Project Title:

Molecular basis of fungicide resistance in *Cercospora beticola.*

Project Number/Description:

Continuation of a previously funded project.

Project Leader:

Melvin Bolton – Research Plant Pathologist
USDA Northern Crop Science Laboratory
Fargo, ND 58105-5677

(701) 239-1373
Melvin.Bolton@ars.usda.gov

Other Personnel Involved:

Ms. Rebecca Spanner (NDSU Department of Plant Pathology, Ph.D. candidate)
Ms. Subi Shrestha (USDA/NDSU, Post-doctoral scientist)
Mr. Jonathan Neubauer (USDA – ARS, Technician)
Dr. Gary Secor (NDSU Department of Plant Pathology, Professor)

Project Location:

The greenhouse, growth chambers, and laboratory to be used in the proposed project are located at the USDA Northern Crop Science Laboratory, Fargo.

Objectives:

As part of our ongoing work on fungicide resistance management, we are interested in studying the molecular basis of fungicide resistance in *Cercospora beticola*. **This project is critical for fungicide resistance management, maintaining fungicide efficacy, and developing tools that increase the accuracy and speed at which fungicide-resistant isolates are identified.** Our current focus is the molecular basis of resistance to triazole fungicides such as tetraconazole (Eminent, Minerva Duo), prothioconazole (Proline), and difenoconazole (Inspire). Additionally, we wish to test

three novel chemistries for their ability to provide synergy with Eminent under field conditions. Finally, we wish to test whether new strategies utilizing RNAi technology may be used for CLS disease management.

Therefore, the objectives for the 2018-2019 funding cycle are:

1. Identify and validate candidate genes from a genome wide association study on tetraconazole resistance.
2. Validate previously-identified chemosensitization agents using greenhouse and field inoculation studies.
3. Identify novel *C. beticola* gene targets and test efficacy of dsRNA directed towards these targets for CLS management.

Materials and Methods:

Objective 1:

Genome wide association studies (GWAS) identifies genetic variations in individuals to see if they are associated with a trait. GWAS has become a popular approach in human medical research to find genetic mutations associated with a particular disease. These studies have become possible because new technology has allowed high-quality sequencing to be available at a relatively low cost. We have developed a high-quality reference genome sequence of a triazole-sensitive *C. beticola* isolate. Last year we sequenced the genomes of 32 triazole-resistant strains and compared them against 32 triazole-sensitive strains. Using our developed GWAS pipeline, we identified a mutation

associated with tetraconazole resistance in a gene encoding a "Multifacilitator superfamily transporter" (Fig. 1).

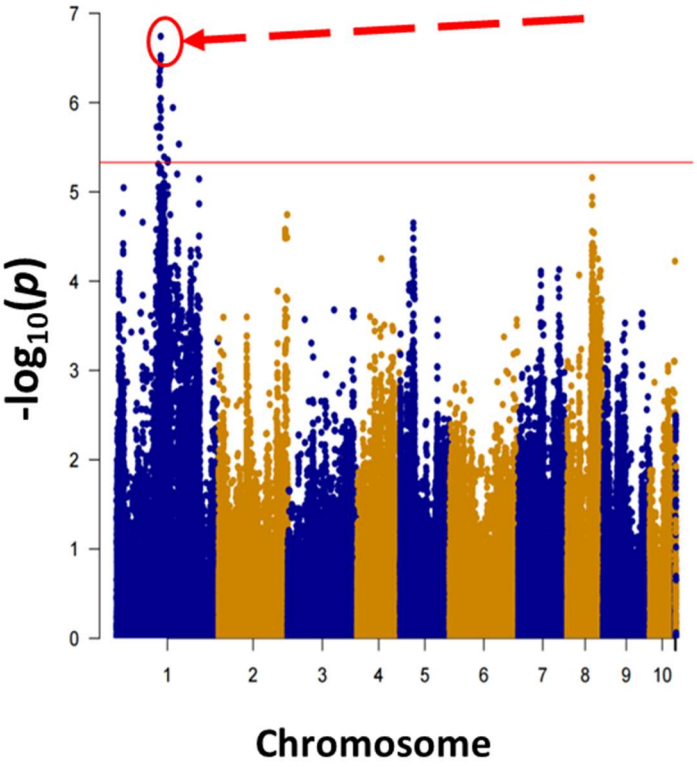

Figure 1. Manhattan plot for tetraconazole resistance (EC50 values) using a general linear model with three principal components. The false discovery rates adjusted to *p*-value cutoff of 0.05 (red line). Red arrow and circle indicates the mutation in CBET3_01079, a gene encoding a multifacilitator superfamily transporter.

