

SENSITIVITY OF *CERCOSPORA BETICOLA* TO FOLIAR FUNGICIDES IN 2014

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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 two to four fungicide applications are made during this time 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 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 for management 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 2014, extensive sensitivity monitoring was conducted for Tin, Eminent, Inspire, 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* to two triazole (DMI) fungicides: Eminent (tetraconazole) and Inspire (difenoconazole)
- 3) Test *Cercospora beticola* isolates for the presence of the G143A mutation that confers resistance to Headline (pyraclostrobin) fungicide using a PCR test
- 4) 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 2014, with financial support of the Sugarbeet Research and Extension Board of MN and ND, we received 1173 *C. beticola* isolates collected from throughout the sugarbeet production regions of ND/MN for sensitivity testing to Tin, Eminent, Inspire, and Headline. Of these isolates, 868 were from commercial fields and 305 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$. Germination of 100 random spores on the Tin amended water agar was counted 16 hrs later and percent germination calculated. Germinated spores are considered resistant.

For triazole fungicide sensitivity testing, a radial growth procedure was 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 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. An RF (resistance factor) was calculated by dividing the EC₅₀ 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. This could 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 2014, disease pressure was moderate, and conditions were favorable for disease the second half of the season. The majority of the CLS samples were delivered to our lab at the end of the season in late September and early October. Approximately 868 field samples representing all production areas and factory districts were tested for sensitivity to four fungicides. Additional samples (n=305) 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 spots due to another disease and were not *Cercospora* leaf spot. It was interesting to note that this year a relatively high number of leaves submitted were affected by *Alternaria* leaf spot.

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%, and declined to less than 10% from 2002 to 2011. From 2012 to 2014 there was a small increase in resistance (**Figure 1**). In 2014, the incidence of isolates resistant to Tin at 1.0 ppm was 16.4% (**Figure 1**). The increase may be due to the increased use of Tin plus Topsin 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 of *C. beticola* isolates to Eminent has been relatively stable, with average RF values approximately doubling from 1998 to 2010 (**Figure 2**). Beginning in 2011, resistance began to increase based on RF values (**Figure 2**). We know from lab and greenhouse trials that EC₅₀ values >1.0 are considered resistant, and diseases losses will occur at these levels even when Eminent is applied. The average RF value was 12.7 in 2011, 34.4 in 2012, and in 45.8 in 2013 (**Figure 2**). In 2014, the average RF value was 54.5 (**Figure 2**), a thirty-fold increase in over 10 year average of 1.8 from 1999 to 2010. In

2002, 1.2 % of the isolates tested had an EC₅₀ value of >1. 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 to 43% in 2014.

Similarly, based on average RF values, resistance to Inspire also increased. The average RF value for Inspire was 2.1 in 2007, and remained low through 2010 (**Figure 2**). From 2011 to 2013 the RF values ranged from 7.2 to 19.7. In 2014, the average RF value was 68.3, a ten-fold increase in resistance over the previous six year average of 6.6 (**Figure 2**). 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%.

Resistance to both triazole fungicides increased in all factory districts, except East Grand Forks where there was a decline in RF value to Eminent (**Figures 3 and 4**). 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. Beginning in 2012, a PCR based molecular procedure has been used to test for the presence of the G143A mutation in *C. beticola*. The presence of this mutation indicates resistance to Headline. The remainder of the composite spore sample containing approximately 2500-5000 spores of *C. beticola* was used for this procedure. 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 (**Figure 5**). The G143A mutation was first detected in the RRV production area in 2012 and increased slightly in 2013 and again in 2014. Resistance to Headline in 2014 increased with a commensurate decrease in sensitivity (**Figure 5**). Samples with an R rating (all spores resistant) were found in all factory districts (**Figure 6**) and ranged from 2.2 to 24.9 percent (**Figure 6**). Samples with S rating ranged from 38.3% to 88.2%. 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.

In order to understand laboratory testing results indicating resistance with field observations of no disease, two special studies were conducted in 2014 based on discussions with agronomy staff at all three cooperatives.

