# SENSITIVITY OF *CERCOSPORA BETICOLA* TO FOLIAR FUNGICIDES IN 2012

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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, especially if they are applied frequently over a period of years. Loss of disease control can result when fungicides become less sensitive. Because both *C. beticola* and the fungicides use to manage it have a history of fungicide resistance, it is important to monitor our *C. beticola* population for changes in sensitivity to the fungicides used for Cercospora leaf spot management in order to achieve maximum disease control. We began testing *C. beticola* populations for changes in sensitivity to tin in 1996, and expanded sensitivity testing to additional fungicides in subsequent years. From 1997-2000 we evaluated sensitivity of *C. beticola* to tin and Topsin. We utilized our extensive culture collection of *C. beticola* isolates from 1997-2000 to establish baseline sensitivities to Eminent, Headline and Gem and to evaluate shifts in sensitivity to tin and Topsin. Fungicide sensitivity monitoring 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 has been conducted in the years 2003-2011. In 2012, extensive sensitivity monitoring was conducted for Tin, Eminent, Inspire, and Headline.

## OBJECTIVES

The 2012 objectives were:

1) Monitor changes in sensitivity of *Cercospora beticola* isolates to Tin (triphenyl tin hydroxide) and 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 2012, with financial support of the Sugarbeet Research and Extension Board of MN and ND, BASF Corporation, and Syngenta Crop Protection, we tested 1414 *C. beticola* isolates collected from throughout the sugarbeet production regions of ND/MN for sensitivity to Tin, Eminent, Inspire, and Headline. Of these isolates, 1127 were from commercial fields and 287 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 and combined into a single composite of approximately 2500 spores/600 µl . From this composite of spores, 100 µl was transferred to two Petri plates containing water agar amended with Tin at 1 ug/ml or non-amended (water agar alone).

For Tin sensitivity testing, a bulk spore germination procedure was used. Germination of 100 random spores on the Tin amended water agar was counted 16 hrs after plating and percent germination calculated. Germination on non-amended media was calculated and this plate was used as a source of single spore sub-cultures for subsequent Eminent, Inspire testing.

For triazole fungicide sensitivity testing, a standard radial growth procedure for *C. beticola* was used. A single spore subculture from the non-amended media 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 Eminent and Inspire. 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.

For the first time in 2012, we used a PCR based molecular procedure was used to detect 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 identified 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 100 µl for tin testing. The total DNA is tested by real time PCR using primers specific for the G143A mutation, and we are able to estimate the number of spores 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 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 idea whether resistance is present in a particular field.

**RESULTS AND DISCUSSION**

In 2012, disease pressure was relatively common with moderate severity despite the dry conditions, probably because of prolonged dew that favored infection. As in past years, the majority of the CLS samples were delivered to our lab at the end of the season in September and October. Approximately 1127 field samples representing all production areas and factory districts were tested for sensitivity to five fungicides in 2012. Additional samples (n=287) from fungicide trial plots of Dr. Mohamed Khan, NDSU, were also tested for sensitivity to the same 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 done, 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 were not Cercospora 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 was reduced. The incidence of isolates with resistance to Tin at1.0 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% and in 2011 was 10.3%.(**Figure 1**). In 2012, the incidence of isolates resistant to tin at 1.0 ppm increased to 12.9% (**Figure 1**). The increase may be due to the increased use of tin plus Topsin in 2012 because of triazole resistance concerns. This increase 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. The rapid increase of resistance from 2009 to 2011is not surprising, since resistance to benzimidazole fungicides is statble and does not revert to sensitivity quickly, and resistance returns quickly when benzimidazole fungicides are used again. In 2012, only a few random samples were tested for Topsin sensitivity; full scale testing will resume again in 2013.

