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 14α-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 that 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**
