

SENSITIVITY OF *CERCOSPORA BETICOLA* TO FOLIAR FUNGICIDES IN 2013

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Leaf spot, caused by the fungus *Cercospora beticola*, is an endemic disease of sugar beet produced in the Northern Great Plains area of North Dakota and Minnesota. It causes a reduction in photosynthetic area thereby reducing both yield and sucrose content of the beets. 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 two to four fungicide applications are made during this time for disease control. 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 adapt to repeated fungicide exposure and become less sensitive to the fungicides used to control them, and increased disease losses can result when fungicides become less sensitive. Because both *C. beticola* and the fungicides used to manage it have histories of fungicide resistance, 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 2013, extensive sensitivity monitoring was conducted for Tin, Topsin, Eminent, Inspire, and Headline.

OBJECTIVES

The 2013 objectives were:

- 1) Monitor changes in sensitivity of *Cercospora beticola* isolates to Tin (triphenyl tin hydroxide) and Topsin (thiophanate methyl) and compare to previous years
- 2) Monitor changes in sensitivity of *Cercospora beticola* to two triazole (DMI) fungicides: Eminent (tetraconazole) and Inspire (difenoconazole) and compare to previous years.
- 3) Test *Cercospora beticola* isolates for the presence of the G143A mutation that confers resistance to Headline (pyraclostrobin) fungicide using a newly developed PCR test
- 4) Distribute results of sensitivity monitoring in a timely manner to the sugar beet 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 2013, with financial support of the Sugarbeet Research and Extension Board of MN and ND, we received 951 *C. beticola* isolates collected from throughout the sugarbeet production regions of ND/MN for sensitivity to Tin, Topsin, Eminent, Inspire, and Headline. Of these isolates, 711 were from commercial fields and 240 from research plots. 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.

Sugar beet leaves with *Cercospora* leaf spot (CLS) were collected from commercial sugar beet 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. The spores were mixed in

water, and a composite of 200 µl of the spore suspension was transferred to each of three Petri plates containing water agar amended with Tin at 1 µg/ml, and amended with Topsin at 5 µg/ml or non-amended (water agar alone).

For Tin and Topsin sensitivity testing, a bulk spore germination procedure was used. Germination of 100 random spores on the Tin and Topsin amended water agar was counted 16 hrs after plating and percent germination calculated. Germinated spores are considered resistant.

For triazole fungicide sensitivity testing, a radial growth procedure was used. A single spore subculture 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 EC₅₀ value for each isolate; EC₅₀ 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 EC₅₀ values mean reduced sensitivity to the fungicide.

For Headline 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 was developed cooperatively between scientists at the USDA-ARS and NDSU and detects a specific mutation, G143A, which results in total resistance to Headline. This mutation has been previously identified in Headline resistant isolates from Michigan and Italy. DNA is extracted from the remaining composite of spores from the five spots from five leaves sample after removal of the 200 µl for tin and Topsin testing. The total DNA is tested by real time PCR using primers specific for the G143A mutation. The test enables us to estimate the percentage of spores collected with the resistance 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. This will allow fields to be tested in advance to determine if Headline can be efficaciously applied. Each sample tested contains approximately 2500-5000 spores and the DNA pool for testing 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

In 2013, disease pressure was relatively low due to the dry conditions and high temperatures. Cercospora disease again developed late in the season due to the formation of dew. The majority of the CLS samples were delivered to our lab at the end of the season in late September and early October. Approximately 711 field samples representing all production areas and factory districts were tested for sensitivity to five fungicides in 2013. Additional samples (n=240) from fungicide trial plots of Dr. Mohamed Khan, NDSU, were also tested for sensitivity to these fungicides. For this report, only results from the field samples are included; the fungicide trial plot results are not included. A few samples that were submitted were not tested because the spores did not germinate. We postulate that the fields from which these samples were collected had recently been treated with a fungicide that interfered with spore germination in the lab, or that the leaves collected had bacterial leaf spot and not Cercospora leaf spot. It was interesting to note that some of the leaves submitted were affected by Alternaria leaf spot which is not normally seen in our area.