Based on average $EC_{50}$ values, overall resistance of *C. beticola* isolates to Eminent was generally stable from 1998 to 2010 but increased dramatically the past two years (**Figure 2**). The average $EC_{50}$ 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, in 2010 was 0.26, and in 2011 was 1.40 µg/ml. In 2012, the average $EC_{50}$ value increased to 3.78, almost a thirteen-fold increase in resistance over the previous fourteen year average of 0.28 **(Figure 2)**. In 2002, 1.2 % of the isolates tested had an $EC_{50}$ 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% in 2010 an 35.5% of the isolates in 2011. In 2012, 65.4% of the isolates tested had an $EC_{50}$ value >1.0 ppm, some >10.0 ppm.

Based on average $EC_{50}$ values, sensitivity to Inspire also increased. The average $EC_{50}$ values for Inspire were 0.15 in 2007, 0.20 in 2008, 0.10 in 2009, 0.17 in 2010, and 0.51 in 2011 (**Figure 3)**. In 2012, the average $EC_{50}$ value increased to 0.75 **(Figure 3)**, more than a three-fold increase in resistance over the previous four year average of 0.22. In 2009, the percent isolates in 2009 isolates with $EC_{50}$ values >1.0 ppm to Inspire was 0.5%, in 2010 was 8.4%, and in 2011 was 9.5%, with a few >10 ppm.

Resistance to Eminent and Inspire increased similarly in all factory districts (**Figures 4 and 5**). In general, we found no differences in $EC_{50}$ values between lower leaves (early infection) and upper leaves (recent infection) in 2011. Resistance in the US, defined as $EC_{50}$ >1, correlates with reduced disease control in field and greenhouse trials we have conducted. 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

In 2012, the spore germination test to determine sensitivity of *C. beticola* isolates to Headline was abandoned and replaced with a PCR based procedure that detect the presence of the G143A mutation that imparts absolute resistance to Headline. Consequently, $EC_{50}$ values are not reported for 2012. Instead field samples are reported that the G143A mutation was detected in each field population of over 2500 composite spores. In 2012, of the 1127 field samples tested, 12 samples tested positive for the G143A

mutation. Of these samples, three were completely resistant (100% of the spores in the sample had the G143A mutation), six were R/S (more than 50% of the spores in the sample had the G143A mutation) and three were S/R (less than 50% of the spores in the sample had the G143A mutation). Samples with the G143A mutation were found in the East Grand Forks, Hillsboro, Moorhead, Minn-Dak and SMBSC districts, but not in the Drayton or Crookston factory districts. This same mutation was found in MI sugarbeet fields with widespread resistance in 2011, and also found at a high incidence in sugarbeet fields in Italy 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 that enhances or reduces the competiveness or ability to overwinter of isolates with the G143A mutation. We are currently trying to find the answer to this question. However, based on data from MI and Italy, it appears that isolates that have the G143A mutation can survive and increase in the population.

**SUMMARY**

1. Resistance to Tin at 1.0 µg/ml has almost disappeared in our region, 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. In 2011 and 2012, there was an increase in isolates resistant to tin, ostensibly due to an increase in tin application. In 2012, 10.3% of the isolates were resistant to tin.

2. Sensitivity to Eminent was relatively stable based on average $EC_{50}$values and the number of isolates with an $EC_{50}$ > 1.0 µg/ml from 2003-2010. In the past two years, sensitivity to both Eminent and Inspire fungicides has increased, more so for Eminent than Inspire.

3. For the first time in 2012 the G143A mutation that results in resistance to Headline was found in CLS samples collected from ND and MN. This is a stable and absolute mutation that has the potential to become severe and must be monitored for vigilantly.

4. It appears that so far 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. However our monitoring program has detected several shifts toward decreased sensitivity to all fungicides used for control, and this need continued monitoring.

5. Both alternation and combinations of fungicides with different modes of actions will be necessary to prevent further reduction in sensitivity of *C. beticola* to currently registered fungicides. New fungicides with new modes of action should be tested for efficacy for registration.

