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# Fungicide Sensitivity Characteristics of *Cercospora beticola* Isolates Recovered from the High Plains of Colorado, Montana, Nebraska, and Wyoming.

## 2. Mancozeb, Propiconazole, and Azoxystrobin.

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### ABSTRACT

Sugarbeet (*Beta vulgaris* L.) growing areas in northeastern Colorado, southeastern Montana and southwestern Nebraska were surveyed in 1998 through 2001 to determine if insensitive *Cercospora beticola* isolates were present in fields. One field in southeast Wyoming was included in the 1999 and 2000 surveys. A total of 328 isolates recovered from 110 fields in 1998, 305 isolates recovered from 101 fields in 1999, 255 isolates recovered from 72 fields in 2000, and 256 isolates recovered from 85 fields in 2001 was tested in this study. The radial growth of each isolate on potato dextrose agar (PDA) was compared to its radial growth on PDA amended with 5  $\mu\text{g mL}^{-1}$  mancozeb, 1 and 10  $\mu\text{g mL}^{-1}$  propiconazole and 1 and 10  $\mu\text{g mL}^{-1}$  azoxystrobin. The overall percentages of isolates that grew in the presence of 5  $\mu\text{g mL}^{-1}$  mancozeb were 92% in 1999, 88% in 2000 and 90% in 2001. Overall percentages of isolates that grew in the presence of 1  $\mu\text{g mL}^{-1}$  propiconazole were 2% in 1999, 0.4% in 2000 and 0% in 2001. The overall percentages of isolates that grew in the presence of 1  $\mu\text{g mL}^{-1}$  azoxystrobin were 99.7% in 1998 and 98% in both 2000 and 2001. Data reported here establishes baseline characteristics of the current *C. beticola*

**population infecting sugarbeet in the High Plains regions  
of Colorado, Montana, Nebraska and Wyoming.**

**Additional Key Words:** *Beta vulgaris*, Cercospora leaf spot, disease management, survey.

*Cercospora beticola* Sacc. is the causal agent of Cercospora leaf spot (CLS) in sugarbeet (Whitney and Duffus, 1986). The fungus is widely distributed in the High Plains production region. If CLS lesions cover more than 3% of the foliage this causes a marked reduction in root weight and sugar yield (Windels *et al.*, 1998).

Foliar fungicides frequently are used to help manage CLS. However, sugarbeet growers in many regions of the world have observed the emergence of fungicide tolerant and/or resistant isolates of *C. beticola* (Campbell *et al.*, 1998). Resistance to benzimidazole and tolerance to triphenyltin hydroxide fungicides has been detected in many sugarbeet growing areas of the United States (Campbell *et al.*, 1998) and more recently in the High Plains regions of Colorado, Montana, Nebraska and Wyoming (Briere *et al.*, 2001).

An effort to identify new fungicide chemistries that suppress CLS development is ongoing. For example, recently published research has reported the field screening of fungicides for CLS control in Wyoming (Stump *et al.*, 1999 and 2000) and Minnesota (Khan *et al.*, 2000a,b,c). These reports reveal that the efficacy of recently developed fungicide formulations containing triazole (e.g. propiconazole or tetraconazole) or azoxystrobin was similar to those of traditional fungicide chemistries against sensitive isolates. New fungicide chemistries that can be incorporated into current CLS management programs are important tools that may be used to reduce or delay the development of fungicide resistance in the pathogen population.

Surveys that measure fungicide sensitivities in the pathogen population may detect emerging fungicide insensitivity before it is widespread, thus, permitting more appropriate fungicide use or greater reliance on other disease suppression tactics. Therefore, the goal of our survey was to determine the baseline fungicide sensitivity of the *C. beticola* population in the High Plains region so that emerging insensitivity may be detected by future surveys. This report summarizes results for propiconazole and azoxystrobin sensitivity before their widespread use on sugarbeet. We also measured the current baseline sensitivity to mancozeb, a fungicide used along with benzimidazole and triphenyltin hydroxide for many years in the same region. Baseline sensitivities to benzimidazole and triphenyltin hydroxide were previously reported (Briere *et al.*, 2001).