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 isolates with resistance to Tin at 1.0 µg/ml increased between 1997 and 1999, but the incidence of 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 isolates resistant to Tin at 1.0 µg/ml was 64.6%, in 1999 was 54.3%, in 2000 was 17.7%, in 2001 was 14.9%, in 2002 was 9.0%, in 2003 was 1.1%, in 2004 was 1.1%, in 2005 was 0.97%, in 2006 was 0.0%, in 2007 was 5.1%, in 2008 was 0%, in 2009 was 2.0%, in 2010 was 1.4%, in 2011 was 10.3% and in 2012 was 12.9%. In 2013, the incidence of isolates resistant to tin at 1.0 ppm

increased slightly to 14.7 % (**Figure 1**). The increase may be due to the increased use of tin plus Topsin in 2013 because of triazole resistance concerns. The trend for increased tin resistance is a beginning concern that deserves watching, as tin is an important component of fungicide resistance management program.

Resistance to the benzimidazole fungicide Topsin became widespread in *C. beticola* in the 1980's in many sugar beet production areas of the US, including the Northern Great Plains. In 1998, 70.8% of the samples were resistant to Topsin at >5.0 µg/ml when tested using a bulk spore germination procedure; in 1999, 71.3% of the samples were resistant; in 2001, 56.4% of the samples were resistant; in 2003, 71.3% of the samples were resistant; in 2004, 78.3% of the isolates were resistant, and in 2009, 14% of the samples were resistant and in 2011 53.2% of the samples were resistant. In 2013, the incidence of isolates resistant to Topsin at 5.0 ppm increased to 74.5% (**Figure 2**). It appears that incidence of isolates resistant to Topsin has increased dramatically since 2009 and has returned to levels found in 1998 and 1999. This rapid increase is not surprising, since resistance to benzimidazole fungicides is a stable resistance that remains in the population and returns quickly when benzimidazole fungicides are used again

Resistance of *C. beticola* isolates to Eminent has been relatively stable, with average EC₅₀ values approximately doubling from 1998 to 2010 (**Figure 3**). The average EC₅₀ value of field-collected isolates collected in 2002 was 0.21µg/ml, in 2003 was 0.12, in 2004 was 0.24, in 2005 was 0.29, in 2006 was 0.14, in both 2007 and 2008 was 0.20, in 2009 was 0.25, and in 2010 was 0.26 (**Figure 3**). Beginning in 2011, resistance began to dramatically increase based on EC₅₀ values. We know from lab and greenhouse research that EC₅₀ values >1.0 are considered resistant, and diseases losses will occur at these levels even when Eminent is applied. The average EC₅₀ value in 2011 was 1.40 and 2012 was 3.78 µg/ml (**Figure 3**). In 2013, the average EC₅₀ value was 5.03 µg/ml, a nine-fold increase in over the previous thirteen year average of 0.56 (**Figure 3**). In 2002, 1.2 % of the isolates tested had an EC₅₀ value of >1 compared to 6.0% of the isolates in 2003, 10.8% of the isolates in 2004, 12.4% of the isolates in 2005, 7.3% of the isolates in 2006, 9.5% of the isolates in 2007, 12.4% of the isolates in 2008, and 6.6% of the isolates in 2009, 19% of the isolates in 2010, 35.5% in 2011 and 65.4 % in 2012. In 2013, 59.7 % of the isolates tested had an EC₅₀ value >1.0 ppm, but the percent of isolates with EC₅₀ value >10.0 ppm increase from 18% in 2012 to 29% in 2013.