6. 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
- Fungicide combinations such as triazole + EBDC, tin + Topsin, Headline + tin, triazole + Topsin etc.
- 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
- 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 2012 at 1.0 µg/ml as measured by bulk spore germination

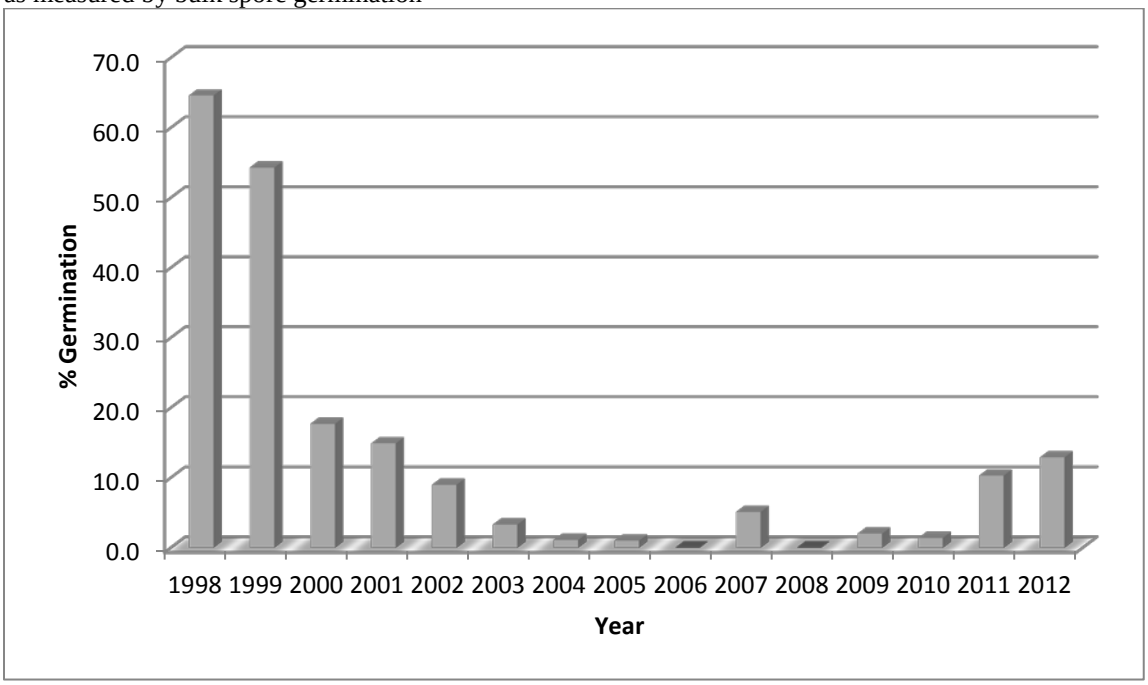


Figure 2. Average $EC_{50}$ values of *C. beticola* isolates collected in ND and MN from 1997-2012 to Eminent