For results reported herein, sensitivity is defined as the amount (in percent) of the reduction of radial growth in the presence of fungicide relative to growth of the same isolate in the absence of fungicide. Conversely, insensitivity describes a *C. beticola* isolate's radial growth (in percent) on fungicide amended medium.

## MATERIALS AND METHODS

Sugarbeet leaves with CLS lesions were collected by Western Sugar Company personnel during routine field visits throughout the High Plains growing region. Leaf samples were collected from 110 fields in 1998, 101 fields in 1999, 72 fields in 2000, and 85 fields in 2001. Collections were made from Colorado (Weld, Logan, Morgan, and Sedgwick Counties), Montana (Big Horn, Yellowstone, Rosebud, Treasure, and Billings Counties), Nebraska (Morrill, Scotts Bluff, Box Butte, Keith, Perkins, Deuel, and Cheyenne Counties) and Wyoming (Goshen County). Most leaves were collected from mid-August to mid-September and all collections were made prior to root harvest. Immediately after collection, leaves were placed in a labeled envelope and shipped to a central laboratory in Alliance, NE. Upon receipt, leaves were air-dried at room temperature and stored for several months before *Cercospora* recovery was attempted. Because collections were only possible when symptomatic leaves were encountered, the survey was biased towards fields that had evident *Cercospora* leaf spot lesions present.

*C. beticola* was isolated from the stored leaves by placing sections of symptomatic leaf tissue into a 300 mL beaker. The beaker was covered with a fine mesh screen and placed under cool running tap water for 1 hour to aid removal of surface contaminants and to rehydrate the leaf tissue. Washed leaf sections were blotted on a paper towel and the central portion of lesions that contained stromata was excised with a flamed scalpel. The excised lesions were surface disinfested in 2% sodium hypochlorite for 30 to 60 seconds and rinsed in sterile distilled water for 1 minute. The excised lesion tissue was plated onto 2% water agar and then incubated at 22°C with a 12 hr photoperiod for 3 to 4 days. After incubation, excised lesion tissue was examined microscopically for the presence of slow-growing white mycelia characteristic of *C. beticola*. Mycelia from the edge of presumptive *C. beticola* colonies were removed and transferred onto half-strength Difco® potato dextrose agar (PDA). These cultures were incubated as described above. Resultant colonies were subcultured by hyphal-tip transfers onto amended Sugar Beet Leaf

Extract Agar (SBLEA) and incubated for 12 to 14 days at 22°C with a 12 hr photoperiod. The SBLEA (E. G. Ruppel, personal communication) was prepared by adding 250 g of sugarbeet leaves (previously washed and frozen) to 1 L of distilled water and boiling the mixture for 40 minutes in a microwave oven. The mixture was filtered through gauze and the filtrate volume was adjusted to 1 L with distilled water and then amended with 4 g L<sup>-1</sup> of glucose to promote mycelial growth. Extensive sporulation occurred after incubation of cultures on SBLEA and these cultures were stored at 5°C until needed. Each stored culture served as the source of each particular isolate for all subsequent experiments.

**Fungicide Sensitivity Tests:** Media for testing fungicide sensitivity was made by amending full-strength PDA prepared in 2 L Erlenmeyer flasks. Each flask received a stir bar to facilitate mixing of the amended medium while dispensing. The PDA was autoclaved at 121°C and 20 psi for 25 minutes and then cooled to approximately 48°C. Stock suspensions of 50 µg mL<sup>-1</sup> of mancozeb (Dithane® DF, Rohm and Haas), 50 µg mL<sup>-1</sup> of propiconazole (Tilt®, Syngenta) or 50 µg mL<sup>-1</sup> of azoxystrobin (Quadris®, Syngenta) prepared in sterile distilled water were added to the cooled medium to achieve the concentrations listed below. Fifteen mL of cooled amended medium was dispensed into each petri dish with the aid of an automatic dispensing unit. The poured plates were left to dry in the hood for 24 hours before use. The final fungicide concentrations tested were 5 µg mL<sup>-1</sup> mancozeb (1999, 2000, 2001), 1 µg mL<sup>-1</sup> (1999, 2000, 2001) and 10 µg mL<sup>-1</sup> (1998, 1999, 2000) propiconazole and 1 µg mL<sup>-1</sup> (1998, 2000, 2001) and 10 µg mL<sup>-1</sup> (1998, 1999, 2000) azoxystrobin.

