Fungicide Sensitivity Characteristics of *Cercospora beticola* Isolates Recovered from the High Plains of Colorado, Montana, Nebraska, and Wyoming. 1. Benzimidazole and Triphenyltin Hydroxide.

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ABSTRACT

The appearance of *Cercospora beticola* isolates with insensitivity to triphenyltin hydroxide (TPTH) or benzimidazole (BM) fungicides is a problem in many sugarbeet growing areas of the world including the United States. In 1998 and 1999 sugarbeet growing areas in northeastern Colorado, southeastern Montana and southwestern Nebraska were surveyed to determine if insensitive isolates were present. One field in southeast Wyoming was included in the 1999 survey. There were 328 isolates recovered from 110 fields in 1998 and 305 isolates from 101 fields in 1999. Radial growth of isolates on potato dextrose agar amended with 1, 5, and 10μg mL^{-1} TPTH and 5μg mL^{-1} BM was compared to growth in the absence of fungicide. Percent inhibition of radial growth in the presence of 1μg mL^{-1} TPTH ranged from 17% to 100% in 1998 and 37% to 100% in 1999. More inhibition of radial growth was observed as the concentration of TPTH increased. Results for 5.0μg mL^{-1} BM revealed that 72 of the 328 isolates grew with 20% or less inhibition in 1998 and 87 of 305 isolates grew with 40% or less inhibition in 1999. Insensitivity to BM is more pronounced in Colorado and Nebraska when compared to Montana, presumably due to greater fungicide use in the more southerly production areas. Data reported here establishes baseline characteristics of the current *C. beticola* population.

Additional Key Words: *Beta vulgaris*, *Cercospora beticola*, systemic fungicides, protectant fungicides, disease management, leaf spot.

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, sugar yield and increased impurities during processing (Windels *et al.*, 1998).

Sugarbeet growers in many regions of the world have observed the emergence of fungicide tolerant and/or resistant isolates of *C. beticola*. Resistance to benzimidazole fungicides was detected in Greece in 1972 (Georgopoulos and Dovas, 1973) and triphenyltin hydroxide (TPTH) tolerance was subsequently found in Greece, Italy and Yugoslavia in the late 1970s and early 1980s (Giannopolitis, 1978; Cerato and Grassi, 1983; and Maric *et al.*, 1984). In the United States, resistance to benzimidazole fungicides was first detected in 1973 (Ruppel and Scott, 1974). More recently, Bugbee (1995) demonstrated that many *C. beticola* isolates recovered from sugarbeet fields in Minnesota were tolerant to TPTH. Campbell *et al.* (1998) also reported tolerance to TPTH and resistance to benzimidazole in North Dakota and Minnesota.

The High Plains region includes northeastern Colorado, eastern Montana, western Nebraska and Wyoming. During the past several years, the incidence and severity of CLS have increased in the High Plains.

The terms tolerance and resistance are sometimes used interchangeably. For this paper, the term insensitivity is used to describe a *C. beticola* isolate's ability to grow on a fungicide-amended medium. The objective was to survey representative fields throughout the High Plains to determine current baseline fungicide insensitivity in the *C. beticola* population.

MATERIALS AND METHODS

Symptomatic leaves with CLS lesions (circular leaf spots with tan to grayish centers and brown to reddish borders) were collected by Western Sugar personnel during routine field visits. Because collections were only possible when symptomatic leaves were encountered, the survey was biased towards fields that had some amount of Cercospora leaf spot present. 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. Leaf samples were collected from 110 fields in 1998 and 101 fields in 1999 throughout the High Plains growing regions: from Colorado (Weld, Logan, Morgan, and

Sedgwick Counties); Montana (Big Horn, Yellowstone, Rosebud, Treasure, and Billings Counties); Nebraska (Morrill, Scottsbluff, Box Butte, Keith, Perkins, Deuel, and Cheyenne Counties); and Wyoming (Goshen County) in 1999. Upon receipt, leaves were scrutinized to verify the presence of presumptive Cercospora lesions. Leaves with presumptive Cercospora lesions were air-dried at room temperature and stored for several months before Cercospora recovery was attempted.

