SENSITIVITY OF *CERCOSPORA BETICOLA* TO FOLIAR FUNGICIDES IN 2016

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Leaf spot, caused by the fungus *Cercospora beticola*, is an endemic disease of sugarbeet produced in the Northern Great Plains area of North Dakota and Minnesota that reduces both yield and sucrose content. The disease is controlled by crop rotation, resistant varieties and timely fungicide applications. *Cercospora* leaf spot usually appears in the last half of the growing season, and multiple fungicide applications are necessary for disease management. Fungicides are used at high label rates and are alternated for best efficacy, but in recent years, mixtures are becoming more common. The most frequently used fungicides are Tin (triphenyl tin hydroxide), Topsin (thiophanate methyl), Eminent (tetraconazole), Proline (prothioconazole), Inspire (difenoconazole) and Headline (pyraclostrobin). All fungicides are applied alone, except Topsin, which is applied as a tank mix with Tin.

Like many other fungi, *C. beticola* has the ability to become less sensitive (resistant) to the fungicides used to control them after repeated exposure, and increased disease losses can result. Because both *C. beticola* and the fungicides used for management have histories of fungicide resistance in our production areas and others, it is important to monitor our *C. beticola* population for changes in sensitivity to the fungicides in order to achieve maximum disease control. We have monitored fungicide sensitivity of field isolates of *C. beticola* collected from fields representing the sugarbeet production area of the Red River Valley region to the commonly used fungicides in our area annually since 2003. In 2016, extensive sensitivity monitoring was conducted for Tin, Topsin, Eminent, Inspire, Proline and Headline.

OBJECTIVES

1) Monitor changes in sensitivity of *Cercospora beticola* isolates to Tin (triphenyl tin hydroxide)

2) Monitor changes in sensitivity of *Cercospora beticola* isolates to Topsin (thiophanate methyl)

3) Monitor changes in sensitivity of *Cercospora beticola* to three triazole (DMI) fungicides: Eminent (tetraconazole) and Inspire (difenoconazole) and Proline (prothioconazole)

4) Test *Cercospora beticola* isolates for the presence of the G143A mutation that confers resistance to Headline (pyraclostrobin) fungicide

5) Distribute results of sensitivity monitoring in a timely manner to the sugarbeet industry in order to make fungicide recommendations for disease management and fungicide resistance management for Cercospora leaf spot disease in our region.

METHODS AND MATERIALS

In 2016, with financial support of the Sugarbeet Research and Extension Board of MN and ND, we tested 1326 *C. beticola* field isolates collected from throughout the sugarbeet production regions of ND/MN for sensitivity testing to Tin, Topsin, Eminent, Inspire, Proline and Headline. For this report we use the commercial name of the fungicides, but all testing was conducted using the technical grade active ingredient of each fungicide, not the formulated commercial fungicide. The term µg/ml is equivalent to ppm.

Sugarbeet leaves with Cercospora leaf spot (CLS) were collected from commercial sugarbeet fields by agronomists from American Crystal Sugar Company, Minn-Dak Farmers Cooperative and Southern Minnesota Beet Sugar Cooperative representing all production areas in ND and MN. Leaves were delivered to our lab, and processed immediately to insure viability of spores. From each field sample, *C. beticola* spores were collected from a minimum of five spots per leaf from five leaves and mixed to make a composite of spores. A subsample of the spore composite was transferred to a Petri plates containing water agar amended with Tin at 1 ug/ml and a second subsample of the spore composite was transferred to a Petri plates containing water agar amended with Topsin at 5 ug/ml. Germination of 100 spores on Tin and Topsin amended water agar plates were counted 16 hours later and percent germination calculated. Germinated spores are considered resistant.

For triazole fungicide sensitivity testing, a radial growth procedure is used. A single spore subculture from the composite was grown on water agar medium amended with serial ten-fold dilutions of each technical grade triazole fungicide from 0.01 – 10.0 ppm. A separate test was conducted for each triazole fungicide. After 15 days, inhibition of radial growth was measured, and compared to the growth of *C. beticola* on non-amended water agar medium. This data was used to calculate an EC_{50} value for each isolate; EC_{50} is a standardized method of measuring fungicide resistance and is calculated by comparing the concentration of fungicide that reduces radial growth of *C. beticola* by 50% compared to the growth on non-amended media. Higher EC_{50} values mean reduced sensitivity to the fungicide. An RF (resistance factor) was calculated by dividing the EC_{50} value by the baseline value so fungicides can be directly compared.

For Headline resistance testing we used a PCR based molecular procedure to test for the presence of a specific mutation in *C. beticola* that imparts resistance to Headline. This procedure detects a specific mutation, G143A, which results in total resistance to Headline. DNA is extracted from the remaining spores in the composite and tested by real time PCR using primers specific for the G143A mutation. The test enables us to estimate the percentage of spores with the G143A mutation in each sample. The PCR test has advantages over the previously used spore germination procedure. The procedure can be completed in one day, compared to 14 days for the spore germination procedure. Each sample tested contains approximately 2500-5000 spores and the DNA from this spore pool will test for the G143A mutation from each spore. The spore germination test we previously used only tested one spore per five spot/five leaf sample. The PCR test is also more sensitive and requires less interpretation than the previously used spore germination test. The PCR test will estimate the incidence of resistance in the population of spores tested, and give a better indication whether resistance is present in a field.