Based on average EC₅₀ values, resistance to Inspire also increased. The average EC₅₀ values for Inspire were 0.15 in 2007, 0.20 in 2008, 0.10 in 2009, 0.17 in 2010, 0.51 in 2011, 0.75 µg/ml in 2012 (**Figure 3**). In 2013, the average EC₅₀ value increased to 1.38, almost a 4.5 fold increase in resistance over the previous six year average of 0.31 µg/ml (**Figure 3**). The percent isolates in 2009 isolates with EC₅₀ values >1.0 ppm to Inspire was 0.5%, in 2010 was 8.4%, in 2011 was 9.5%, in 2012 was 9.0% and in 2013 increased to 25.7%.

In 2013, resistance to both triazole fungicides increased in all factory districts (**Figures 4 and 5**). 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. 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. It will be critical to monitor resistance to triazole fungicides in the RRV region due to their widespread use and increased resistance in recent years. It may be prudent to pursue registration of fungicides with new modes of action and/or fungicide mixtures to help manage fungicide resistance

Based on EC₅₀ 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. The percentage of isolates with EC₅₀ values >1 ppm to Headline was 0.5 % in 2009, 2.3% in 2010 and 3.7% in 2011. In 2012 and 2013, a PCR based molecular procedure was used to test for the presence of the G143A mutation in *C. beticola*. The presence of this mutation indicates resistance to Headline. As previously described, a bulk sample that contains approximately 2500-5000 spores of *C. beticola* was used to perform this test. The results are placed in four categories based on an estimate of the percentage of spores with the G143A mutation: S = no resistance detected, all spores sensitive; S/r most spores sensitive but a few resistant spores detected; S/R = equal number of sensitive and resistant spores detected, and R = all spores resistant (**Figure 6**). In 2012, S = 98%, S/r = 0.6%, S/R = 0.3%, R/S=0.5% and R=0.4%. In 2013, the resistance

increased slightly: S = 86.6%, S/r = 5.8%, S/R = 4.5% and R = 3.1% (**Figure 6**). Samples with slight to moderate levels of resistance (S/r) were found in all factory districts, while samples with high levels of resistance were found only in the Crookston, Moorhead and Minn-Dak factory districts. This same mutation was found in Italy and MI sugarbeet fields with widespread resistance in 2011. It will be critical to continue monitoring 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 data from MI and Italy, it appears that isolates with the G143A mutation are stable and can survive and increase in the population.

SUMMARY

1. Resistance to Tin at 1.0 µg/ml almost disappeared in our region from 2003-2010, presumably because of the use of alternate fungicides that has resulted in the reduction in the number of Tin applications from 2.14 in 1998 to less than one each year since 2001. However, since 2011 there has been an increase in isolates resistant to tin, ostensibly due to an increase in tin application. In 2013, 14.6% of the isolates were resistant to tin.

2. Resistance to Topsin has increased from a low in 2004 to levels of resistance as high as was present in 2003-2004. This increase is probably due to more Topsin applications as a result of increased Eminent resistance. This is not good news, but is not surprising.

3. Sensitivity to Eminent increased dramatically from 2011-2013 and, is at an all-time high in 2013. Resistance to Inspire has similarly increased from 2011-2013, but the resistance factor for Inspire is much lower than for Eminent. Triazole resistance appears to be present in all factory districts, including those that did not recommend the use of triazole fungicides in 2013. The high levels of resistance are a serious concern for the industry as the triazole fungicides are widely used as part of the fungicide resistance strategies in place.

4. The number of isolates with the G143A mutation that results in absolute resistance to Headline increased from 2012 to 2013. Resistance to Headline to not disappear with time, and if the increasing trend to resistance continues, this could have a serious impact on CLS management. It will be critical to continue monitoring 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.

5. It appears that the fungicide resistance management plan that we are following has been working since there have been no fungicide failures in our area due to fungicide resistance. Our monitoring program has detected several shifts toward decreased sensitivity to all fungicides used for control.

6. Combinations of fungicides with different modes of actions may be necessary to prevent reduced sensitivity of *C. beticola* to currently registered fungicides. New fungicides with new modes of action should be tested for efficacy for registration, if there are any.