The first study was conducted to determine if resistance levels found in lab testing may be exaggerated due to preferential sampling of highly infested local infections (hot spots) from overwintering resistant populations that do not represent an entire field. These are also referred to as typhoid Mary (TM) outbreaks. In 2014, TM samples were collected and tested separately from samples collected throughout fields to see if there are resistance differences. A total of 91 TM samples were submitted; 81 were successfully tested for the five fungicides. One sample was resistant to tin, compared to 16.4% of the field samples. The average EC₅₀ value for Eminent of TM isolates with 4.5 compared to 5.0 for field isolates. The percent isolates with EC₅₀ values >10 was 34% for TM isolates compared to 43% for field isolates. The average EC₅₀ value for Inspire of TM isolates with 3.8 compared to 4.8 for field isolates. Sixteen percent of the TM isolates were R for the G143A, compared to 11.2% of the isolates. Based on this preliminary one-year data, it appears that TM isolate resistance does not contribute greatly to artificially increased resistance levels compared to resistance found in scattered field samples.

The second trial was 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. Briefly, sugar beet plants were inoculated in the greenhouse with isolates of *C. beticola* resistant, moderately resistant and sensitive to Eminent, sprayed with field-strength Eminent or water and evaluated for leaf spot disease. Isolates with resistance caused more disease (leaf spots) than sensitive plants treated with Eminent at the field rate, and isolates with resistance can cause as much or disease than the sensitive isolates in plants NOT treated with Eminent. It appears there is not a fitness penalty associated with the resistance to Eminent, but differences in aggressiveness among isolates may account for inconsistency of data. 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 *C. beticola*.

SUMMARY

1. Resistance to Tin at 1.0 µg/ml almost disappeared in our region from 2003-2010, but has increased slightly the past four years. In 2014, 16.4% of the isolates were resistant to tin.
2. Resistance to Topsin was not tested in 2014, as Topsin testing is only conducted in alternate years. It is anticipated that a PCR test will be used for Topsin testing in 2015.
3. Resistance to both Eminent and Inspire, as measured by both resistance factor and percent isolates with EC₅₀ values >10 increased again in 2014. Resistance increases were found in all factory districts
4. The number of isolates with the G143A mutation that results in absolute resistance to Headline increased some in 2014 with an average of 11.2% R across all factory districts. 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 does not appear that preferential collection of early appearing infection clusters (TM) seriously explains the high level of resistance found in lab assay compared to no serious field outbreaks due to fungicide resistance.
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. *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. It appears there is not a fitness penalty associated with the resistance to Eminent, but differences in aggressiveness among isolates may account for inconsistency of data.
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 variety with a KWS resistance rating of 5.0 or less, using spray intervals of 14 days, and applying fungicides to insure maximum coverage

Figure 1. Sensitivity to Tin of *C. beticola* isolates collected in ND and MN from 1998 to 2014 at 1.0 µg/ml as measured as percent spore germination