RESULTS AND DISCUSSION

CLS was severe in 2016, and could be found in almost every plant in every field. The high disease pressure was due to a “perfect storm” of conditions, including early planting, high disease pressure in 2015 that resulted in high spore numbers, a mild winter that allowed more inoculum to survive, warm and wet weather favorable for infection and a late freeze. The early planting and late freeze resulted in a longer than normal growing season and opportunity for disease infection for an extended period of time. planting was generally a month earlier than usual, and consequently, disease pressure was higher than usual as well. While the majority of the CLS samples were delivered to our lab at the end of the season in late September and early October, we received samples earlier in the season. CLS was prevalent, and the company agronomists did not have trouble finding samples for testing. Field samples (n=1326) representing all production areas and factory districts were tested for sensitivity to five fungicides.

TIN. Tolerance (resistance) to Tin was first reported in 1994 at concentrations of 1-2 µg/ml. At these levels, disease control in the field is reduced. The incidence of fields with isolates resistant to Tin at 1.0 µg/ml increased between 1997 and 1999, but the incidence of fields with resistant isolates has been declining since the introduction of additional fungicides for resistance management, including Eminent in 1999, Gem in 2002 and Headline in 2003. In 1998, the percentage of fields with isolates resistant to Tin at 1.0 µg/ml was 64.6%, and declined to less than 10% from 2002 to 2010. From 2011 to 2014 there was an increase in resistance **(Figure 1).** From 2014 to 2015, fields with isolates resistant to Tin increased from16.4% to 38.5% (**Figure 1**), and in 2016 this increased to 46.0% (**Figure 1**). The germination rate of spores from fields with resistant isolates ranged from 1 to 100%, with the average germination rate of 15% (mean) and the most common rate of germination 26% (median). Tin resistance was present in all factory districts ranging from 38.2% to 67.6% of the samples (**Figure. 3**).

TOPSIN. Resistance to Topsin has been present in our area since 1999, and is also common and widespread in European Union production areas. Resistance has historically been >70% but has declined below that level in six of the past twelve years. Topsin resistance, in sugarbeet and other crops, tends to decline when it is not used, but reappears quickly when it is again used in the field. In 2013, the percentage of fields with isolates resistant to Topsin at 5 µg/ml was 74.2%, in 2014 was 73.5% and in 2016 increased to 86.0% (**Figure 2)**. The germination rate of spores from fields with resistant isolates ranged from 1 to 100%, with the average germination rate of 25% (mean) and the most common rate of germination 20% (median). This increase in Topsin resistance is not surprising, since many fungicide applications of Tin plus Topsin were applied in 2016. Resistance to Topsin was high in all factory districts ranging from 73.3 to 92.3% of the samples (**Figure 3**).

DMI (triazoles). Resistance of *C. beticola* isolates to Eminent has been relatively stable, with average RF values approximately doubling from 1998 to 2010 (RF values are the EC_{50} values divided by the baseline values). Beginning in 2011, resistance began to increase based on RF values (**Figure 4**). We know from lab and greenhouse trials that EC_{50} values >1.0 are considered resistant, and diseases losses will occur in a susceptible variety at these levels even when Eminent is applied. The average RF value was 45.8 in 2013 and was 54.5 in 2014 (**Figure 4**), a thirty-fold increase in over 10-year average of 1.8 from 1999 to 2010. Surprisingly, there was a 28% decline to in RF values to Eminent across all factory districts in 2015 (**Figure 4)** with an average RF value of 39.0 across all factory districts**.** In 2016, the RF value across all factory districts continued to decline slightly to an average value of 35.3 **(Figure 4)**. Among factory districts, RF values decreased ranging from 3.2% to 22,5% **(Figure 5)**. This is good news and may indicate the presence of a fitness penalty in resistant isolates, but this must be confirmed.

Similarly, based on average RF values, resistance to Inspire also increased since 2011. The average RF value for Inspire was 2.1 in 2007, and remained low through 2012, but increased to 19.7 in 2013 **(Figure 4)**. In 2014, the average RF value was 68.3, a ten-fold increase in resistance over the previous six-year average of 6.6. There was also a decline in RF values to Inspire across all factory districts in 2015 (**Figure 4)** with an average RF value of 21.2 across all factory districts, a remarkable 69% decline **(Figure 6).** In 2016, the RF value across all factory districts increased slightly to an average value of 30.7 **(Figure 4)**. RF values for Inspire increased in all factory districts ranging from 4% to 121% **(Figure 6)**.

Resistance to Proline, as measured by EC_{50} values, was higher than either Eminent or Inspire. This was observed in every factory district **(Figure 7).** While Proline is not widely used for managing CLS, it does provide an alternative triazole based fungicide for use.

The resistance to the triazole fungicides we see in US isolates of *C. beticola* is related to overexpression of Cyp51 enzyme, and not due to a specific genetic mutation, so it will be difficult to develop a PCR assay for this group of fungicides. In companion studies we have conducted, higher levels of resistance to triazole fungicides are present in *C. beticola* isolates collected from Italy and France than found in the RRV production area. We do not know if the he reduction in the RF values we saw in 2015 will continue in future years and it will be critical to monitor resistance to triazole fungicides in the RRV region due to their widespread use. This is the first report of such a strong decline in RF values for the triazole fungicides which will hopefully continue or at least stabilize in future years.

