SENSITIVITY OF *CERCOSPORA BETICOLA* TO FOLIAR FUNGICIDES IN 2017

Gary Secor[1], Viviana Rivera[1], Melvin Bolton[2]
[1]Department of Plant Pathology, North Dakota State University, Fargo, ND 58108 and [2]USDA-ARS, Northern Crop Science Lab, Fargo, ND 58102

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). In 2017, most of the DMI and QoI fungicides were applied as mixtures with either mancozeb or copper and Topsin is usually 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 other production areas in the US, Europe and Chile, 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 2017, extensive sensitivity monitoring was conducted for Tin, Eminent, Inspire, Proline and Headline.

OBJECTIVES

1) Monitor changes in sensitivity of *Cercospora beticola* isolates to Tin (fentin 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 2017, with financial support of the Sugarbeet Research and Extension Board of MN and ND, we tested 1105 *C. beticola* field isolates collected from throughout the sugarbeet production regions of ND and MN for sensitivity testing to Tin, 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 quickly 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

approximately 2500 spores. A subsample of the spore composite was transferred to a Petri plates containing water agar amended with Tin at 1 ug/ml. Germination of 100 spores on Tin amended water agar plates were counted 16 hours later and percent germination calculated. Germinated spores are considered resistant. Sensitivity to Topsin is tested alternate years and was not tested in 2017.

For triazole fungicide sensitivity testing, a radial growth procedure is used. A single spore subculture from the spore 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 use 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 complete resistance to Headline. DNA is extracted from the remaining spore 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. 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 of Headline resistance in a field.

RESULTS AND DISCUSSION

CLS in 2017 was common but much less severe in than in 2016, and CLS in general was well managed by the fungicide programs that were used. The majority of the CLS samples were delivered to our lab at the end of the season in late September and early October. Almost all samples arrived in excellent condition and delivered as fresh samples. Field samples (n=1105) representing all production areas and factory districts were tested for sensitivity to five fungicides: tin, tetraconazole (Eminent), difenoconazole (the most active part of Inspire), prothioconazole (Proline) and pyraclostrobin (Headline). Three additional DMI, one SDHI ande one QoI fungicides not registered in the US for CLS were tested for activity against *C. beticola* in lab trials. One new DMI and one new QoI appeared to have good activity against *C. beticola.*

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 the number of fields with resistance **(Figure 1),** and from 2015 to 2017, the incidence of fields with isolates resistant to Tin increased from 38.5% to 97% (**Figure 1**). The severity of resistance (as expressed as germination rate of spores from fields with resistant isolates) ranged from 1 to 100%, with the average germination rate ranging from 16 to 28% during the five year period of 2012 to 2017 **(Figure 2)**. The incidence of fields with tin resistance increased in all factory districts, with the lowest incidence in the Drayton, East Grand Forks and Hillsboro factory districts (**Figure. 3**). The low severity of resistance (<30%) may be the reason that tine is still an effective fungicide for managing CLS despite widespread incidence of resistance to tin.

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 2014, the percentage of fields with isolates resistant to Topsin at 5 µg/ml was 73.5% and in 2016 increased to 86.0% (data not shown). The incidence of resistance as measured by germination rate of spores from fields with resistant isolates ranged from 1 to 100%, with the average germination rate of 25%. Most applications of Topsin are as tank mixtures with Tin, which seems to be an effective management practice. Sensitivity to Topsin is measured in alternate years and was not tested in 2017.

DMI (triazoles). Sensitivity of *C. beticola* isolates to the DMI fungicides Eminent and Inspire, as measured by Resistance Factor values(RF), only doubled from 2007 to 2010, with average RF values <3 (RF values are the calculated EC_{50} values divided by the baseline values). From 2011 to 2014, RF values of both Eminent and Inspire increased to 54.5 and 68.3 respectively (**Figure 4**). Surprisingly, in 2015 there was a 29% and 69% decline to in RF values to Eminent and Inspire respectively across all factory districts to average RF values of 39.0 and 21 (**Figure 4).** In 2016, the RF value of Eminent declined slightly and increased slightly for Inspire across all factory districts **(Figure 4)**. In 2017, RF values for both Eminent and Inspire increased **(Figure 4)**, ranging from 27.1 in the Moorhead district to 57.0 in the Hillsboro district **(Figure 5)**.

