Growth Promotion May Compensate for Losses Due to Moderate Aphanomyces Root Rot

Michael S. Metzger[1,3] and John J. Weiland[1,2]

[1]Department of Plant Pathology, North Dakota State University, Fargo ND, 58105 USA and [2]USDA-ARS, Red River Valley Agricultural Research Center, Sugarbeet and Potato Research Unit, Fargo, ND 58105 USA. [3]Current Address: Minn-Dak Farmers Coop, Wahpeton, ND 58075 USA. Corresponding author: John Weiland (john.weiland@ ars.usda.gov)

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ABSTRACT

A two-year study investigated the use of chemically-induced resistance and biocontrol bacteria for reducing sugarbeet root rot disease caused by the oomycete organism *Aphanomyces cochlioides*. Stand establishment, yield, and quality analysis of sugarbeet from replicated field plots, as well as root rot of seedlings grown in controlled conditions, were analyzed. Bacterial isolates AMMDR1 of *Burkholderia cepia* and PRA25rifz of *Pseudomonas fluorescens* were tested for their ability to inhibit reductions in stand and yield caused by *A. cochlioides*. A commercially available inducer of systemic resistance (harpin protein formulated as Messenger™) also was tested in the field for the ability to reduce root rot disease, whereas the inducers harpin, salicylic acid, and riboflavin were tested in growth-chamber studies. Field and growth chamber data combined suggested that a subset of the biological treatments in combination with chemical treatments enhanced root yield and recoverable sugar over control treatments even when stand and root rot ratings were unimproved. Integration of induced resistance and biocontrol with cultural practices, chemical treatments, and heritable resistance may lead to

improved control of *Aphanomyces* diseases of sugarbeet.

Additional key words: *Beta vulgaris*, *Aphanomyces cochlioides*, biocontrol, *Pseudomonas fluorescens*, *Burkholderia cepacia*, harpin, induced systemic resistance.

Seedling damping off and chronic root rot of sugarbeet caused by *Aphanomyces cochlioides* Drecsh. (Dreschler, 1928) has caused historic and recent losses to producers in the Red River Valley of Minnesota and North Dakota and other production regions of the United States. The pathogen is an oomycete, distantly related to *Pythium* and *Phytophthora* (Drechsler 1929, Dick, 1990). Zoospores are the infectious entity produced by the pathogen, whereas oospores are the stable resting stage of the organism, capable of surviving many years in the infested soil (Papavizas and Ayers, 1974; Park and Grau 1992). Seedling root rot disease is favored by warm, wet soils (Duffus and Ruppel, 1993); hence stand establishment is improved in soils known to contain the pathogen when seed is sown early in the spring when temperatures are cooler. Chronic root rot typically occurs in June and July in Minnesota and North Dakota following wet periods with seasonable temperatures. In 2000 alone, estimates of nearly 20% locally (Fargo, ND) and 4% total, of the sugarbeet hectares in the American Crystal Sugar Company's factory districts were abandoned due to the chronic root rot caused by this pathogen (A. Cattanach, American Crystal Sugar Company, pers. comm.).

Although resistance active in young and mature beets has been characterized (Coe and Scheider, 1966), seedlings must be protected from *A. cochlioides* by seed treatment with the chemical hymexazol (Tachigaren™; Windels, 1990; Payne and Williams, 1990). Since protection of the crop by this solitary compound is considered precarious, new measures for controlling black root and root rot disease continue to be investigated. Biocontrol using beneficial bacteria or fungi offers one avenue for disease control that potentially would expedite product registration and provide season-long crop protection (Cook and Baker, 1983; Handelsman and Stabb, 1996). Biocontrol studies using bacterial species antagonistic to *A. cochlioides* have been described (Jacobsen et al., 2000; Williams and Asher, 1996; Kristek et al., 2006), but to date none are used in major sugarbeet producing regions. This is due in part to the lack of bacterial strains proven to provide protection under a wide range of environments, to the formulation of such organisms to meet industry and regulatory standards and practices, and to education regarding the most effective way to implement a biocontrol partner in an integrated

disease-management scheme (Cook and Baker, 1983; Weller, 2007).