HEADLINE. Based on EC_{50} values using spore germination testing, sensitivity of C. *beticola* to Headline remained relatively stable from 2003-2009 with only a seven-fold decrease in sensitivity. Beginning in 2012, a PCR based molecular procedure was used to test for the presence of the G143A mutation in *C. beticola* using the remainder of the composite spore sample containing approximately 2500-5000 spores. The presence of this mutation indicates absolute resistance to Headline. The results are placed in five categories based on an estimate of the percentage of spores with the G143A mutation: S = no spores with G143A; S/r = <50 of the spores with G143A; S/R = equal number of spores with G143A; R/s >50% of the spores with G143A; and R = all spores with G143A**.** The G143A mutation was first detected in the RRV production area in 2012 and increased from 2013 to 2015. Resistance to Headline in 2016 increased dramatically with a commensurate loss in sensitivity Across all factory districts in 2016, only 10% of the isolates collected had all spores without the G143A mutation; the G143A mutation was found in 90% of the samples, and 49.7% of the samples has >50 of the spores with the G143A mutation (**Figure 8**). Samples with an R rating (all spores resistant) were found in all factory districts ranging 27.2% to 50.5%. (**Figure 9**). Samples with S (all spores sensitive) ranged from 0% to 8.0%. Based on this data, the QoI fungicides Headline and Gem will likely not control CLS and will not be widely used in 2017. Although this is a stable mutation, we will continue to partially monitor for resistance to Headline in the RRV production area, particularly because Headline is often the only fungicide used, and is used annually even in the absence of disease. We do not know if there is a fitness penalty associated with the G143A mutation, but based on observation in MI and Italy, Austria and Serbia, where QoI resistance due to the G143A mutation is widespread. it appears that isolates with the G143A mutation are stable and can survive and increase in the population.

An increasing concern is the development of *C. beticola* isolates with resistance (reduced sensitivity) to more than one fungicide. Of the isolates tested in 2016:

91.3% were resistant to Headline
24.4% were resistant to all three DMI's (Eminent, Inspire, Proline)
19.7% were resistant to tin plus a DMI
28.1% were resistant to Topsin plus a DMI
2.1% were resistant to Headline plus a DMI
0.1% were resistant to tin plus Headline plus a DMI
12.2% were resistant to Topsin plus a DMI plus Headline
18.4% were resistant to tin plus Topsin plus a DMI
14.4% were resistant to tin plus a DMI plus Topsin plus Headline

In 2015, 4.9% of the isolates tested were resistant to all four fungicide groups.

Previously we conducted a greenhouse trial to determine if isolates of *C. beticola* with high levels of resistance results in decreased disease control by field application rates of Eminent compared with isolates sensitive to Eminent. Results of this work showed that the break point for causing more disease was the EC_{50} value of >1 µg/ml. At this value, there was significantly more disease when the field rate of Eminent was used. This trial was conducted using a CLS susceptible variety. We repeated this study using a CLS resistant variety to see if the break point results were the same or not. The break point for disease loss for a CLS resistant variety increased to the EC_{50} value of 10 µg/ml. After this level of resistance, there was a significant loss in disease control. This study suggests that variety resistance increases the level *of C. beticola* isolated resistance necessary for disease loss five-fold. A solid recommendation for CLS management will be to use varieties with good CLS resistance, and to find higher levels of resistance in future years. The use of varieties with increased levels of resistance will be important to manage CLs in future years and breeding for CLS resistance should be encouraged. Differences in aggressiveness among isolates may account for inconsistency of data and should be considered during resistance breeding. Measuring disease loss due to fungicide resistance is difficult, and additional work is necessary to confirm and document the results of these preliminary trials with CLS and Eminent resistant to *C. beticola*.

SUMMARY

1. Resistance to Tin at 1.0 µg/ml almost disappeared in our region from 2003-2010, but has increased since 2011, probably due to increased use. In 2016, isolates from 46% of the fields samples had some resistance to tin, with a median germination rate of 26%. Tin resistance was found in all factory districts.

2. Resistance to Topsin at 5.0 µg/ml continues to be present in our region at high levels. In 2016, isolates from 86.0% of the field samples had some resistance to Topsin, with a median germination rate of 20%. Topsin resistance was found in all factory districts.

3. Resistance to both Eminent and Inspire, as measured by RF values, declined again in 2016 in all factory districts. Proline had higher resistance values than Eminent or Inspire.

4. The number of isolates with the G143A mutation that results in resistance to Headline increased again in 2016 across all factory districts. Approximately 90% of the fields sampled have some level of resistance to Headline, and approximately 50% of the fields sampled have >50% of the spores resistant to Headline. These findings may limit the effective use of Headline for CLS management in 2017.

5. The incidence of *C. beticola* isolates with resistance to multiple fungicides is a concern. Some isolates (4.9%) have resistance to five fungicides.