The RF values of *C. beticola* isolates to Proline from 2016 to 2017 were 6.5 and 9.1 respectively, much lower than either Eminent or Inspire RF values **(Figure 4),** and was observed in every factory district **(Figure 5).** Proline has become more widely used for managing CLS in recent years.

The resistance to the triazole fungicides we see in US isolates of *C. beticola* is due 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 RF values indicates a fitness penalty or not, but it will continue to be important to monitor resistance to triazole fungicides in the RRV region due to their widespread use. We are testing other DMI fungicides in our lab for their activity against *C. beticola*, but unfortunately, most of them are not registered for CLS management.

HEADLINE. 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 incidence of this mutation has increased in the population of isolates we test every year since then. Resistance to Headline in 2017 was similar to 2016. Across all factory districts in 2017, 10.9% of the isolates collected had all spores without the G143A mutation; the G143A mutation was found in 89.1% of the samples, and 64.2% of the samples had >50 of the spores with the G143A mutation (**Figure 6**). Samples with an R rating (all spores resistant) increased from 40.0% to 55.8% **(Figure 6).** Resistance (R) was detected in all factory districts ranging 45.6% in the East Grand Forks district to 70.3% in the Moorhead district (**Figure 7)**. Samples with S (all spores sensitive) ranged from 3,0% in the SMBSC district to 10.9% in the Moorhead district. Based on this data, the QoI fungicides Headline and Gem will likely not control CLS and will not be widely used in the near future. 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 2017:

25.9% were resistant to Eminent > 1ppm
47.1% were resistant to Inspire > 1ppm
97% were resistant to tin >1 ppm
89.1% were resistant to Headline
27.7% were resistant to tin plus a DMI
14.0% were resistant to tin plus Headline plus a DMI

In 2016, 14.4% of the isolates tested were resistant to tin plus a DMI plus Headline.

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 2017, isolates from 97% of the fields samples had some resistance to tin (incidence), with a mean germination rate of 28% (severity). Tin resistance was found in all factory districts.

2. Topsin was not tested in 2017.

3. Resistance to both Eminent and Inspire, as measured by RF values, increase slightly in 2017 in all factory districts. The RF values for Proline were much lower that either Eminent or Inspire.

4. The incidence of isolates with the G143A mutation that results in resistance to Headline remained about the same in 2017 as it was 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 future years.

5. The incidence of *C. beticola* isolates with resistance to multiple fungicides is a concern. About 14 % of our isolates have resistance to five fungicides.

6. *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

7. We recommend continuing disease control recommendations currently in place including fungicide rotation, using high label rate of fungicides, mixtures with mancozeb or copper, 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 appears that early fungicide applications in 2017 helped manage CLS and early applications should continue in 2018. Improved disease control may be possible with improvements in fungicide coverage using proper spray nozzles and spray parameters such as ground speed, timing and gallonage.

Figure 1. Incidence of fields with *C. beticola* resistant to Tin at 1.0 µg/ml as measured by spore germination collected in ND and MN from 1998 to 2017