7. Continue to use disease control recommendations currently in place including:

- Fungicide rotation
- Only one triazole per season
- Only one strobilurin (QoI) per season
- A good three spray program is triazole, tin, strobilurin
- Using the high label rate of all fungicides
- Scout at end of the season to decide the necessity of a late application; CLS developed late in recent years
- NDAWN daily infection values, row closure, first appearance of disease and the calendar are all used to determine first fungicide application
- Use fungicide resistance maps for fungicide selection
- Use a variety with resistance to CLS; KWS rating of 5.0 or less
 - ▶ Is it time to increase genetic resistance to CLS?

- Spray intervals of 14 days
- Apply fungicides in a manner to insure maximum coverage; the fungicides used for Cercospora leaf spot control are protectants; better coverage results in better control. Fungicides must be in place before *C. beticola* inoculum arrives.

Figure 1. Sensitivity to Tin of *C. beticola* isolates collected in ND and MN from 1998 to 2013 at 1.0 µg/ml as measured as percent spore germination

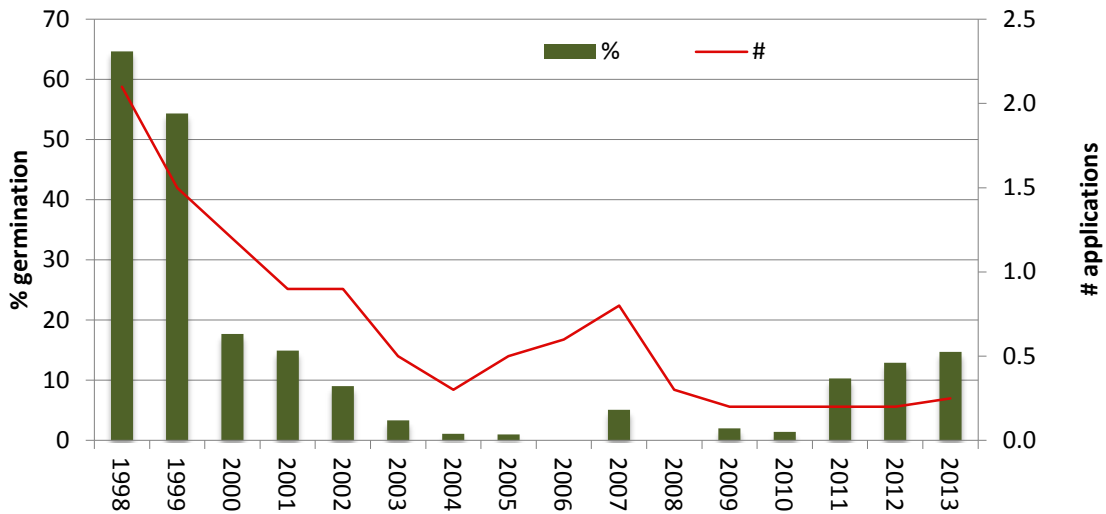


Figure 2. Percent germination of *Cercospora beticola* isolates collected in ND and MN from 2003 to 2013 on medium amended with Topsin at 5 µg/ml.