7. *C. beticola* isolates with resistance caused more disease (leaf spots) than sensitive plants treated with Eminent at the field rate in greenhouse trials, and isolates with resistance can cause as much or disease than the sensitive isolates in plants not treated with Eminent. There is a difference between CLS susceptible and resistant varieties disease loss based on isolate resistance to Eminent. The EC_{50} value break point for significant disease loss for a susceptible variety is 1.0 µg/ml for the susceptible varieties compared to a break point of 10.0 µg/ml for a resistant variety

8. We recommend continuing disease control recommendations currently in place including fungicide rotation, using high label rate of fungicides, scouting at end of the season to decide the necessity of a late application, using fungicide resistance maps for fungicide selection, using a resistant variety, spray intervals of 14 days, and applying fungicides to insure maximum coverage. It may be useful in 2017 to begin fungicide applications earlier than in past years.

Figure 1. Sensitivity to Tin of *C. beticola* isolates collected in ND and MN from 1998 to 2016 at 1.0 µg/ml as measured by percent spore germination

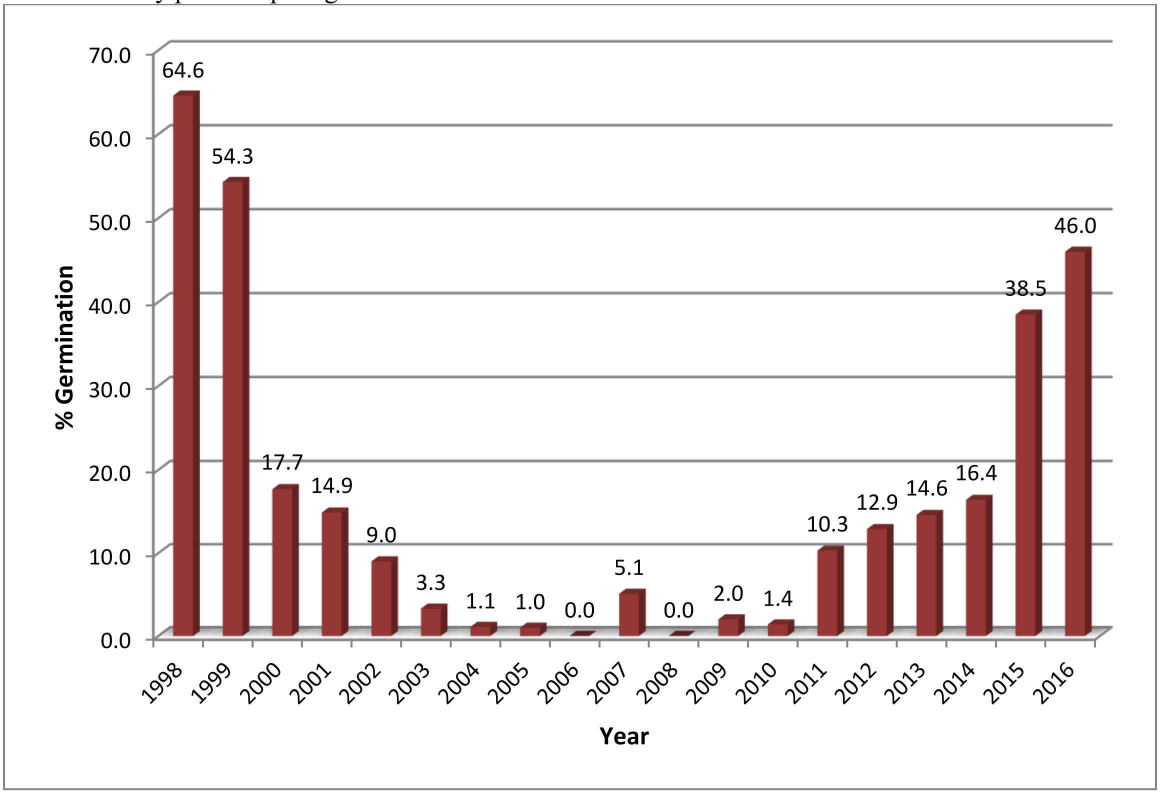

Figure 2. Sensitivity of *C. beticola* isolates collected in ND and MN from 1998 to 2016 to Topsin at 5.0 µg/ml as measured by percent spore germination