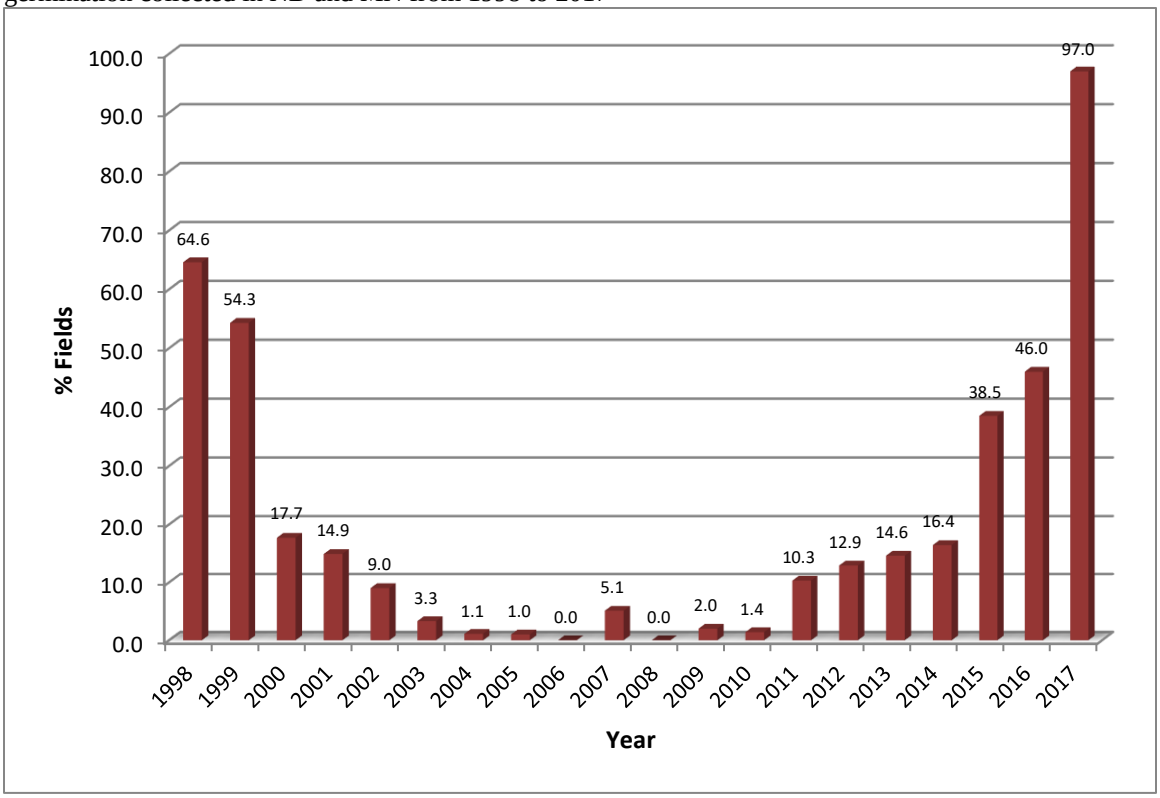

Figure 2. Incidence and severity of Tin resistance in *C. beticola* isolates collected from sugarbeet fields in ND and MN from 2003 to 2017

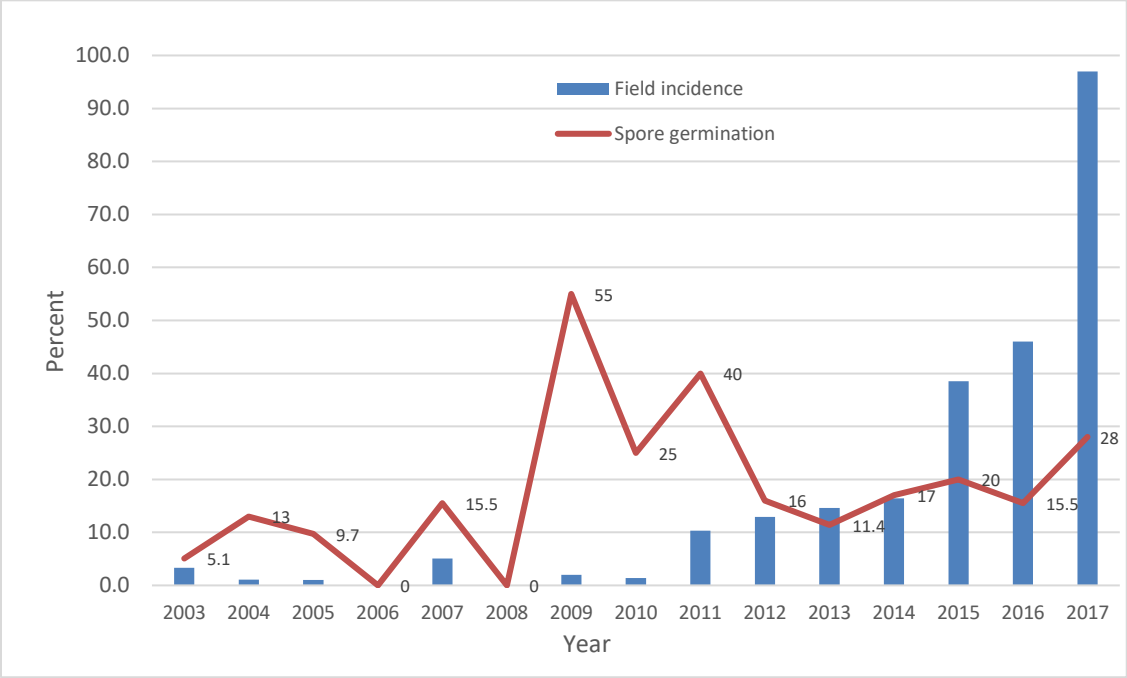

Figure. 3. Incidence of fields with *C. beticola* isolates collected in ND and MN resistant to Tin from 2013 to 2017 by factory district