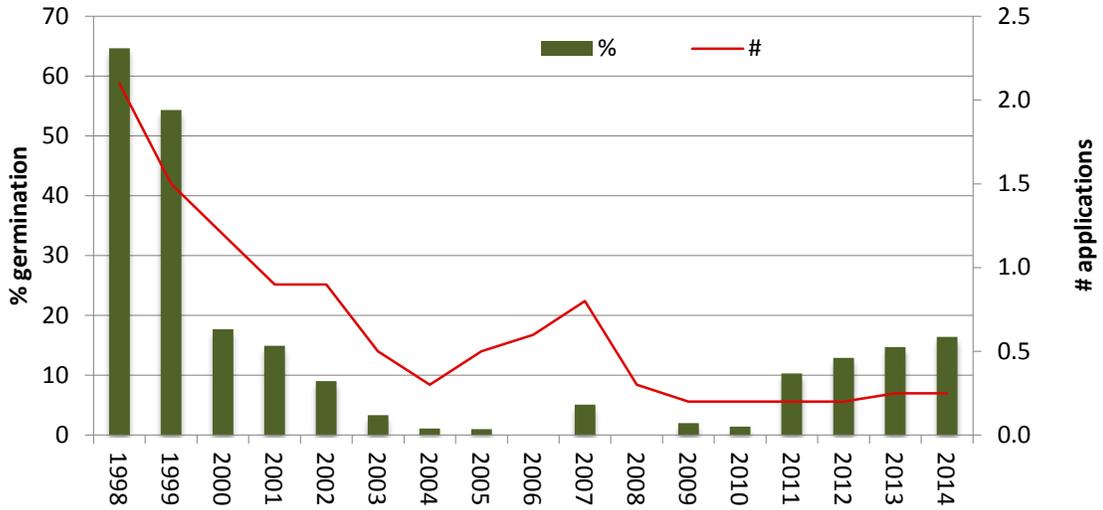


Figure 2. Resistance Factor of *C. beticola* isolates collected in ND and MN from 1997-2014 to Eminent(tetraconazole) and Inspire (difenoconazole)

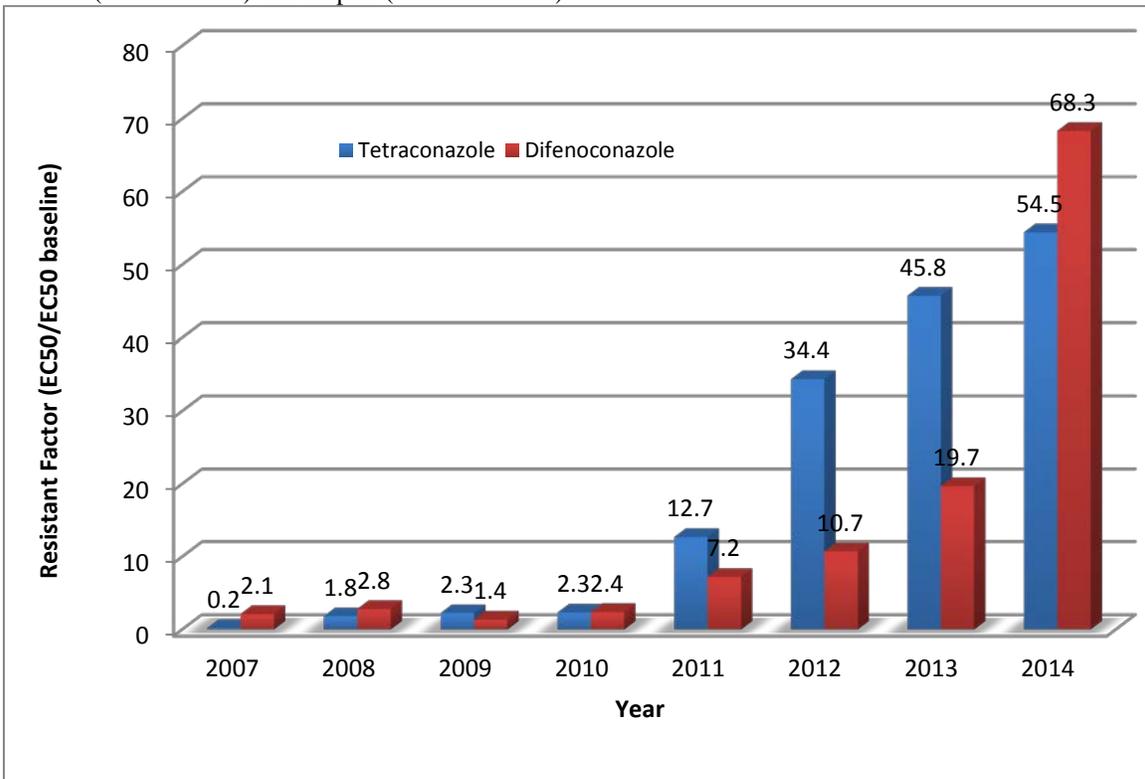


Figure 3. Resistance Factor of *C. beticola* isolates collected in 2009-2014 to Eminent by factory district