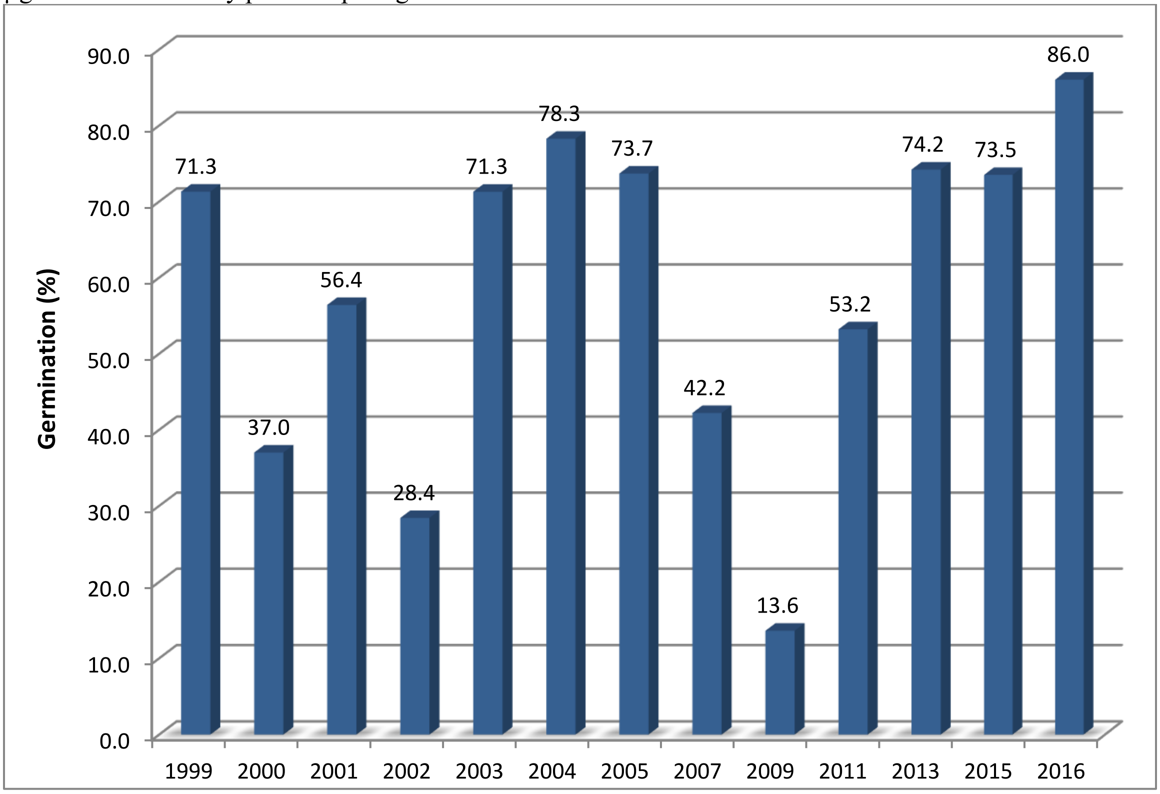

Figure. 3. Sensitivity of *C. beticola* isolates to Tin and Topsin in 2016 by factory district

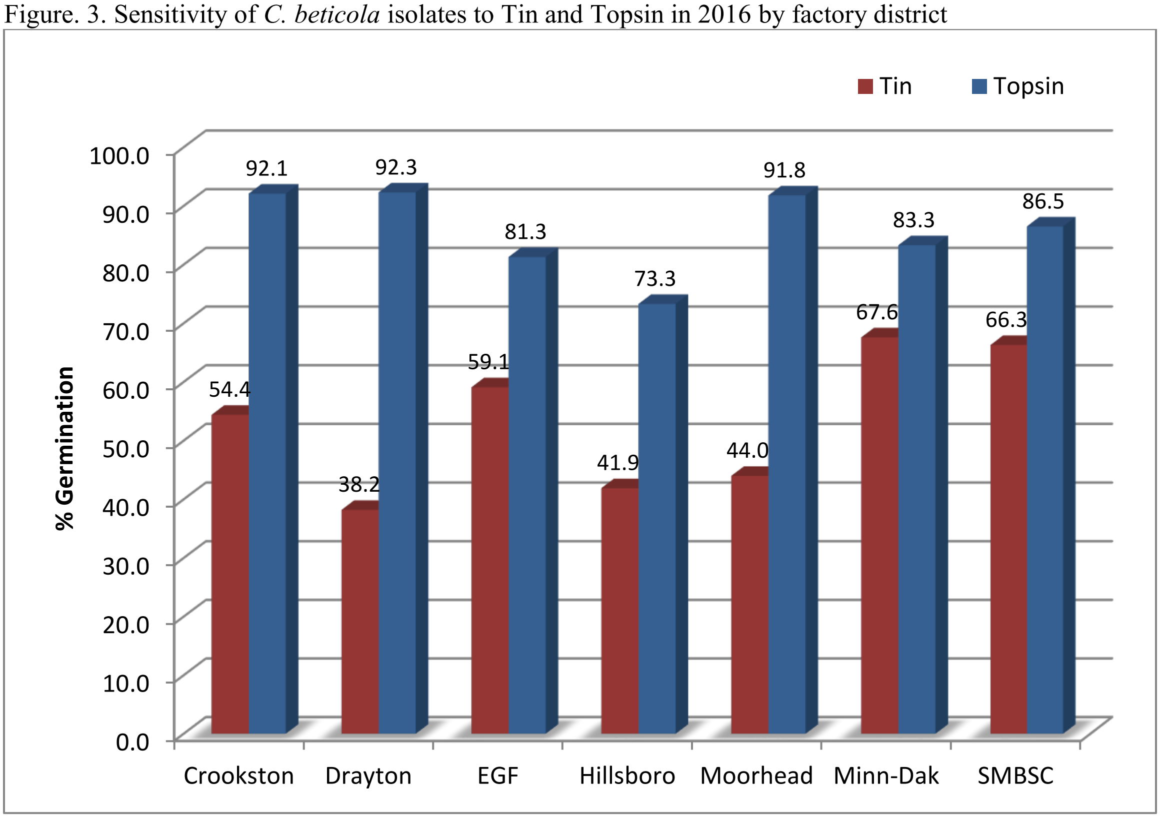

Figure. 4. Resistance Factor of *C. beticola* isolates collected in ND and MN from 1997-2016 to Eminent (tetraconazole) and Inspire (difenoconazole)