Isolation of *C. beticola* from the stored leaves was done 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 portions of lesions that contained stromata were excised with a flamed scalpel. Working in a laminar flow hood, the excised lesions were surface sterilized 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 mycelium characteristic of *C. beticola*. Mycelia from the edge of presumptive *C. beticola* colonies was removed and transferred onto half-strength Difco® potato dextrose agar (PDA). These cultures were incubated as described above. Colonies were subcultured by hyphal-tip transfers onto amended Sugarbeet Leaf Extract Agar (SBLEA) and incubated as described above for 12 to 14 days. The SBLEA (E. G. Ruppel, personal communication) was prepared by adding 250g of sugar beet leaves to 1L of distilled water and heating the mixture in a microwave oven for 40 minutes on the high setting. The mixture was then filtered through cheese cloth and the filtrate volume was adjusted to 1L with distilled water. The SBLEA was amended with the addition of 4g/L of glucose to promote mycelial growth. Extensive sporulation occurred after 12 to 14 days of incubation and these cultures were stored at 5°C until needed. Each culture served as the source of each particular strain for all subsequent experiments.

Fungicide Sensitivity Tests:

The media for testing fungicide sensitivity was made by first preparing PDA in 2L Erlenmeyer flasks. Each flask received a stir bar before autoclaving to aid in the 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 100 ppm of triphenyltin hydroxide (TPTH; Supertin® 80WP, Griffin L.L.C.) and 50 ppm of methyl benzimidazolecarbamate (BM; Benlate®, du Pont de Nemours and Company) prepared in sterile distilled water were added 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 concentrations of amended media prepared were TPTH 1μg mL^{-1}, TPTH 5μg mL^{-1}, TPTH 10μg mL^{-1} and BM 5μg mL^{-1}. Each isolate tested on fungicide-amended media was subcultured from the stored source plate onto PDA and incubated for 12 to 14 days at 23°C with a 12 hr photoperiod. Conidial suspensions from each isolate were prepared by pipetting 1 mL of sterile distilled water on a 1 x 2 cm section of the colony. This area of the colony was lightly rubbed with a sterile glass rod to loosen conidia from the mycelia. The conidial suspension 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 1.0 μL aliquots of the conidial suspension. Approximately 40 isolates were tested in each batch. *Cercospora beticola* strains with known insensitivity to TPTH and BM (provided by J. J. Weiland, USDA, Fargo, N.D.) were used as insensitive controls in each batch. Inoculated plates were incubated at 22°C with a 12 hr photoperiod. The colony diameter of each colony was measured after 7 days.

The percent inhibition of each test isolate grown on each amended medium was compared to its growth on non-amended PDA. The diameter of the three inoculation sites was measured with a metric ruler the first year and a digital caliper the second year. The mean value for all three colonies for each treatment was computed. The diameter of the initial inoculum drop on the plate was approximately 3mm and this diameter was subtracted from the mean colony diameter for both the amended and non-amended control for each isolate before computing percent inhibition. The percent inhibition for each isolate was then calculated according to Bugbee (1995) with the following equation, [(non-amended control - amended / non-amended control) X 100]. Colonies with diameters greater than 3mm were considered insensitive.

RESULTS AND DISCUSSION

Recovery of *C. beticola* from rehydated leaves proved problematic at first. Initial recovery efforts concentrated on excising the whole lesion including a thin area of green tissue bordering the lesion. However, other fungi and bacteria competing with *C. beticola* complicated recovery. Subsequent recovery efforts concentrated on excising only the central portion of the lesion which included only necrotic tissue and stromata of *C. beticola*. This method greatly increased the efficiency of *C. beticola* isolation. Also, because stromata are known to be important overwintering structures for this fungus (Whitney and Duffus, 1986), our survey represented the *C. beticola*

population likely to overwinter and initiate infection during the next growing season. Since each isolate recovered came from a distinct lesion, we assumed it was distinct from its neighbors. A total of 328 and 305 isolates were recovered from field samples in 1998 and 1999, respectively.