Stored isolates to be tested on fungicide amended media were subcultured onto PDA and induced to sporulate as described in Briere *et al.*, (2001). A conidial suspension from each isolate was prepared by pipetting 1 mL of sterile distilled water onto approximately a 1 x 2 cm section of the colony. This area of the colony was lightly rubbed with a sterile glass rod to dislodge conidia from the mycelial mat. The conidial suspension, which also contained mycelial fragments, was collected with an Eppendorf Repeater Plus® pipettor fitted with a sterile 0.1 mL pipette tip. For each isolate, non-amended PDA and amended PDA plates were inoculated with three equally spaced 1 µL aliquots of the conidial suspension and incubated for 7 days at approximately 23°C.

The resultant colony diameter for each inoculation site was measured with a digital caliper and the mean value for the three inoculation sites was computed for each isolate. The percent inhibition of radial growth of each test isolate grown on fungicide-amended PDA was compared to its growth on non-amended PDA after 7 days. Because

the diameter of the initial inoculum drop was approximately 3 mm ( $\pm$  0.1 mm, 95% CI), 3 mm was subtracted from the mean colony diameter for each isolate before computing the percentage of growth inhibition in the presence of fungicide (Briere *et al.*, 2001). Isolates producing colonies with diameters greater than 3 mm after 7 days of incubation had some degree of "insensitivity" to the fungicide present in the amended medium.

## RESULTS AND DISCUSSION

Stromata are known to be important overwintering structures for *C. beticola* (Whitney and Duffus, 1986). Therefore, our survey represents the *C. beticola* population likely to overwinter and initiate infection during the next growing season. A total of 328, 305, 255 and 256 isolates was tested from field samples collected in 1998, 1999, 2000 and 2001, respectively. The average colony diameter after 7 days of growth on non-amended PDA was 16.04 mm ( $\pm$ 0.21 mm, 95% CI) for all isolates tested from 1998 through 2001.

Results for *C. beticola* isolates grown on PDA amended with 5  $\mu\text{g mL}^{-1}$  of mancozeb are shown in Table 1. Results for 1999 revealed that 24 isolates did not grow in the presence of 5  $\mu\text{g mL}^{-1}$  mancozeb and the remaining isolates grew with 26% to 99% inhibition. Although the sample size from Montana was smaller than other states surveyed, all isolates from Montana grew to varying degrees in the presence of mancozeb. Results for isolates recovered during the 2000 survey revealed that 31 isolates did not grow in the presence of 5  $\mu\text{g mL}^{-1}$  mancozeb. The growth of the remaining isolates ranged from no inhibition detected to 98% inhibition. All isolates recovered from Colorado and Wyoming grew to varying degrees in the presence of mancozeb. A total of 25 isolates in the 2001 survey did not grow on the 5  $\mu\text{g mL}^{-1}$  mancozeb amended medium. Ten isolates that grew with 20 percent or less inhibition (at least 80% growth) were detected among the 816 isolates tested during the three year survey. The frequency of detection was two of 297 isolates tested from Colorado, four of 112 isolates from Montana, four of 378 isolates from Nebraska, and zero of 29 isolates from Wyoming.

Isolate growth in the presence of 1  $\mu\text{g mL}^{-1}$  propiconazole is summarized in Table 2. A total of 300, 254 and 256 isolates recovered in 1999, 2000 and 2001, respectively, had no growth on the amended medium. The percent inhibition of the remaining isolates ranged from 44% to 97% in 1999 and one isolate had 46% inhibition in 2000. None of the isolates grew on the 1  $\mu\text{g mL}^{-1}$  propiconazole amended medium during the 2001 survey.