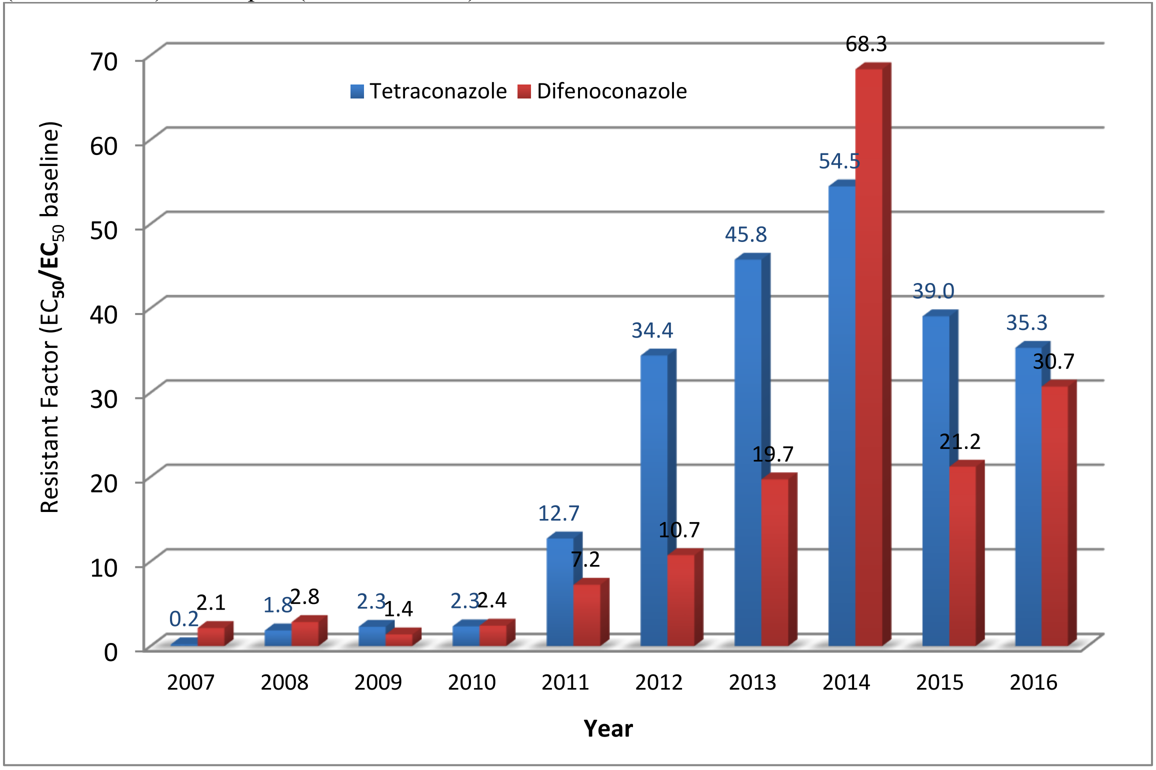

Figure 5. Resistance Factor of *C. beticola* isolates collected in 2013-2015 to Eminent by factory district