Increasing TPTH concentration increased inhibition of radial growth of *C. beticola* isolates (Tables 1 and 2). Results for 1998 (Table 1) reveal that all isolates except one grew in the presence of 1 μg mL^{-1} TPTH. Growth rate data for the remaining 327 isolates ranged from 17% to 93% inhibition relative to the non-amended control. In the presence of 5.0 μg mL^{-1} TPTH, 161 of the 328 isolates grew with a range of 62% to 99% inhibition. At 10.0 μg mL^{-1} TPTH, 156 of the 328 isolates grew with a range of 72% to 99% inhibition. The 1999 survey (Table 2) revealed that 303 of the 305 isolates grew in the presence of 1.0 μg mL^{-1} TPTH with growth rates that ranged from 37% to 94% inhibition. At 5.0 μg mL^{-1}, 25 of the 305 isolates grew with a range of 88% to 98% inhibition and at 10.0 μg mL^{-1}, two of the 305 isolates grew with a range of 92% to 93% inhibition.

Results for 1998 revealed that 72 of the 328 isolates grew with 20% or less inhibition in the presence of 5.0 μg mL^{-1} BM (Table 3). The 1999 survey revealed that 87 of 305 isolates grew with 40% or less inhibition in the presence of 5.0 μg mL^{-1} BM. During 1998, 66 isolates recovered from 17 Montana fields were all sensitive to 5.0 μg mL^{-1} BM. However, the 1999 survey revealed 4 of 22 isolates from 5 fields tested were insensitive to 5.0 μg mL^{-1} BM. All four insensitive isolates were recovered from the same field. Fungicide activity in each batch of media was verified with the known TPTH and BM insensitive isolates. The TPTH insensitive isolate was inhibited 13% to 18% (average 16%) in the presence of 1.0 μg mL^{-1} TPTH. The BM insensitive isolate was inhibited 0% to 17% (average 8%) in the presence of 5.0 μg mL^{-1} BM.

Data summarized in the tables reveals the spectrum of fungicide sensitivity measured in the High Plains *C. beticola* population. The baseline sensitivity for this population before exposure to TPTH and BM is not known because surveys were not conducted before their historical use. Although fungicide use for CLS management was typically sporadic, within the last several years fungicide application has become more routine due to an increased awareness of CLS and the need for disease management (S.N. Godby, personal communication). Although sugarbeet crop failure due to fungicide insensitivity has not been conclusively demonstrated in any field in the High Plains region, it is assumed that selection for TPTH and BM insensitivity is occurring. For example, the number of BM insensitive isolates recovered is proportionately greater in Colorado and Nebraska compared to Montana. This may be due to more frequent application of BM fungicide in more southerly production areas (S.N. Godby, personal communication). The same trend is

Table 1. Sensitivity of *Cercospora beticola* to three concentrations of TPTH. Isolates were recovered from symptomatic leaves collected in 1998 from Colorado, Montana and Nebraska.

	TPTH, μg mL^{-1}											
Percent	1.0				5.0				10.0			
Inhibition*	CO	MT	NE	Tot.	CO	MT	NE	Tot.	CO	MT	NE	Tot.
0 – 10	0	0	0	0	0	0	0	0	0	0	0	0
11 - 20	1	0	1	2	0	0	0	0	0	0	0	0
21 - 30	1	0	0	1	0	0	0	0	0	0	0	0
31 - 40	23	22	16	61	0	0	0	0	0	0	0	0
41 - 50	27	27	39	93	0	0	0	0	0	0	0	0
51 - 60	23	7	21	51	0	0	0	0	0	0	0	0
61 - 70	20	6	27	53	0	0	1	1	0	0	0	0
71 - 80	22	2	20	44	1	0	1	2	0	0	1	1
81 - 90	11	2	7	20	56	12	28	96	6	3	5	14
91 - 99	0	0	2	2	21	13	28	62	32	2	107	141
100	0	0	1	1	50	41	76	167	90	61	21	172
Total Tested	128	66	134	328	128	66	134	328	128	66	134	328

State codes: CO= Colorado, MT= Montana, NE= Nebraska

*Percent Inhibition: Mean colony diameter was first computed for both the amended and non-amended control for each isolate and 3mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated according to Bugbee (1995); [(non-amended control-amended/non-amended control) X 100]

Table 2. Sensitivity of *Cercospora beticola* to three concentrations of TPTH. Isolates were recovered from symptomatic leaves collected in 1999 from Colorado, Montana, Nebraska and Wyoming.