Multifacilitator superfamily transporter genes are known as molecular pumps. We hypothesize that resistant strains have a unique mutation that makes them better equipped to "pump" the fungicide from the cell. Although our results suggest that we have identified genes associated with DMI resistance, **we need to validate these results by sequencing strains from wider geographical areas to ascertain whether the results are specific to the southern Red River Valley or are more general in nature**. Moreover, it is likely that DMI resistance in *C. beticola* is due to the effect of mutations in several genes. Consequently, we propose a new goal of sequencing 60 sensitive and 60

resistant strains. By nearly doubling our current genome inventory with strains collected from a wider geographical area, GWAS results will be validated and new genes may be identified. Additional strains have been partially collected or donated by the Secor lab (NDSU). Ph.D. candidate Rebecca Spanner will continue to carry out the computational analysis of genome sequence data to identify mutations associated with tetraconazole resistance.

Objective 2:

Chemosensitization agents (CAs) are compounds that have been developed in cancer research that render cancer cells more sensitive to chemotherapeutic drugs. In agricultural settings, CAs increase pathogen fungicide sensitivity. For example, addition of the CA thymol (2-isopropyl-5-methylphenol) together with azoxystrobin (Quadris) greatly enhanced azoxystrobin efficacy to inhibit growth of the plant pathogens *Stagonospora nodorum*, *Bipolaris sorokiniana*, *Phoma glomerata*, and *Alternaria* spp. (Dzhavakhiya et al., 2012). Fungicide-resistant strains of *Penicillium expansum* were rendered fungicide-sensitive when fungicides were augmented with CAs that target the fungal oxidative stress-response pathway (Kim et al., 2010). 2-hydroxy-4-methoxybenzaldehyde was shown to possess chemosensitizing ability by magnifying the efficacy of cell-wall targeting fungicides in *Aspergillus* and *Penicillium* species (Kim et al., 2015). More recently, bacterial lipopetides have been used to chemosensitize *Fusarium graminearum* to DMI fungicides in greenhouse and field studies for Fusarium head blight management (Kim et al., 2017). Since there are very few fungicides available for CLS, the identification of a CA that provides synergy with a labeled fungicide may be a means to increase fungicide efficacy while decreasing the risk for fungicide resistance development. Since triazoles may potentially be applied at a lower rate when tank-mixed with a CA, sugarbeet growers may save money while simultaneously managing fungicide resistance.

In the last funding cycle, we identified CAs that inhibit growth of the fungus *in vitro* and in greenhouse studies. Importantly, we identified a specific adjuvant that maximizes the chemosensitization activity of both CAs. In repeated greenhouse studies, the

combination of Eminent, adjuvant, and either CA resulted in significantly less disease that Eminent and adjuvant alone. In this fundying cycle, **we wish to test two CAs for their ability to manage CLS under field conditions**. Field inoculations of *C. beticola* and chemical applications will be carried out by Mr. Mike Mezger (Minn-Dak Famers Coop) with assistance from the Project Leader and his staff.

In addition, in the last three months we have identified another candidate chemisty for CLS disease management. In greenhouse studies, the addition of this chemistry (hereafter known as compound "G") to Eminent resulted in nearly no disease. However, we also tested compound G alone, which showed that this chemisty has fungicidal activity by itself. Consequently, it cannot be termed a CA since CAs are not fungicidal by themselves. However, compound G is a common food additive. Consequently, it is hypothesized that regulatory barriers for compound G may not be high since it is considered safe for human consumption. In this funding cycle, **we wish to test compound G for the ability to manage CLS under field conditions**.

Objective 3:

A new potential strategy to manage fungal diseases has emerged through the foliar application of double-stranded RNA (dsRNA) to crop plants to invoke a gene silencing mechanism called RNA interference (RNAi). For example, the wide host-range fungal pathogen *Botrytis cinerea* was shown to take up foliar-applied dsRNA, which subsequently caused silencing of the target gene and reduction of disease (Wang et al., 2016). Recently, spray application of barley leaves with dsRNA targeting *F. graminearum* Cyp51 coding genes was shown to inhibit growth of the pathogen (Koch et al., 2016). Consequently, spray-induced gene silencing (SIGS) technology may be a new strategy for crop protection against fungal pathogens (Wang and Jin, 2017). A major benefit of SIGS is the easy design of fungal targets. Plant pathogens readily evolve resistance to both fungicides and host resistance genes. However, novel fungicide chemistries are slow to reach the marketplace and incorporating resistance into crop plants can take several years. In contrast, dsRNA targets can be developed very quickly and in response to evolving pathogen populations. However, the limited stability of

dsRNA under environmental conditions carries risk that dsRNA will break down on the plant surface before the signals have been delivered to the pathogen. However, loading dsRNA into clay "nanosheets" has been shown to promote the uptake of dsRNA into plant cells and protect dsRNA from degradation for up to 30 days (Mitter et al., 2017).