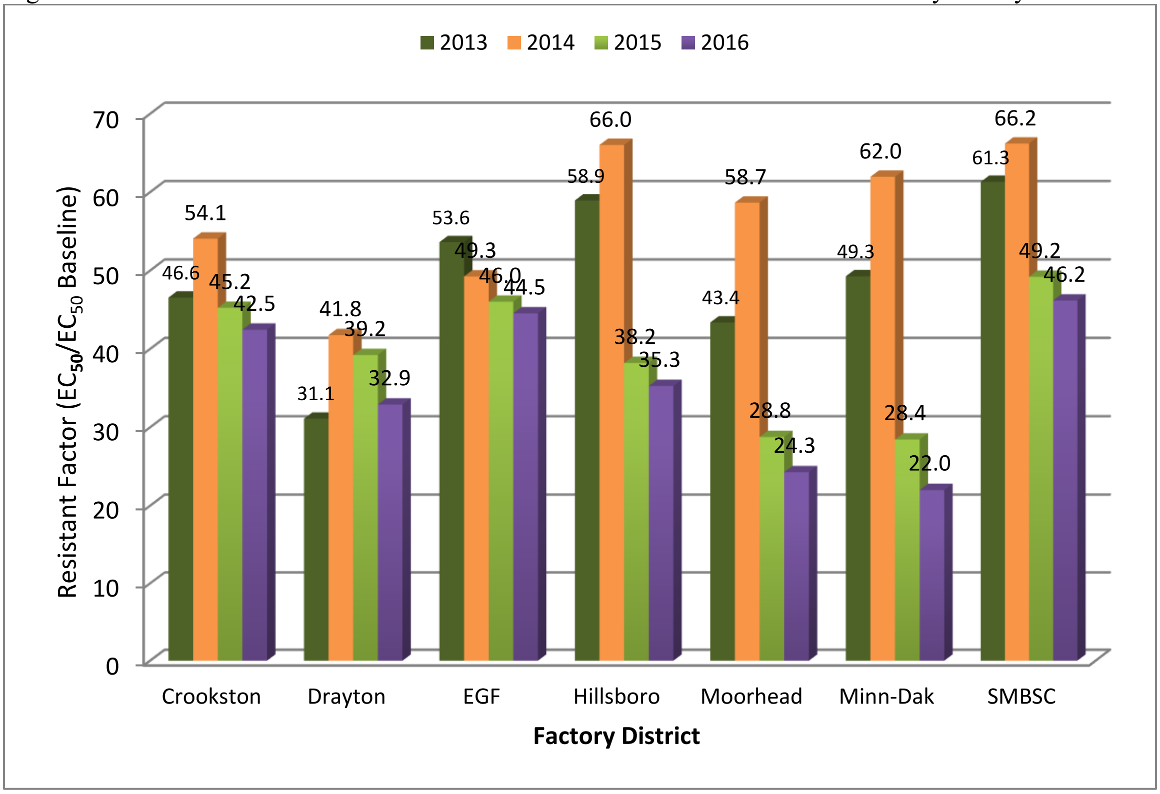

Figure 6. Resistance Factor of *C. beticola* isolates collected in 2013-2015 to Inspire by factory district

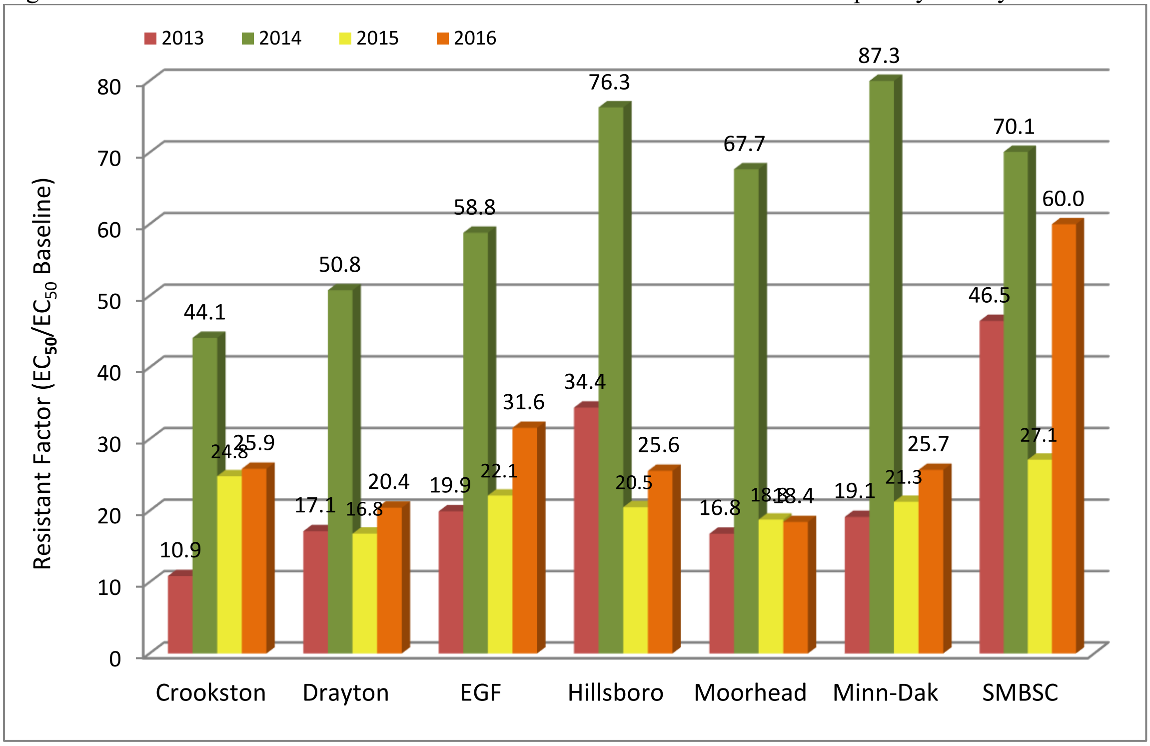

Figure 7. Sensitivity of *C. beticola* isolates collected in 2016 to Eminent, Inspire and Proline by factory district as measured by EC_{50} values

