for low concentration of tetracanozol.

Results from heterologous transcription did not support our hypothesis that the silent mutation at codon 170 could be associated with \textit{C. beticola} DMI resistance, because transformed yeast R-1 strains showed low ED₅₀ 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 \textit{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 \textit{C. beticola} DMI resistance [33]. The silent mutation, however, was found from three variants of \textit{C. beticola} isolates that showed either low or high EC₅₀ values, and it was suggested that the mutation was likely not associated with an overexpression in the \textit{CbCyp51} gene [33]. The first incidence of DMI resistance was reported for two \textit{C. beticola} isolates (UW11-60 and UW11-81) from the Central High Plains region, but the genetic analysis of the \textit{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 \textit{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 tetracanozol.

We could not develop a diagnostic assay for the rapid detection of \textit{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 [14,15,33]. Currently, screening for \textit{C. beticola} for DMI resistance could effectively be performed using fungicide sensitivity.

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