We have initiated studies to consider the possibility that dsRNA may be used to silence *C. beticola* genes and therefore may be a promising technique for disease management. In earlier work from our lab, we identified genes in DMI-resistant *C. beticola* strains that were induced in response to tetraconazole (Bolton et al., 2016). We chose three genes from that study and produced corresponding dsRNA, which were tested for their ability to limit the growth of a DMI-resistant *C. beticola* strain *in vitro* (Fig. 2).

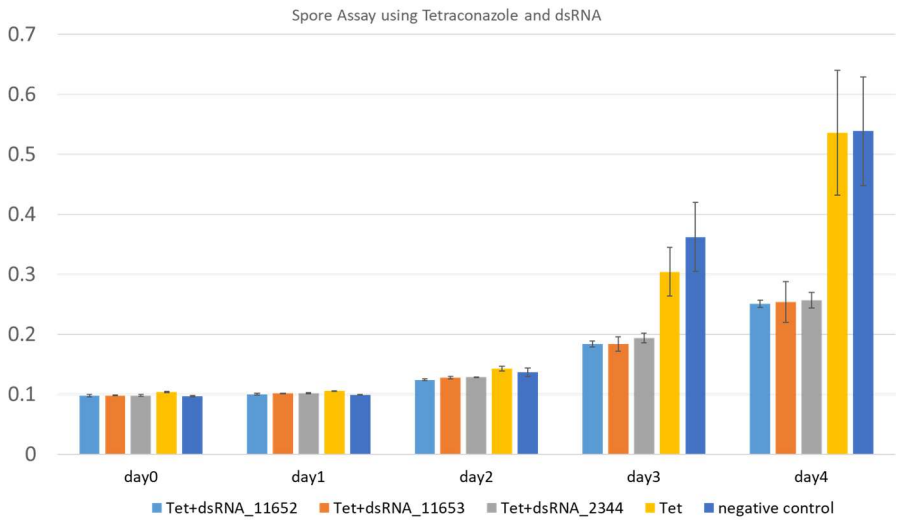

Figure 2. Growth of DMI-resistant *C. beticola* in the presence of dsRNA and tetraconazole *in vitro*. After four days, the DMI-resistant *C. beticola* strain grows equally well in the presence (yellow) or absence (dark blue) of tetraconazole. However, wells which contained tetraconazole and dsRNA targeting three different *C. beticola* genes (light blue, orange, gray) had a significant reduction in growth.

In this funding cycle, **we wish to identify and test additional targets for *C. beticola* gene silencing**. Those targets that result in the most significant decrease in *C. beticola* growth will then be tested in greenhouse SIGS assays to ascertain whether foliar application of dsRNA to sugarbeet leaves can silence *C. beticola* genes and consequently, reduce growth and disease.

Literature Cited

Bolton, M.D., Ebert, M.K., Faino, L., Rivera-Varas, V., de Jonge, R., van de Peer, Y., Thomma, B.P.J.J., and Secor, G.A. RNA-sequencing of *Cercospora beticola* DMI-sensitive and -resistant isolates after treatment with tetraconzole identifies common and contrasting pathway induction. Fungal Genetics and Biology 92: 1-13.

Dzhavakhiya, V., Shcherbakova, L., Semina, Y., Zhemchuzhina, N., and Campbell, B. 2012. Chemosensitization of plant pathogenic fungi to agricultural fungicides. Frontiers in Microbiology 3: 1-9.

Kim, J.H., Campbell, B.C., Mahoney, N., Chan, K.L., Molyneux, R.J., and Xiao, C.L. 2010. Use of chemosensitization to overcome fludioxonil resistance in *Penicillium expansum*. Letters in Applied Microbiology 51: 177-183.

Kim, J.J., Chan, K.L., and Mahoney, N. 2015. Augmenting the activity of monoterpenoid phenols against fungal pathogens using 2-hydroxy-4-methoxybenzaldehyde that target cell wall integrity. International Journal of Molecular Sciences 16: 26850-26870.

Kim, K., et al., 2017. Chemosensitization of *Fusarium graminearum* to chemical fungicides using cyclic lipopeptides produced by *Bacillus amyloliquefaciens* Strain JCK-12. Frontiers in Plant Science 8: 2010.

Koch, A. et al., 2016. An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. PLoS Pathogens 12: e1005901.