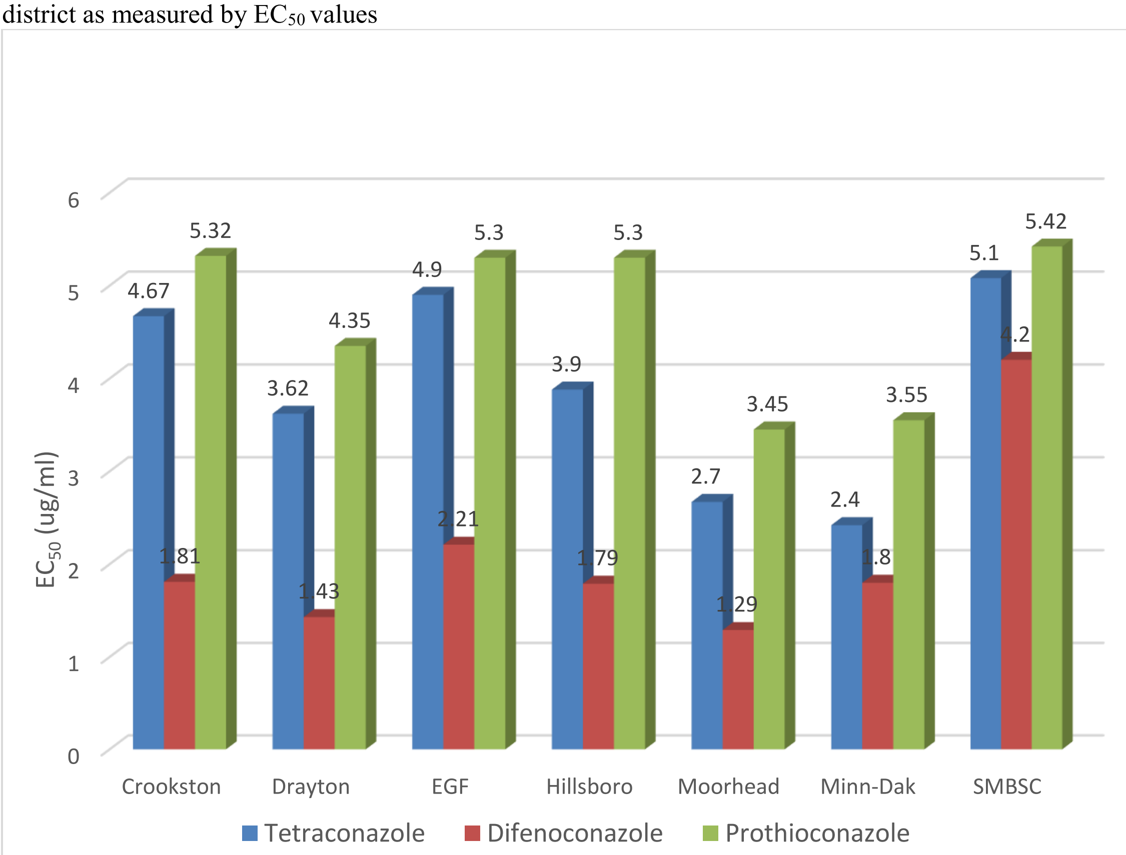

Figure 8. Sensitivity of *C. beticola* isolates collected in ND and MN to Headline from 2012 to 2016 measured by the percentage of spores with G143A mutation

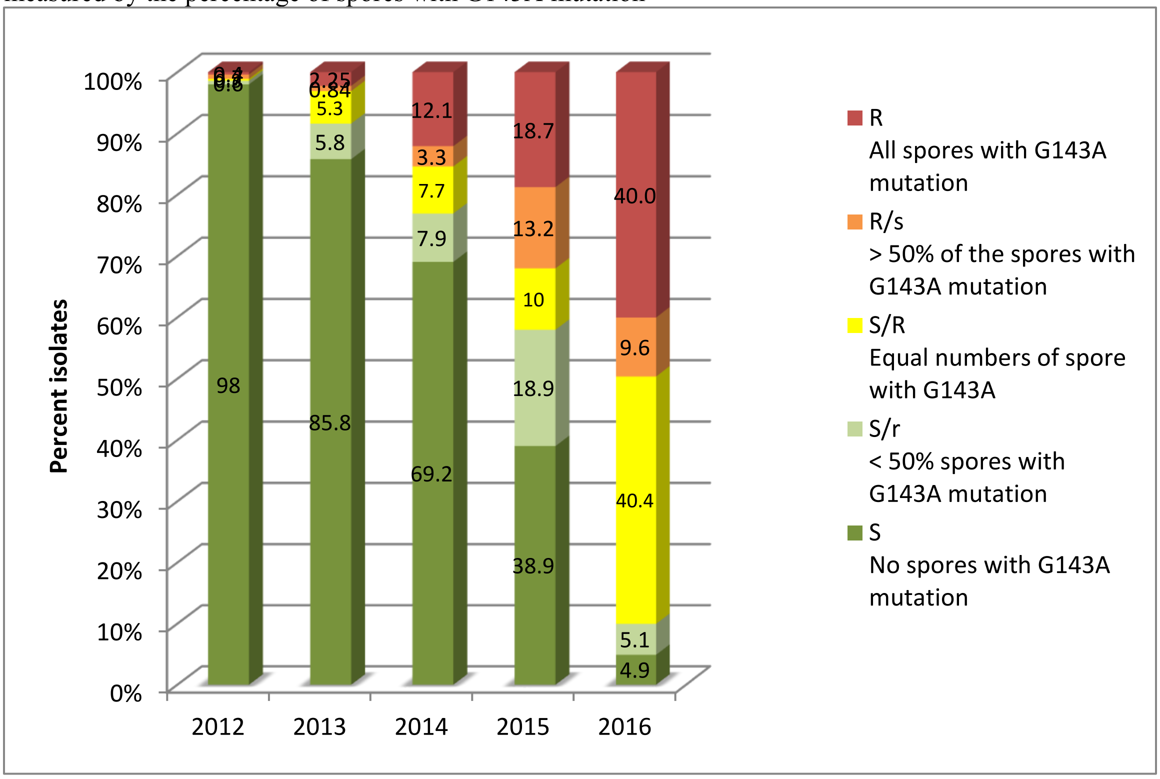

Figure 9. Sensitivity of *C. beticola* isolates collected in ND and MN in 2016 to Headline by factory district as measured by the percentage of spores with G143A mutation