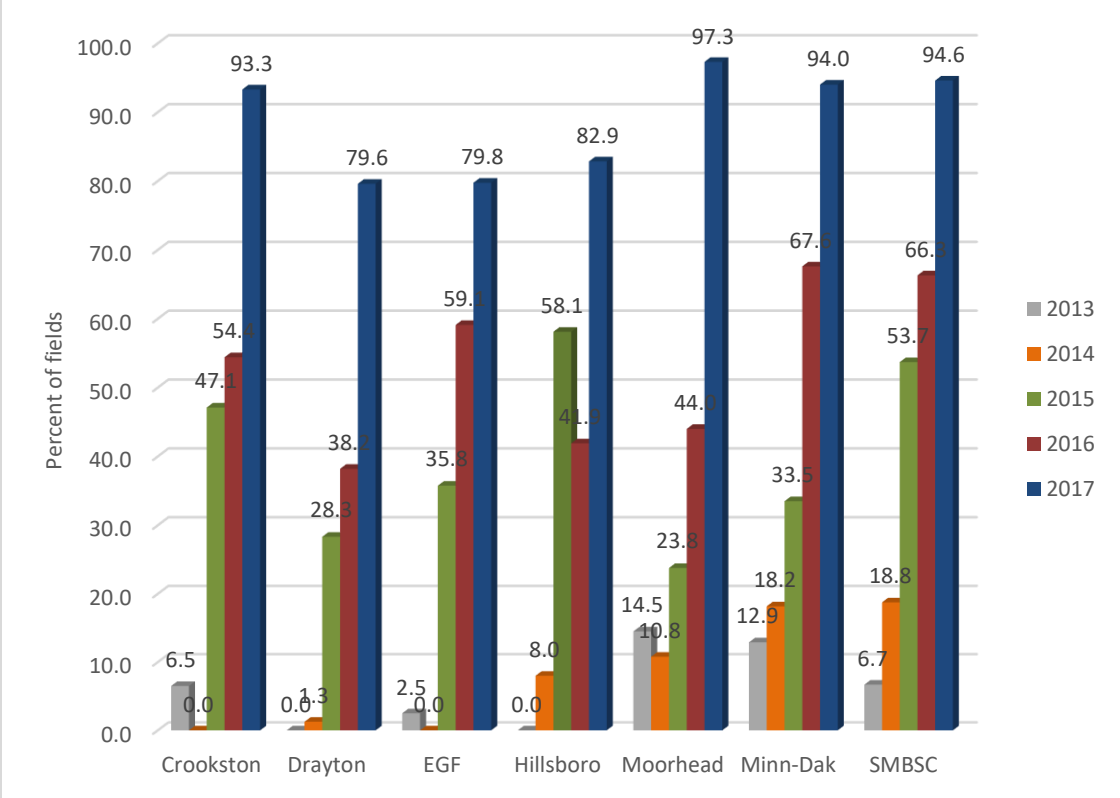

Figure 4. Resistance Factor values of *C. beticola* isolates collected in ND and MN from 2007-2017 to Eminent (tetraconazole), Inspire (difenoconazole) and Proline (prothioconazole)