Wang, M. and Jin, J. 2017. Spray-induced gene silencing: a powerful innovative strategy for crop protection. Trends in Microbiology 25: 4-6.

Wang, M., Weiberg, A., Lin, F-M., Thomma, B.P.H.J., Huang, H-D., and Jin, H. 2016. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nature Plants 2: 16151.

Time Line of Anticipated Accomplishments:

It is anticipated that the objectives outlined above will be met in the 2018-2019 funding cycle.

Progress Toward Past Objectives:

With financial support from the Sugarbeet Research and Education Board of Minnesota and North Dakota, we have accomplished the following (most recent first):

- Developed a LAMP-based methodology for quick and easy identification of strobilurin-resistant strains of *C. beticola*. This may allow for in-field identification of strobilurin-resistant strains by agriculturists.
- Performed RNA-sequencing on triazole-resistant and -sensitive strains after exposure to tetraconazole or control, identified genes differentially expressed in both strains, developed a candidate gene list involved with triazole resistance.
    - Bolton, MD, Ebert, MK, Faino, L, Rivera-Varas, V, de Jonge, R, Van de Peer, Y, Thomma, BPHJ, Secor GA. (2016) RNA-sequencing of *Cercospora beticola* DMI-sensitive and -resistant isolates after treatment with tetraconazole identifies common and contrasting pathway induction. Fungal Genetics and Biology 92: 1-13.
- Developed a fast DNA isolation protocol using leaf spots harvested from infected leaves (unpublished). Together with real-time PCR technique mentioned above, screened nearly 3,000 isolates collected from the Red River Valley for strobilurin resistance and approximately 100 isolates for benzimidazole resistance.
- Identified the first strobilurin-resistant field isolates of *C. beticola* in the Red River Valley.
    - Bolton, MD, Rivera-Varas, V, Secor, GA, Cattanach, AW, Metzger, MS. (2013) Identification of the G143A mutation in cytochrome *b* associated with QoI resistance in *Cercospora beticola* isolates from the Red River Valley. Plant Health Progress doi:10.1094/PHP-2013-0812-02-RS.

- Identified the first strobilurin-resistant field isolates of *C. beticola* in the United States, which were found in Michigan. Developed an extremely sensitive real-time PCR technique to identify resistant isolates, which decreases the amount of time to identify strobilurin resistance from two weeks to 4 hours. Confirmed that resistance developed nearly simultaneously in several areas of Michigan.
    - Bolton, MD, Rivera-Varas, V, and Secor, GA. (2013) Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. Pest Management Science 69: 35-39.
- Identified the first strobilurin-resistant field isolates of *C. beticola*, which were found in Italy. Cloned the gene encoding cytochrome b, which is targeted by strobilurin fungicides and identified the G143A mutation in resistant isolates. Developed a PCR-based protocol to identify resistant isolates.
    - Birla, K, Rivera-Varas, V, Secor, GA, Khan, MFR, and Bolton, MD. (2012) Characterization of *cytochrome b* from European field isolates of *Cercospora beticola* with quinone outside inhibitor resistance. European Journal of Plant Pathology 134: 475-488.
- Showed that the *Cyp51* gene is over-expressed in triazole-resistant isolates, setting the foundation for PCR-based detection of resistance in *C. beticola*.
    - Bolton, MD, Birla, K, Rivera-Varas, V, Rudoloph, KD, and Secor, GA. (2012) Characterization of *CbCyp51* from field isolates of *Cercospora beticola*. Phytopathology 102: 298-305.
- Showed that triazole-resistant isolates are as aggressive as triazole-sensitive isolates in the absence of a fungicide application. This is important for fungicide resistance management because it suggests that triazole-resistant isolates do not have a fitness penalty and therefore may not die out in the field once established. Also provided evidence that cross-resistance occurs between some triazole fungicides (tetraconazole, prothioconazole, and difenoconazole). Confirmed that EC-50 values identified in the laboratory relate well with fungicide efficacy in the greenhouse.
    - Bolton, MD, Rivera-Varas, V, del Rio, LE, Khan, MFR, and Secor, GA. (2012) Efficacy of variable tetraconazole rates against *Cercospora beticola* isolates with differing *in vitro* sensitivities to DMI fungicides. Plant Disease 96: 1749-1756.

Project Proposal Budget:

Labor:	$26,000**
Equipment (over $250.00):	----
Supplies:	----
Travel:	----
Leases:	----
Other:	
Total requested:	**$26,000**

****Note:** The requested labor amount is to partially support Ph.D. candidate Rebecca Spanner with a NDSU graduate assistantship for one year and to partially support post-doctoral scientist Subi Shrestha for one year. All additional supplies necessary for the successful execution of this proposal will be provided by the USDA.