**Table 1.** Sensitivity of *Cercospora beticola* to 5 µg mL<sup>-1</sup> of mancozeb. Isolates were recovered from symptomatic leaves collected in 1999, 2000 and 2001 from Colorado, Montana, Nebraska and Wyoming.

Percent Inhibition*	1999 Survey					2000 Survey					2001 Survey			
	CO	MT	NE	WY	Tot.	CO	MT	NE	WY	Tot.	CO	MT	NE	Tot.
0 - 10	0	0	0	0	0	1	0	3	0	4	0	0	0	0
11 - 20	0	0	0	0	0	1	0	0	0	1	0	4	1	5
21 - 30	0	0	3	0	3	2	0	3	0	5	1	5	5	11
31 - 40	8	0	14	2	24	7	0	12	0	19	3	9	6	18
41 - 50	9	2	22	11	44	9	5	13	0	27	8	14	13	35
51 - 60	40	5	28	6	79	13	3	16	1	33	24	11	11	46
61 - 70	30	7	19	1	57	11	2	21	0	34	14	9	15	38
71 - 80	13	6	13	2	34	7	4	16	0	27	13	5	5	23
81 - 90	9	2	13	2	26	13	7	27	0	47	13	2	10	25
91 - 99	3	0	10	1	14	11	4	11	1	27	15	3	12	30
100	5	0	17	2	24	0	3	28	0	31	14	0	11	25
Total Tested	117	22	139	27	305	75	28	150	2	255	105	62	89	256

State codes: CO= Colorado, MT= Montana, NE= Nebraska, WY=Wyoming, Tot.=total isolates from all states

\* Percent Inhibition: Mean colony diameter for three replicates was first computed for both the amended and non-amended control for each isolate and 3 mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated; [(non-amended control-amended/non-amended control) X 100]

**Table 2.** Sensitivity of *Cercospora beticola* to 1  $\mu\text{g mL}^{-1}$  of propiconazole. Isolates were recovered from symptomatic leaves collected in 1999, 2000 and 2001 from Colorado, Montana, Nebraska and Wyoming.

Percent Inhibition*	1999 Survey					2000 Survey					2001 Survey			
	CO	MT	NE	WY	Tot.	CO	MT	NE	WY	Tot.	CO	MT	NE	Tot.
0 - 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11 - 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21 - 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31 - 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41 - 50	1	0	1	0	2	0	0	1	0	1	0	0	0	0
51 - 60	0	0	0	0	0	0	0	0	0	0	0	0	0	0
61 - 70	0	0	0	0	0	0	0	0	0	0	0	0	0	0
71 - 80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
81 - 90	1	0	0	0	1	0	0	0	0	0	0	0	0	0
91 - 99	1	0	1	0	2	0	0	0	0	0	0	0	0	0
100	115	22	136	27	300	75	28	149	2	254	105	62	89	256
Total Tested	118	22	138	27	305	75	28	150	2	255	105	62	89	256

State codes: CO= Colorado, MT= Montana, NE= Nebraska, WY= Wyoming, Tot.=total isolates from all states

\* Percent Inhibition: Mean colony diameter for three replicates was first computed for both the amended and non-amended control for each isolate and 3 mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated; [(non-amended control-amended/non-amended control) X 100]

A total of 327, 304 and 254 isolates, recovered in 1998, 1999 and 2000, respectively, did not grow in the presence of  $10 \mu\text{g mL}^{-1}$  propiconazole (data not shown). One isolate for each survey year grew on the amended medium, and these isolates had growth inhibitions of 39%, 42% and 64%, for 1998, 1999 and 2000 respectively. These isolates were recovered from Nebraska in 1998 and 2000 and from Colorado in 1999. The  $10 \mu\text{g mL}^{-1}$  concentration of propiconazole was not tested in 2001.