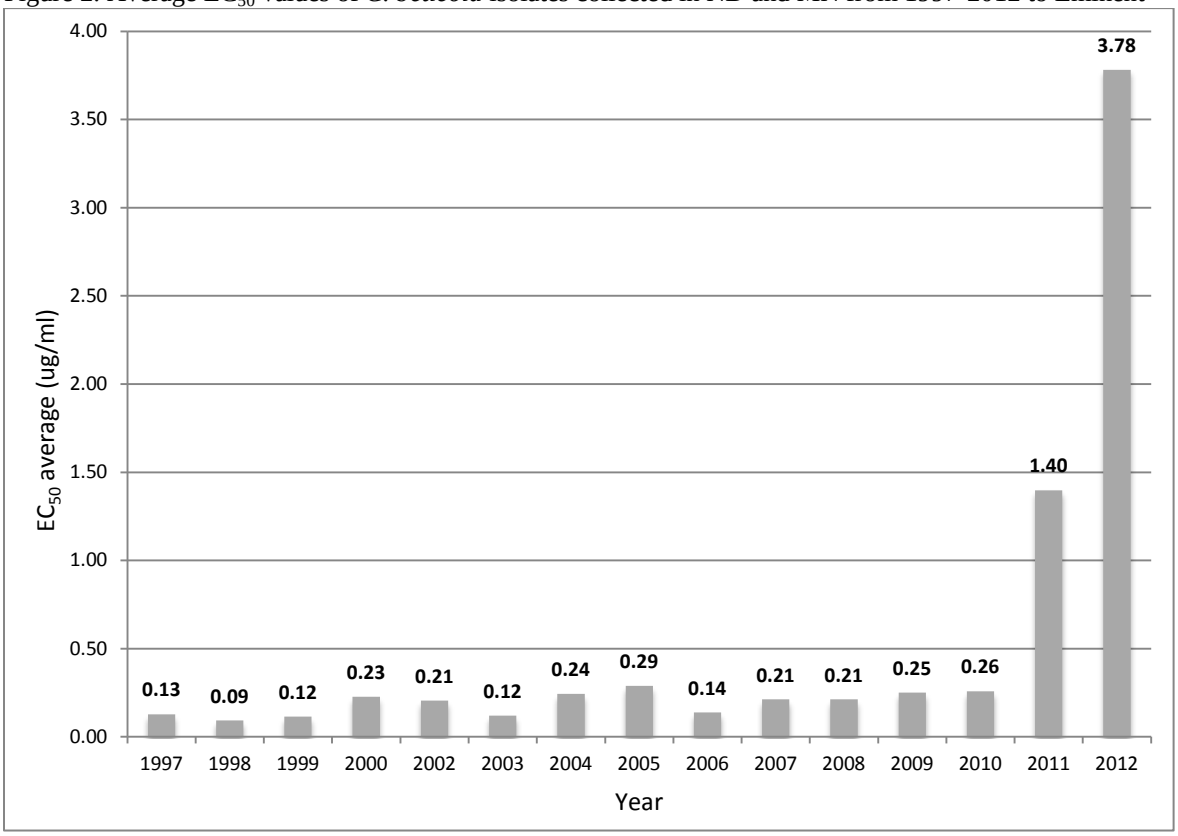

Figure 3. Average $EC_{50}$ values of *C. beticola* isolates collected in ND and MN from 2007-2012 to Inspire