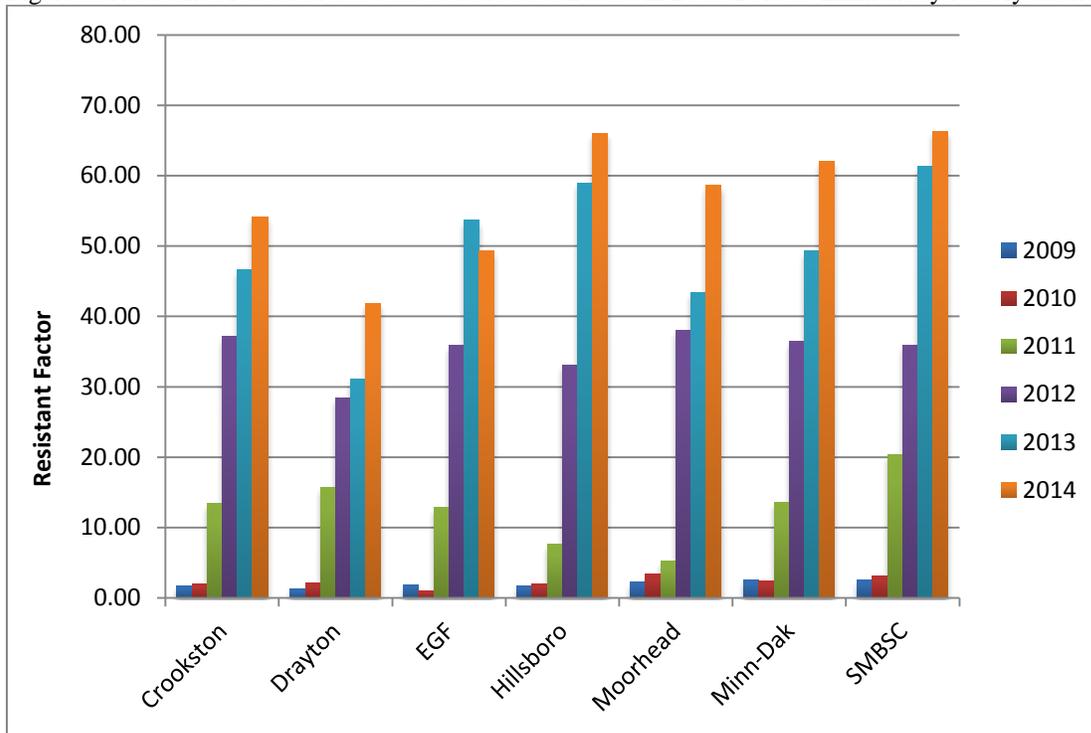


Figure 4. Resistance Factor of *C. beticola* isolates collected in 2009-2014 to Inspire by factory district