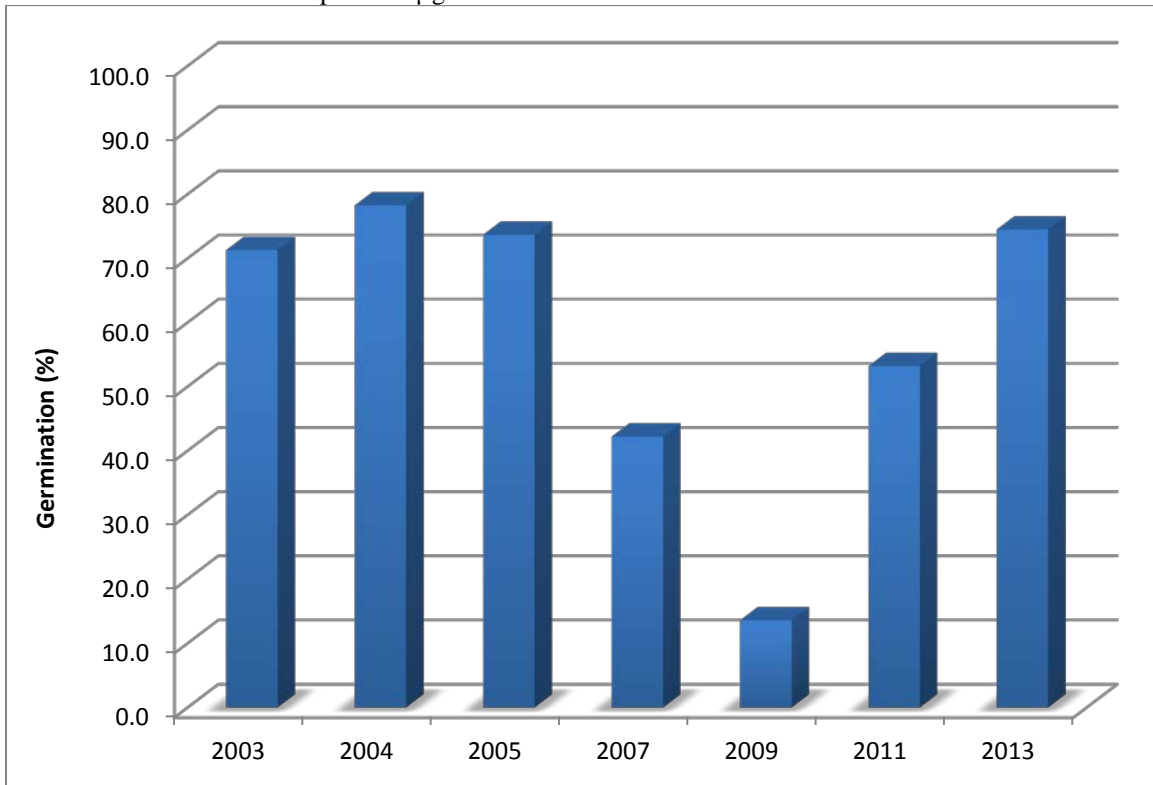


Figure 3. Resistance Factor of *C. beticola* isolates collected in ND and MN from 1997-2013 to Eminent and Inspire