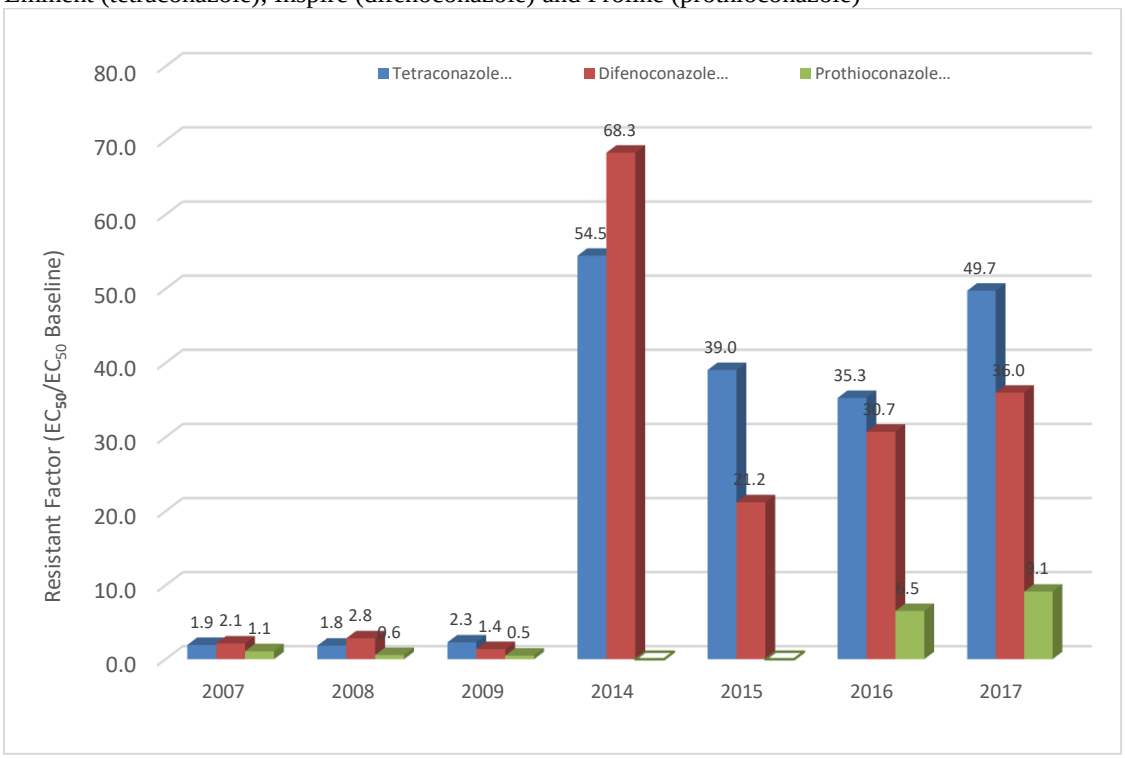

Figure 5. Sensitivity of *C. beticola* isolates collected in 2017 to Eminent, Inspire and Proline by factory district as expressed by Resistance Factor values