Lack of consistent control measures for chronic root rot disease caused by *A. cochlioides* prompted the initiation of a study aimed at the discovery of new, safe components for disease control that would simultaneously accelerate the transfer of any discovered technology to producers. During the 2001 and 2002 growing seasons, two biological control bacteria known to suppress the pathogen *Aphanomyces euteiches* (King and Parke, 1993), the causal agent of pre- and post-emerge damping-off in pea and a close relative of *A. cochlioides*, were field tested along with an inducer of systemic resistance for their ability to control Aphanomyces root rot on sugarbeet in the Red River Valley of Minnesota and North Dakota. The experiments were paralleled by growth chamber tests for systemic inducer and biocontrol protection of sugarbeet seedlings against black root disease.

MATERIALS AND METHODS

Growth Chamber Studies

Seed of Maribo 9369 (American Crystal Sugar Coop, Moorhead, MN), a hybrid susceptible to *A. cochlioides*, was treated with metalaxyl, thiram and hymexazol according to industry standards (McMullen and Bradley 2002), with the application of hymexazol at 45 g per 100,000 seeds. Seed was sown in Sunshine Mix #1 (Sungro Horticulture, Seba Beach, Canada) and sprouted to a stand of 5 seedlings per conetainer (Stuewe and Sons, Corvallis OR) in a greenhouse with an average temperature of 22°C. The bacterial species *P. fluorescens* PRA25rifz and *B. cepacia* AMMDR1 (generously provided by Dr. Jennifer Parke, Oregon State University) were maintained on nutrient agar or in liquid nutrient broth (BD Biosciences, Franklin Lakes, NJ).

The bacterial isolates *B. cepacia* AMMDR1 and *P. fluorescens* PRA25rifz were aseptically cultured in nine-centimeter Petri dishes containing standard nutrient agar. After incubation at 22°C for 24 h, the bacterial isolates were transferred into 500 ml of nutrient broth. The cultures were incubated at 26°C rotating at 100 rpm for 48 h after which they were analyzed for optical absorbance (Ultrospec 4050 spectrophotometer, Cambridge, England). Bacterial cell concentrations of 109 CFU per ml were used for seed treatments in 2001 and concentrations of 10^9 (*B. cepacia*) and 10^7 (*P. fluorescens*) CFU per ml were used in 2002 (Madigan et al. 1997). For the 2001 tests, approximately 300 g of untreated medium-sized Maribo 9369 sugarbeet seed was added to the *B. cepacia* AMMDR1 liquid culture and allowed to soak on an orbital shaker for 2 h at 100 rpm. Seeds were then transferred with a stainless

steel spatula onto several flat 30.48 by 60.96 centimeter plastic trays and placed into a laminar flow hood (Environmental Air Control Inc., Model 6467, Hagerstown, MD) overnight for air-drying. Fungicide coatings on the seeds necessitated treatment of the 2002 bacterial applications differently from 2001 applications. In 2002, 600 g of seed to be treated with bacteria were distributed in a tray, leveled by hand, and placed inside of a fume hood (Model PL-301, Two Rivers, WI) to aid in air-drying. Bacterial suspensions in nutrient broth were sequentially applied (~5 ml per application) to each of the three seed variables (Table 1) at 30 min time intervals utilizing an air-powered liquid atomizer (Model #15, Devilbiss, Somerset, PA) pressurized to 8.28 Pa^4. Seed was allowed to dry in between applications in an effort to retain the chemical fungicide coatings. A total of 10 applications were made amounting to 50 ml of applied bacterial suspension. Seed was dried overnight before packaging and was stored at 4°C for no longer than 20 days before planting.