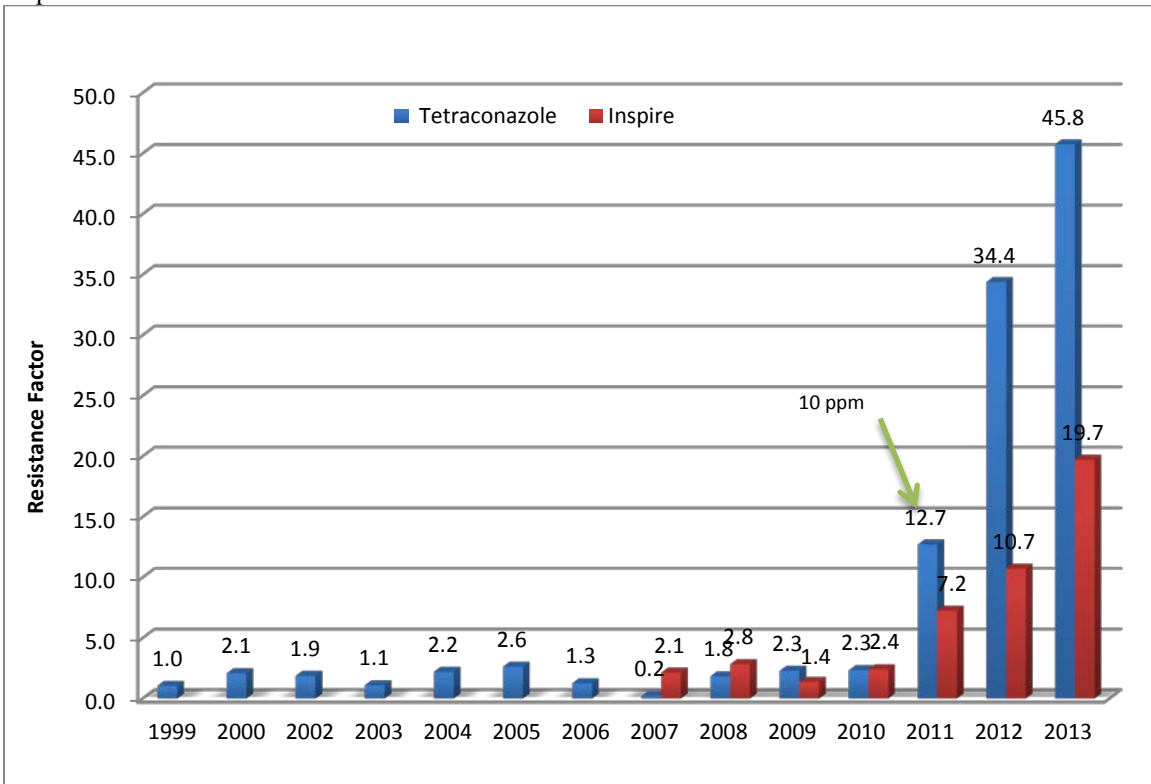


Figure 4. Resistance Factor of *C. beticola* isolates collected in 2009-2013 to Eminent by factory district

