

## PCR-BASED DETECTION OF FUNGICIDE RESISTANCE IN *CERCOSPORA BETICOLA*

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Leaf spot, caused by the fungus *Cercospora beticola*, is an endemic disease of sugarbeets in the North Dakota and Minnesota growing regions. Control measures against the fungus include resistant sugarbeet varieties and crop rotation, but the disease is managed effectively only when combined with timely fungicide applications. However, *C. beticola* is well-known for the ability to develop resistance to fungicides of several chemical classes.

Resistance to site-specific fungicides can be due to mutations in a single gene. In any given population, the frequency of individuals that harbor the mutation increases with the selection pressure that occurs as a result of extensive fungicide use. In *C. beticola*, the development of fungicide-resistance has been closely monitored, and results from sensitivity testing have shown that the North Dakota and Minnesota *C. beticola* population has the ability to adapt and become less sensitive to fungicides in relatively short periods of time (Secor *et al.*, 2008). By monitoring populations for fungicide sensitivity, fungicide recommendations for disease management and fungicide resistance management can be made.

Several of the fungicides used to manage leaf spot fall into two chemical classes. One of these classes is the sterol demethylation inhibitor (DMI) or triazole class that includes fungicides such as Eminent, Proline, Inspire, and Enable. DMI fungicides target an enzyme called 14 $\alpha$ -demethylase (CYP51). Resistance to DMI fungicides has been shown to relate either to specific mutations in the *CYP51* gene, which causes decreased affinity of DMIs to the target enzyme, or over-expression of the *CYP51* gene leading to an over-production of the target enzyme. The *CYP51* gene was recently isolated from *C. beticola* (Nikou *et al.*, 2009). In this study, the authors found that over-expression of the *CYP51* gene correlated with DMI-resistance. However, the study was carried out with very few field isolates that were collected from fields in Greece where fungicide formulations (epoxiconazole) and fungicide application programs are different than those used in North Dakota and Minnesota. Therefore, it is still unclear if mutations in the *CYP51* gene or *CYP51* over-expression will correlate with DMI resistance in Minnesota and North Dakota *C. beticola* isolates.

### **Objectives:**

The objectives for 2010 were to:

1. Clone the full-length *CYP51* gene.
2. Sequence the *CYP51* gene from 25 isolates resistant to DMIs. The same gene will also be sequenced from 25 isolates sensitive to DMI fungicides.
3. Compare the derived DNA sequences of resistant isolates to those from sensitive isolates to identify if specific mutations are associated with resistance.
4. If mutations correlate with fungicide resistance, develop PCR primers that specifically identify only resistant isolates.
5. Develop protocols for DNA extraction and PCR amplification from infected plant leaves to test the feasibility of using PCR to detect fungicide resistance.

### **Materials and Methods:**

#### **Cloning *CYP51* from *C. beticola***

The DNA walking kit (Seegene, USA) was used to clone the full-length *CYP51* gene using the previously cloned partial sequence obtained by Nikou *et al.*, 2009 as the starting point.

### ***CYP51* sequence from DMI resistant and DMI sensitive isolates**

Field isolates were assessed for fungicide sensitivity as described by Secor et al., (2008). Twenty-five isolates with EC50 values greater than 1.0 to both tetraconazole and prothioconazole were considered “DMI-resistant” and chosen for subsequent sequencing experiments. Likewise, twenty-five isolates with EC50 values less than 0.01 to both tetraconazole and prothioconazole were considered “DMI-sensitive.”

The derived ~2400 bp of *CYP51* sequenced was broken into three segments for easy PCR amplification of each segment. Therefore, three PCR products were obtained from each of 50 isolates. These PCR products were sequenced in both directions to generate over-lapping sequences for the entire gene. Gene sequences were analyzed in vector NTI (Invitrogen) to identify gene mutations.

### ***CYP51* gene expression**

To assess whether over-expression of *CYP51* is responsible for resistance to DMI fungicides, RNA was extracted from selected *in vitro* grown cultures and RT-PCR was conducted on the *CYP51* gene using primers designed from *CYP51* sequence data. Gene expression analysis was carried out using the delta delta Ct method.

## **RESULTS**

The full-length *CYP51* gene was cloned from *C. beticola*, including several hundred bp on both sides of the gene, for a total of ~2400 bp of derived sequenced. Primers were designed on this sequence to generate three PCR products for each isolate. The PCR products were sequenced in both directions and were overlapping to ensure that any mistakes during sequencing reactions would not go unnoticed.

The *CYP51* gene was sequenced in all 50 isolates. Several mutations were found in the promoter and coding sequence of the gene. However, in each case, mutations either could not be found in all resistant isolates or some sensitive isolates also harbored the mutation.

Gene expression of the *CYP51* gene was assessed in *in vitro* grown cultures. Resistant isolates had a two-to-three-fold increase in *CYP51* gene expression (not shown).

## **DISCUSSION**

The goal of this project was to develop a PCR-based approach to monitor for DMI-resistance in *C. beticola*. Since current strategies are laborious, time-intensive, and take some level of technical expertise to carry out, the development of a PCR-based approach would provide a distinct advantage to current approaches. However, the derived *CYP51* sequence data will not lend itself to a PCR-based approach because no mutations were found in all resistant isolates. It should be noted that *CYP51* genes are in gene-families. Sequence analysis of other *CYP51* genes may still provide an avenue for PCR-based detection of DMI resistance in *C. beticola*. Some clues as to the molecular basis of DMI resistance were provided by gene expression experiments that showed that DMI resistant isolates had enhanced *CYP51* gene expression. Although this finding if of scientific interest, assessing gene expression in field isolates is more involved than mutation analysis. Therefore, we currently feel that *CYP51* gene expression is also not worth pursuing in terms of PCR-based detection of DMI resistance.

## **LITERATURE CITED**

- Nikou, D., Malandrakis, A., Konstantakaki, M., Vontas, J., Markoglou, A. and Ziogas, B. (2009) Molecular characterization and detection of overexpressed C-14 alpha-demethylase-based DMI resistance in *Cercospora beticola* field isolates. *Pestic Biochem Physiol*, **95**, 18-27.
- Secor, G., Rivera, V. and Khan, M. (2008) Sensitivity of *Cercospora beticola* to foliar fungicides in 2008. *Sugarbeet Res Ext Rept*, **39**, 208-215.