Results for *C. beticola* isolate growth in the presence of  $1 \mu\text{g mL}^{-1}$  of azoxystrobin are shown in Table 3. Results for 1998 revealed 100% inhibition of only one isolate, and that this isolate was recovered from Montana. The growth of the remaining isolates ranged from no inhibition detected to 92% inhibition. Results for isolates recovered during the 2000 survey revealed that four isolates did not grow in the presence of  $1 \mu\text{g mL}^{-1}$  azoxystrobin and that the growth of the remaining isolates ranged from no inhibition detected to 98% inhibition. A total of 6 isolates were recovered during the 2001 survey that did not grow on the  $1 \mu\text{g mL}^{-1}$  azoxystrobin amended medium. Growth of the remaining isolates revealed at least 21% inhibition in the presence of  $1 \mu\text{g mL}^{-1}$  azoxystrobin. The number of isolates that grew with 20% or less inhibition (at least 80% growth) was 24, 7 and 0 respectively for the 1998, 2000 and 2001 surveys.

Results for isolates recovered in 1998, 1999 and 2000 grown in the presence of  $10 \mu\text{g mL}^{-1}$  azoxystrobin are shown in Table 4. A total of two and seven isolates recovered in 1998 and 2000, respectively, did not grow on the amended medium. Growth of the remaining isolates ranged from no inhibition detected to 94% in 1998, 3% to 87% in 1999 and from no inhibition detected to 98% inhibition in 2000. The number of isolates that grew with 20% or less inhibition was 14, 3, and 10 for the 1998, 1999 and 2000 surveys, respectively.

These data reveal that several isolates recovered from Colorado and Nebraska in 2000 grew with less than 20% inhibition in the presence of  $5 \mu\text{g mL}^{-1}$  mancozeb. Mancozeb has been used extensively for many years as well as triphenyltin hydroxide and benzimidazole. The number and levels of insensitivity of isolates over the three years of the survey have been relatively constant. This leads us to believe that the insensitivity to mancozeb remains constant in the surveyed population.

In the first part of this study (Briere *et al.*, 2001), isolates that had simultaneous high insensitivity for both triphenyltin hydroxide and benzimidazole were not observed. The isolates reported here also were tested for growth on triphenyltin hydroxide and benzimidazole amended media. Some isolates insensitive to mancozeb also were insensitive to

**Table 3.** Sensitivity of *Cercospora beticola* to 1  $\mu\text{g mL}^{-1}$  azoxystrobin. Isolates were recovered from symptomatic leaves collected in 1998, 2000 and 2001 from Colorado, Montana, Nebraska and Wyoming.

Percent Inhibition*	1998 Survey				2000 Survey					2001 Survey			
	CO	MT	NE	Tot.	CO	MT	NE	WY	Tot.	CO	MT	NE	Tot.
0 - 10	0	9	2	11	1	0	2	0	3	0	0	0	0
11 - 20	0	4	9	13	1	0	3	0	4	0	0	0	0
21 - 30	1	11	28	40	3	1	3	1	8	0	0	1	1
31 - 40	24	8	46	78	4	2	8	1	15	4	0	1	5
41 - 50	66	15	36	117	29	3	68	0	100	20	10	12	42
51 - 60	31	10	10	51	29	8	34	0	71	25	15	16	56
61 - 70	4	4	2	10	8	7	16	0	31	34	12	31	77
71 - 80	2	3	0	5	0	0	6	0	6	20	12	23	55
81 - 90	0	0	1	1	0	3	7	0	10	2	6	3	11
91 - 99	0	1	0	1	0	3	0	0	3	0	2	1	3
100	0	1	0	1	0	1	3	0	4	0	5	1	6
Total Tested	128	66	134	328	75	28	150	2	255	105	62	89	256

State codes: CO= Colorado, MT= Montana, NE= Nebraska, WY= Wyoming, Tot.=total isolates from all states

\* Percent Inhibition: Mean colony diameter for three replicates was first computed for both the amended and non-amended control for each isolate and 3 mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated; [(non-amended control-amended/non-amended control) X 100]

**Table 4.** Sensitivity of *Cercospora beticola* to 10 µg mL<sup>-1</sup> of azoxystrobin. Isolates were recovered from symptomatic leaves collected in 1998, 1999 and 2000 from Colorado, Montana, Nebraska and Wyoming.