Resistance inducing compounds (RIs) were applied to 14 day-old seedlings 2 days prior to inoculation with *A. cochlioides*. Solutions of the RIs harpin (Messenger™, Eden Biosciences, Bothell, WA), riboflavin, and salicylic acid (SA) were prepared in distilled water at the concentrations of 40 μg/ml (a 10-fold concentrate with respect to the field application rate recommended by the manufacturer), 7 μg/ml (Aver'yanov et al., 2000), and 2.8 mg/ml (Rasmussen et al., 1991), respectively, to a final volume of 50 ml. The RIs were transferred to a pressurized tank sprayer (Stanley Model 7402, Chapin Mfg., Batavia NY) adjusted to 6.9 Pa^4 and applied at a rate of 0.5 ml/conetainer.

Zoospores of *A. cochlioides* isolate 898A(IV) were produced by standard methods (Parke and Grau, 1992) and quantitated microscopically on a haemocytometer. Zoospore suspensions were applied to the conetainers in 5 ml aliquots resulting in seedling exposure to 30, 100, 300, 1000, and 10,000 spores per treatment. Plants were maintained in a growth chamber (Conviron Model PGR15, Winnipeg, Manitoba) at 26 degrees Centigrade under a 16 hr daylength until harvested for disease rating. The root rot index (RRI) described by Beale et al. (1994), calculated as

$$RRI = \frac{\Sigma \text{ (Disease rating X number of plants with rating)}}{\text{(Total number of emerged seedlings X 3)}} \text{ X } 100$$

was used to evaluate seedling damage at 6 days post-inoculation (dpi) using a rating scale of 0 = healthy root, 1 = light brown hypocotyl, water soaked, 2 = hypocotyl brown, moderate amount of constriction, and 3 =

hypocotyl brown, constricted or root dead.

Field Experiments

During both the 2001 and 2002 growing seasons, field plots were established within commercial fields located near Hillsboro, ND and Perley, MN contracted to American Crystal Sugar Company (Moorhead, MN) as Aphanomyces Specialty Sites and chosen for the testing of varietal response to this pathogen. Soils were indexed for Aphanomyces infestation according to Windels and Nabben-Schindler (1991). Seedlings were visually rated on a 0 to 3 scale as above. Root rot index values (0-100 scale, 0=Healthy, 100=Total Mortality) averaged 72 for Hillsboro and 68 for Perley in 2001, while the sites for 2002 averaged 88 and 64, respectively. As in the growth chamber studies, variety Maribo 9369 was used for all treatments and for soil indexing.

Treatments and Plot Design

At both locations and during both years, the experiment was arranged as a randomized complete block design. Each individual plot consisted of four rows, each 15.24 meters long and spaced at the sugarbeet production standard of 55.88 cm apart (0.0034 hectares per individual plot). A 3.05-meter alley for maintenance purposes separated all the ranges.

Three variables were evaluated during the 2001 growing season (Table 1). Each treatment was replicated four times at two separate locations. All seed used in 2001 lacked fungicide treatment. Treatments included an untreated check, weekly foliar treatments of emerged seedlings with the commercially-formulated harpin protein, and seed treated with *B. cepacia* AMMDR1 (Table 1). Seed treatment followed methods detailed above.

Eighteen variables were evaluated during the 2002 growing season (Table 1). Each variable was replicated three times at each location. Base seed treatments in 2002 included untreated seed and seed treated with commercial rates of metalaxyl (M; 113.6 g per cwt.) and thiram (T; 227.2 g per cwt.). Pelleted seed treated with commercial rates of metalaxyl and thiram (hence referred to as MT treated seed) and including hymexazol at a rate of 45g per 100,000 seeds. (referred to as MT + H treated seed) made up the third seed treatment. Biological control treatments for 2002 included the three seed variables listed above combined with the application of *B. cepacia* AMMDR1 and *P. fluorescens* PRA25rifz to the seed, and post-emergence foliar treatments with a formulation of the harpin protein. The three seed variables not receiving any type of biological treatments served as the untreated checks.