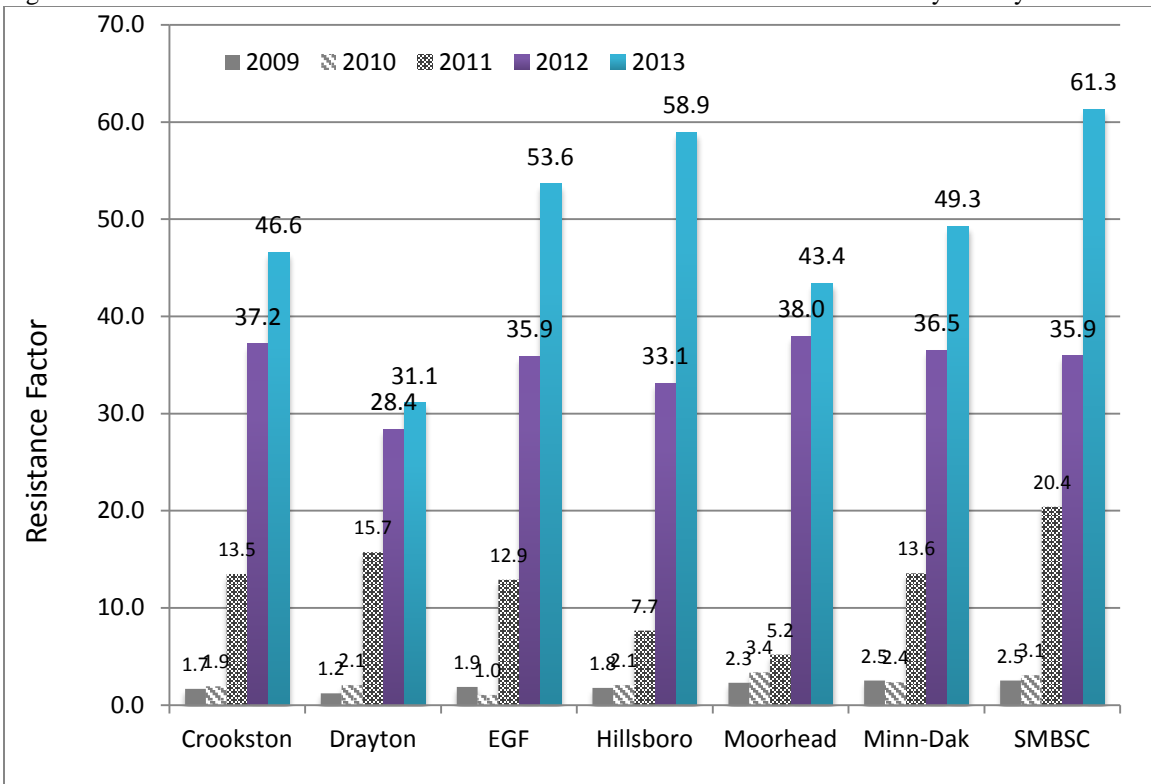


Figure 5. Resistance Factor of *C. beticola* isolates collected in 2009-2013 to Inspire by factory district

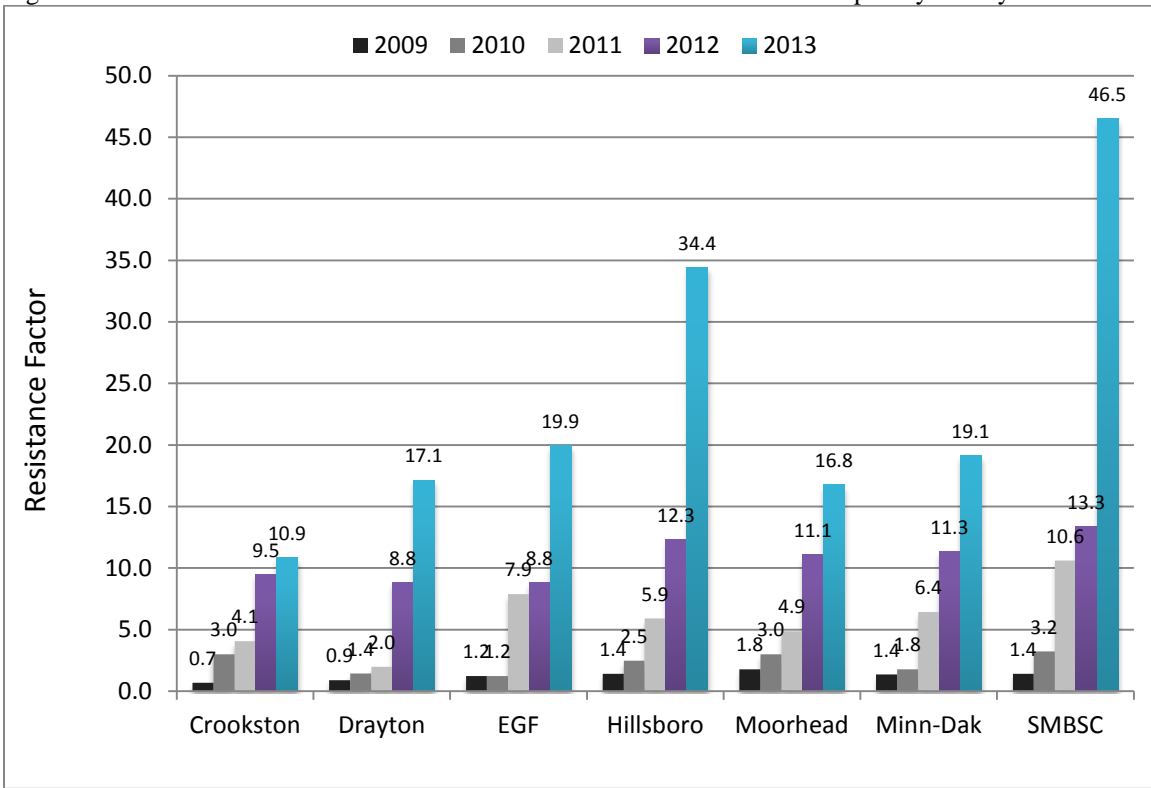


Figure 6. Sensitivity of *C. beticola* isolates collected in ND and NM to Headline from 2012 and 2013 measured by the presence of the QoI mutation G143A