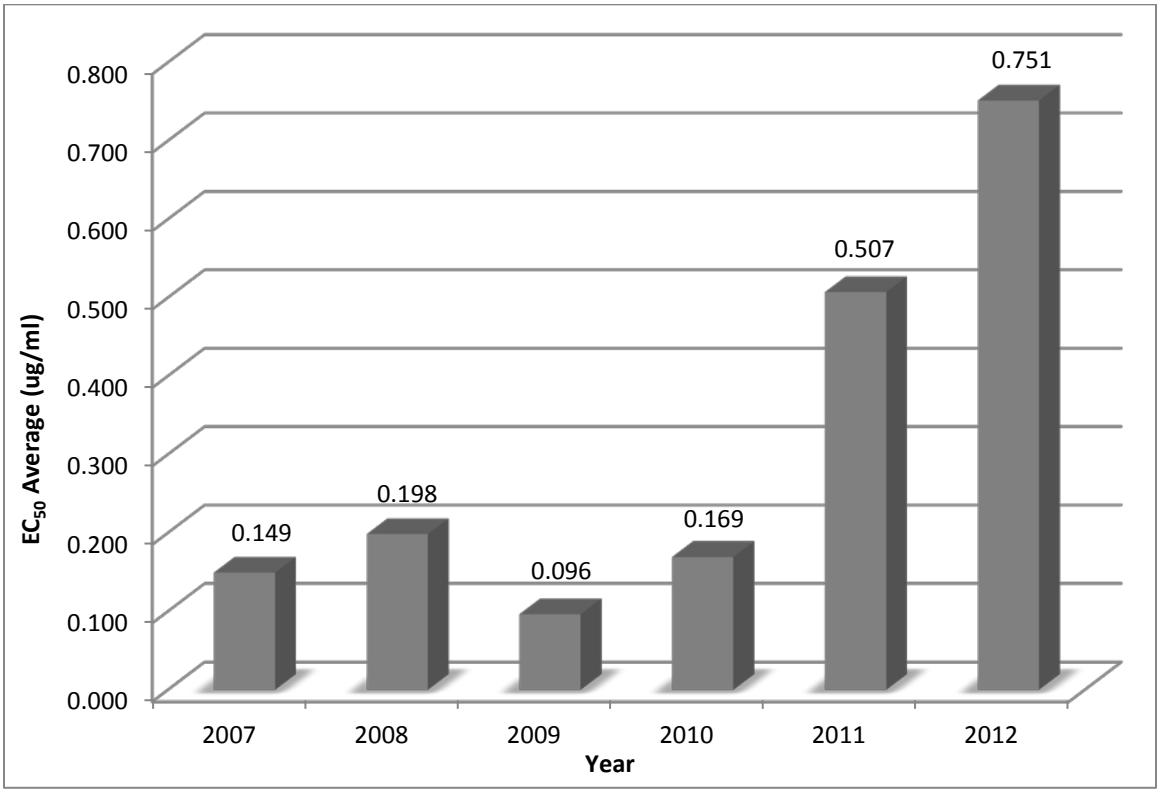


Figure 4. Average EC-50 values of *C. beticola* isolates collected in 2009-2012 to Eminent by factory district

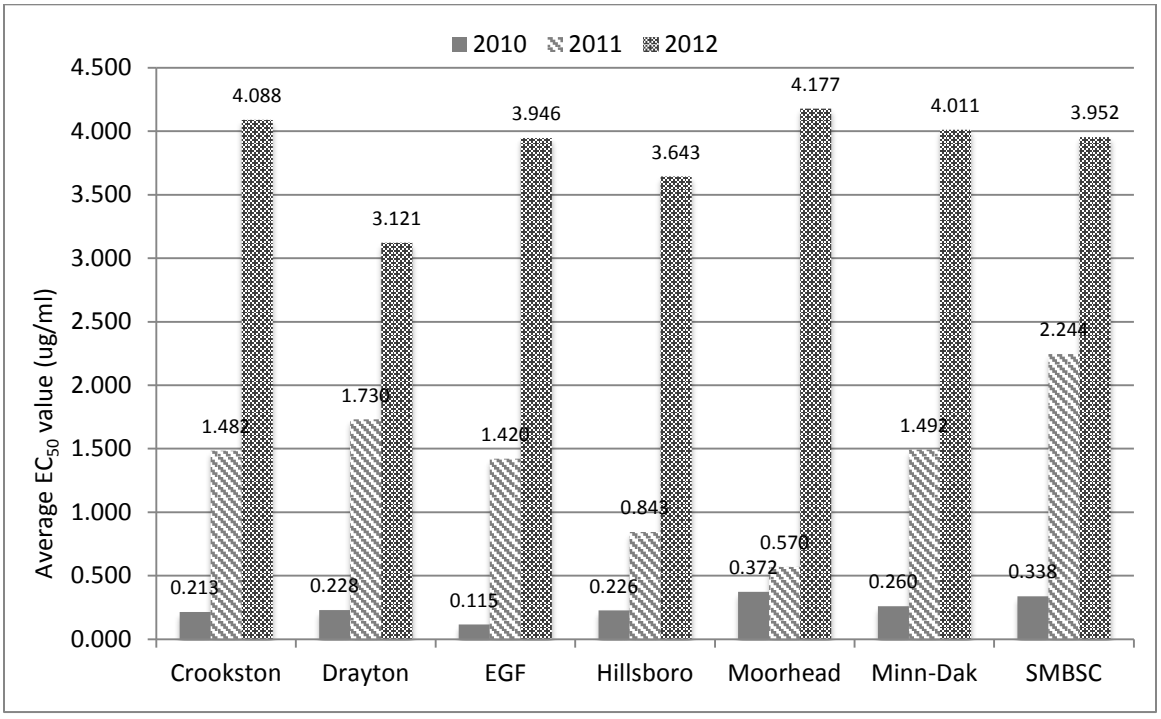

Figure 5. Average $EC_{50}$ values of *C. beticola* isolates collected in 2009-2012 to Inspire by factory district