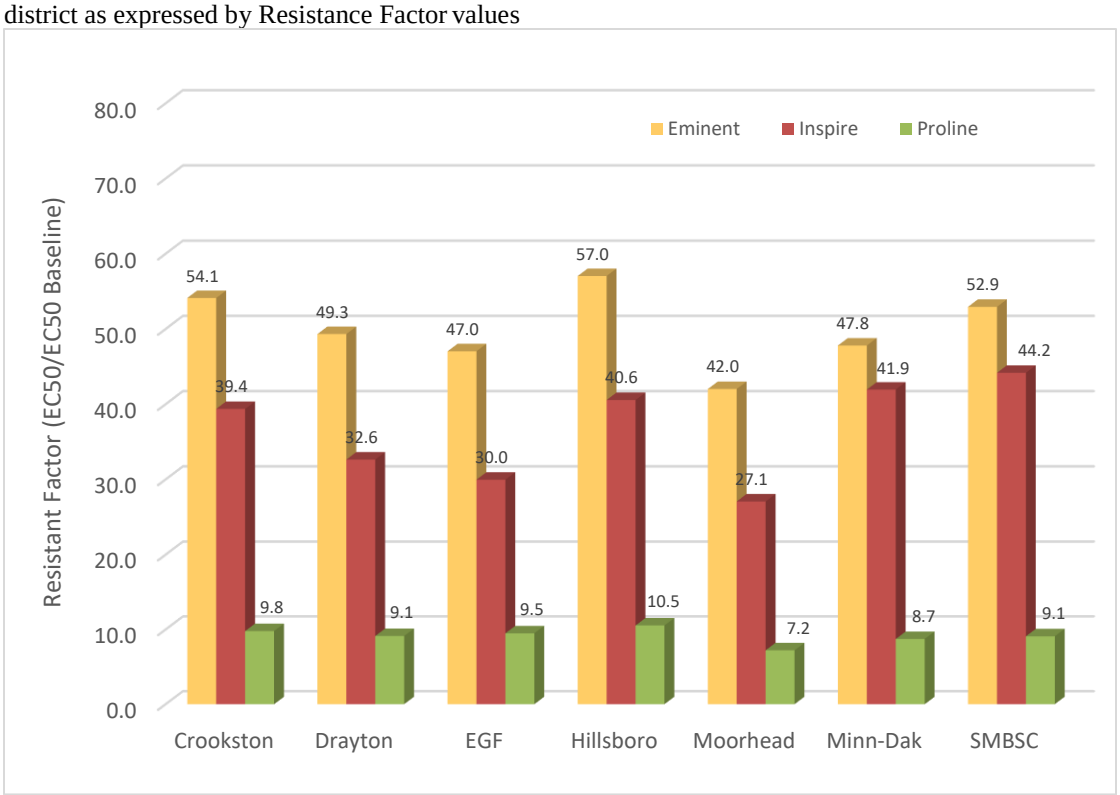

Figure 6. Sensitivity of *C. beticola* isolates collected in ND and MN to Headline from 2012 to 2017 as expressed by the percentage of spores with G143A mutation

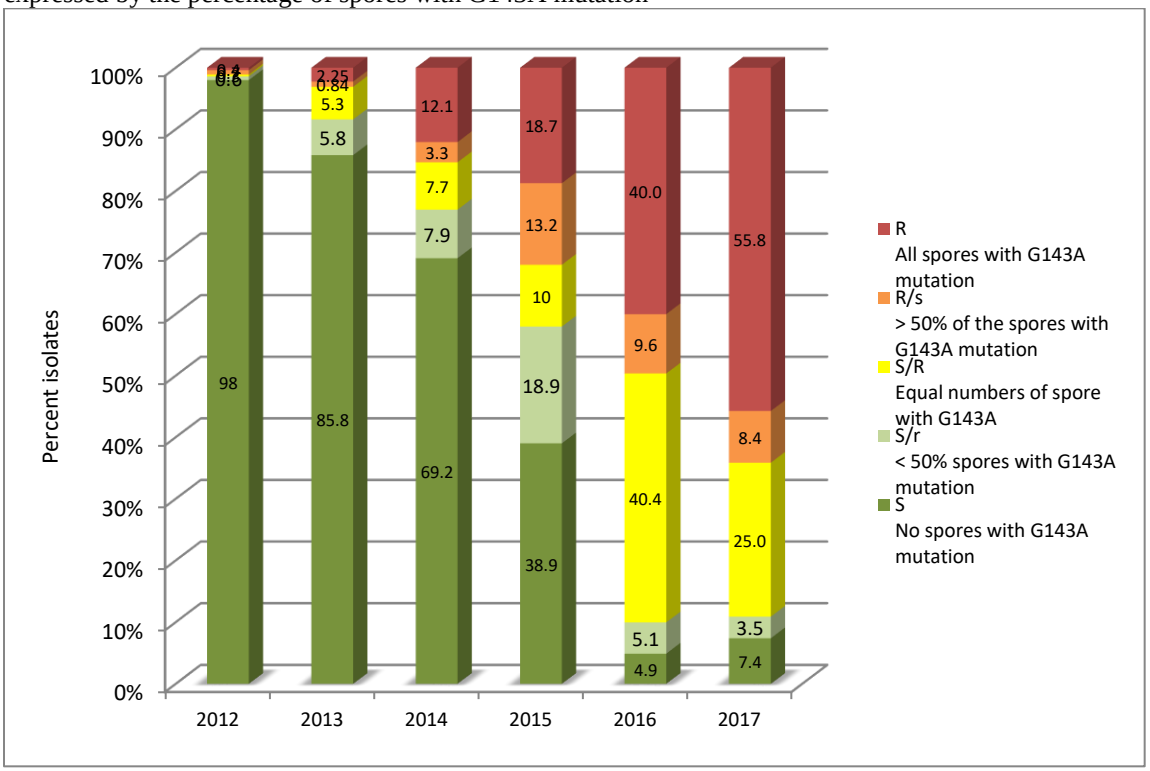

Figure 7. Sensitivity of *C. beticola* isolates collected in ND and MN in 2016 to Headline by factory district as measured by the percentage of spores with G143A mutation