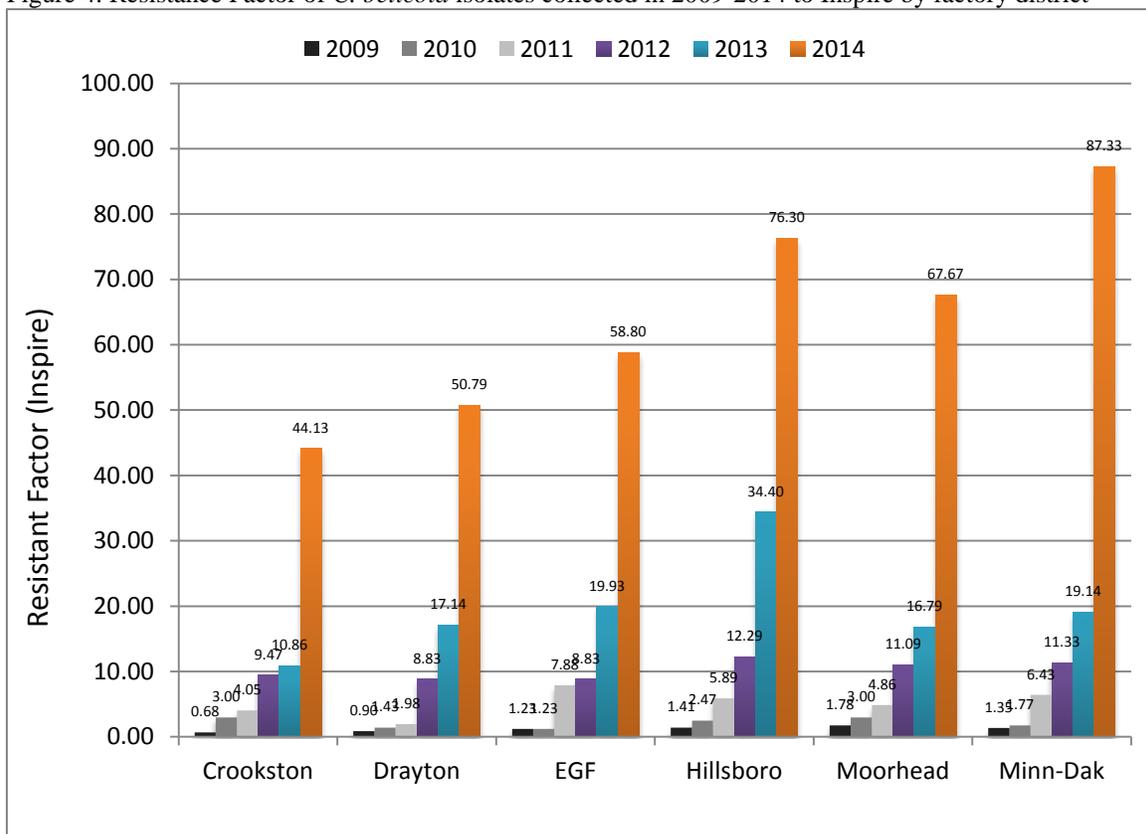
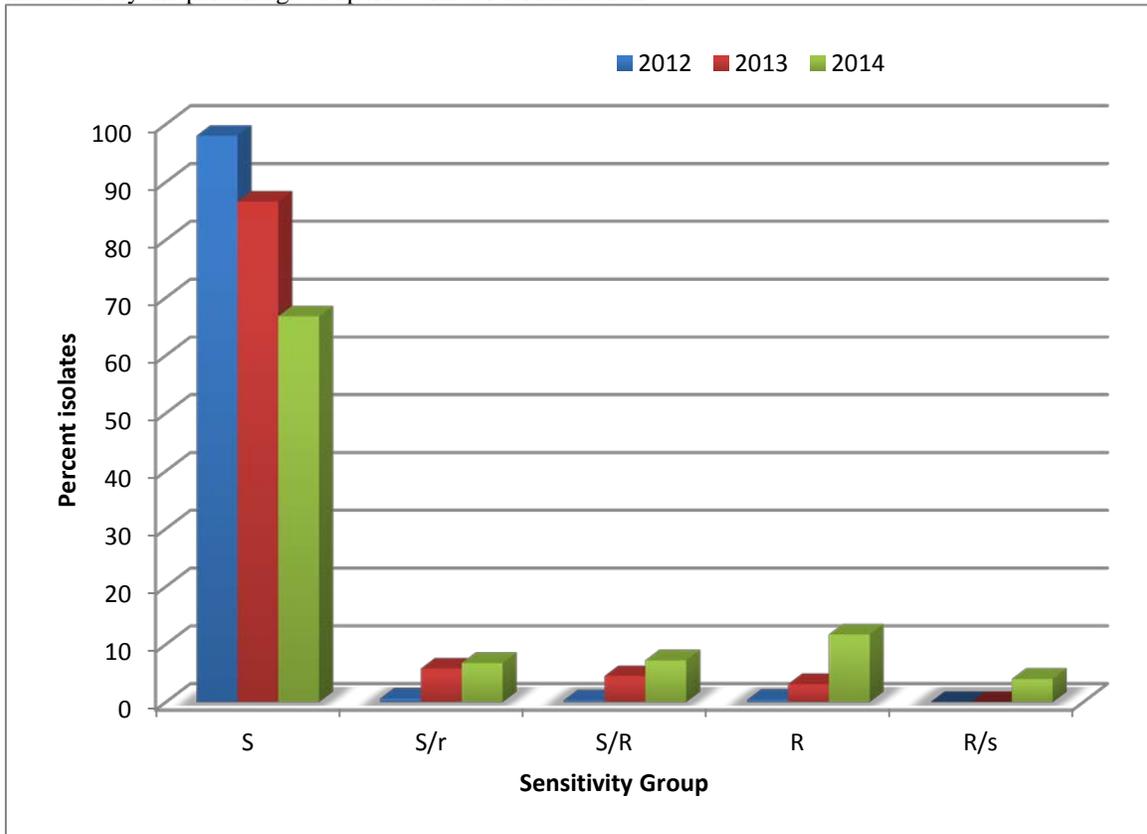
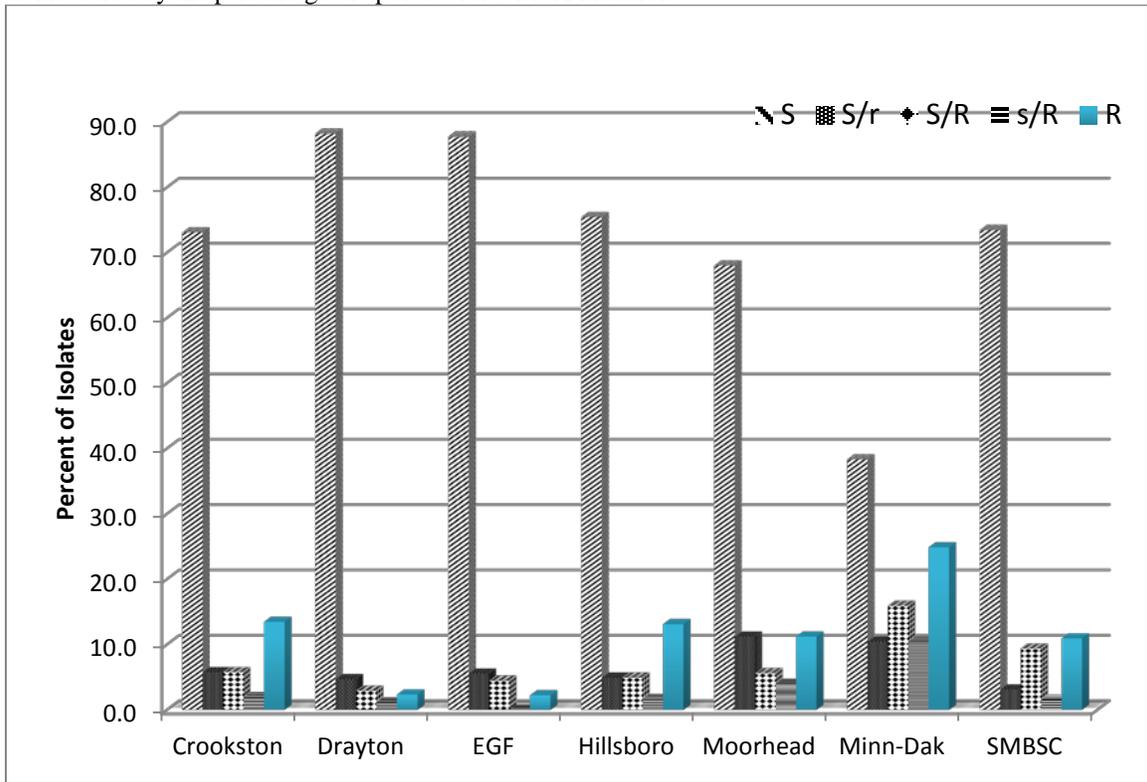


Figure 5. Sensitivity of *C. beticola* isolates collected in ND and MN to Headline from 2012 to 2014 measured by the percentage of spores with G143A mutation



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; R = all spores with G143A

Figure 6. Sensitivity of *C. beticola* isolates collected in ND and MN in 2014 to Headline by factory district as measured by the percentage of spores with 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; R = all spores with G143A