	TPTH, $\mu g\ mL^{-1}$														
Percent	1.0					5.0					10.0				
Inhibition*	CO	MT	NE	WY	Tot.	CO	MT	NE	WY	Tot.	CO	MT	NE	WY	Tot.
0 - 10	0	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	0
21 - 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31 - 40	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
41 - 50	10	0	12	13	35	0	0	0	0	0	0	0	0	0	0
51 - 60	27	2	49	9	87	0	0	0	0	0	0	0	0	0	0
61 - 70	22	2	28	4	56	0	0	0	0	0	0	0	0	0	0
71 - 80	39	10	23	1	73	0	0	0	0	0	0	0	0	0	0
81 - 90	15	8	23	0	46	1	0	3	4	8	0	0	0	0	0
91 - 99	4	0	1	0	5	2	0	5	10	17	0	0	0	2	2
100	1	0	1	0	2	115	22	130	13	280	118	22	138	25	303
Total Tested	118	22	138	27	305	118	22	138	27	305	118	22	138	27	305

State codes: CO= Colorado, MT= Montana, NE= Nebraska, WY= Wyoming

*Percent Inhibition: Mean colony diameter was first computed for both the amended and non-amended control for each isolate and 3mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated according to Bugbee (1995); [(non-amended control-amended/non-amended control) X 100]

Table 3. Sensitivity of *Cercospora beticola* to 5.0 μg mL^{-1} of benzimidazole. Isolates were recovered from symptomatic leaves collected in 1998 and 1999 from Colorado, Montana, Nebraska and Wyoming.

Percent Inhibition*	1998 Survey				1999 Survey				
	CO	MT	NE	Tot.	CO	MT	NE	WY	Tot.
0 - 10	58	0	8	66	15	3	6	0	24
11 - 20	5	0	1	6	17	0	6	0	23
21 - 30	0	0	0	0	19	0	8	1	28
31 - 40	0	0	0	0	3	1	8	0	12
41 - 50	0	0	0	0	0	0	1	0	1
51 - 60	0	0	0	0	0	0	1	0	1
61 - 70	0	0	0	0	0	0	0	0	0
71 - 80	0	0	0	0	0	0	0	0	0
81 - 90	0	0	0	0	0	0	0	0	0
91 - 99	0	0	0	0	0	0	0	0	0
100	65	66	125	256	64	18	108	26	216
Total Tested	128	66	134	328	118	22	138	27	305

State codes: CO= Colorado, MT= Montana, NE= Nebraska, WY= Wyoming

*Percent Inhibition: Mean colony diameter was first computed for both the amended and non-amended control for each isolate and 3mm was subtracted from each value to account for the initial inoculum deposition area. The percent inhibition for each isolate was calculated according to Bugbee (1995); [(non-amended control-amended/non-amended control) X 100]

not readily apparent for TPTH insensitivity. The data set summarized here establishes baseline characteristics of the current *C. beticola* population and will serve as a reference for future surveys.

At the time of leaf collection approximately 25% of the growers reported their current fungicide spray program. In some instances, we were able to isolate TPTH and BM insensitive strains of *C. beticola* from the same field sample despite the growers practice of applying up to three different fungicide chemistries (Supertin, Benomyl and Mancozeb) during the growing season. However, we did not find any single isolate that was insensitive to both TPTH and BM. This result shows that the insensitive strains persist in these fields despite the mixing of fungicide chemistries. It also shows that successive years of fungicide anti-resistance management strategies in the whole High Plains production area may be needed to reduce the numbers of insensitive isolates in the *C. beticola* population. It also emphasizes the need to incorporate new fungicide chemistries into current CLS management programs.

The High Plains production region must continue to develop CLS management programs that minimize the selection pressure exerted by any one fungicide. New fungicide chemistries in development will prove useful for resistance management programs when used to supplement fungicides currently utilized in CLS management programs. The survey reported here revealed that insensitive *C. beticola* isolates are present in the production region and, as a result, educational programs were developed that alert growers to the potential ramifications of this finding. The goal is to develop a robust integrated pest management approach for CLS management in the High Plains that includes use of resistant varieties, cultural practices that reduce inoculum, disease forecasts, and the appropriate use of fungicide as it relates to efficacy and fungicide resistance management.

ACKNOWLEDGMENT

The authors gratefully acknowledge Steve Godby and Western Sugar Company's fieldmen for collecting leaf samples during the course of this two year study. The Western Sugar Company-Grower Joint Research Committee provided partial funding for this research project.

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