Percent Inhibition*	1998 Survey				1999 Survey					2000 Survey				
	CO	MT	NE	Tot.	CO	MT	NE	WY	Tot.	CO	MT	NE	WY	Tot.
0 - 10	0	7	4	11	1	0	1	0	2	1	0	4	0	5
11 - 20	0	1	2	3	1	0	0	0	1	2	0	3	0	5
21 - 30	2	5	10	17	1	0	4	0	5	2	1	1	1	5
31 - 40	17	11	52	80	20	1	32	2	55	5	1	1	1	8
41 - 50	53	14	51	118	68	3	68	10	149	12	1	14	0	27
51 - 60	45	6	14	65	24	8	27	16	75	17	2	29	0	48
61 - 70	8	11	0	19	2	3	4	0	9	23	2	55	0	80
71 - 80	3	3	0	6	0	5	1	0	6	10	12	24	0	46
81 - 90	0	5	0	5	0	1	2	0	3	3	2	9	0	14
91 - 99	0	1	1	2	0	0	0	0	0	0	5	5	0	10
100	0	2	0	2	0	0	0	0	0	0	2	5	0	7
Total Tested	128	66	134	328	117	22	138	27	305	75	28	150	2	255

State codes: CO= Colorado, MT= Montana, NE= Nebraska, WY= Wyoming, Tot.=total isolates from all states

\* Percent Inhibition: Mean colony diameter for three replicates was first computed for both the amended and non-amended control for each isolate and 3 mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated; [(non-amended control-amended/non-amended control) X 100]

either benzimidazole or triphenyltin hydroxide. Köller and Wilcox (2001) studied fungicide resistant populations of the apple scab fungus, *Venturia inaequalis*, and proposed that resistance to one class of fungicides can accelerate the speed by which mutants resistant to a second independent class of fungicides are selected. Thus, they concluded that selection for the trait of fungicide resistance also might select for individuals with increased genetic plasticity and increased ability for adaptation to environmental challenges, including fungicide exposure.

Tests with propiconazole revealed consistent suppression of isolate growth with few exceptions. Based on survey data from 1999 through 2001 and field trials of fungicide efficacy (Stump *et al.*, 1999, 2000; Khan, 2000a,b,c), propiconazole appears to be a good candidate for CLS suppression and a good fungicide-partner for fungicide-resistance management programs.

Reaction of *C. beticola* isolates to azoxystrobin appeared to be uniform over the three years of the survey. However, the increase of azoxystrobin concentration from 1 to 10  $\mu\text{g mL}^{-1}$  did not greatly affect the growth of isolates. A possible explanation is that increasing the azoxystrobin concentration did not increase the soluble fraction of azoxystrobin in the medium and only increased the insoluble fraction. The material safety data sheet for azoxystrobin reports an azoxystrobin solubility of 6  $\text{mg L}^{-1}$  water at 20°C (Syngenta Crop Protection, Inc., Greensboro, NC 27419). Enhancing the solubility of azoxystrobin in the growth medium may improve the ability to differentiate between sensitive and insensitive *C. beticola* isolates during future surveys. However, two other strobilurin formulations (Flint® and Headline®) were included in the 2001 survey (Briere *et al.*, unpublished) and the isolates displayed levels of insensitivity on those amended media similar to that observed for azoxystrobin. This leads us to conclude that the solubility of the formulation may not totally explain the high levels of insensitivity to azoxystrobin. A defined growth medium rather than a general growth medium such as PDA may more be more effective for differentiating *in vitro* fungal growth response in the presence of strobilurin fungicide. Also, the only variable we measured was vegetative growth of isolates on the amended medium. Therefore, additional measurable factors that may be affected by exposure to azoxystrobin, such as conidial formation and germination, also may be appropriate for studies that determine baseline sensitivity. However, the results for azoxystrobin insensitivity reported here are useful in that they provide the current vegetative growth sensitivity baseline for the High Plains region.

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