Table 1. Treatments used in the evaluation of induced resistance and biocontrol for the protection of sugarbeet against Aphanomyces root rot.[†]

Applied Treatment	Seed Treatment	Growth Chamber	2001 Field	2002 Field
B. cepacia - AMMDR1	Raw Seed		X	X
	Apron/Thiram			X
	Tach (45g)			X
Messenger - Micro Rate	Raw Seed			X
	Apron/Thiram			X
	Tach (45g)			X
Meddenger - 8x	Raw Seed			X
	Apron/Thiram			X
	Tach (45g)			X
Messenger - 12x	Raw Seed		X	X
	Apron/Thiram			X
	Tach (45g)			X
P. flourescens - PRA25rifz	Raw Seed			X
	Apron/Thiram			X
	Tach (45g)			X
Untreated Check	Apron/Thiram			X
	Raw Seed	X	X	X
	Tach (45g)	X		X
	P. flourescen	X		
	B. cepacia	X		
Riboflavin	Raw Seed	X		
	Tach (45g)	X		
	P. flourescen	X		
	B. cepacia	X		
Salicylic Acid	Raw Seed	X		
	Tach (45g)	X		
	P. flourescen	X		
	B. cepacia	X		
Messenger - 1x	Raw Seed	X		
	Tach (45g)	X		
	P. flourescen	X		
	B. cepacia	X		

[†] Treatments were applied at both the Perley and Hillsboro locations in both years.

[‡] Treatments of *B. cepacia* and *P. fluorescens* were applied to the seed: all other treatments were applied to seedling or young plant foliage.

Plot Planting, Plot Maintenance, and Stand Counts

The Hillsboro and Perley Aphanomyces Specialty locations were planted on 16 May and 20 May 2001, respectively, while the 2002 plots were seeded on 18 May and 7 May. Due to a killing frost, the 2002 Perley location was replanted on 29 May. Plots were managed to minimize weed populations (Dexter, et al., 1997), Cercospora leaf spot disease (Windels et al., 1998), and insect damage (Khan, 2006) using standard industry practices. Herbicide applications were made within 12 days of respective plots' planting date.

In both 2001 and 2002, stand counts were taken at 15, 30, and 45 days after planting, as well as a final count during harvest. Multiple seedlings are usually counted as a single plant if they emerge less than 2.54 cm apart (Steen, 2001). Due to lower than average populations (less than 150 plants per 30.48 m), however, multiple seedlings were counted as two plants regardless of distance.

Harpin Applications

A solution of harpin (11.45 liters containing 4 μg harpin/ml) was transferred to a modified backpack sprayer pressurized to 6.9 Pa[4] (in 2001) and 13.8 Pa[4] (in 2002) with CO_2. The solution was applied at the labeled rate of 0.011 kg ha^{-1} using 93.5 L ha^{-1} of water. The sprayer was calibrated to spray 4 rows in unison applying the harpin solution at a rate of 2.17 liters every 60 seconds.

Beginning immediately after seedling emergence, harpin was applied either on a weekly basis or according to scheduled herbicide treatments. The 2001 Hillsboro site received its first application on 23 May while applications at Perley were initiated one week later on 30 May. Research sites for 2002 received their first applications on 29 May at Hillsboro while application at Perley was on 22 May. Having been replanted on 29 May, weekly treatments for the 2002 Perley location were reinitiated on 5 June. After their first application, selected plots at the 2001 locations received an application every seven days for twelve consecutive weeks while selected 2002 plots received applications every seven days, continuing in a consecutive pattern varying from 4, 8, and 12-week intervals. Plots in 2002 labeled as "Messenger – Micro Rate" received foliar harpin applications within one hour after the post-emerge herbicides and every week thereafter for four consecutive weeks. The latter applications were designed to determine the potential for tank mixing the product with herbicide.

Plot Harvest

The 2001 plots were harvested on 25 September at Hillsboro and

on 28 September at Perley while the 2002 plots were harvested on 24 September and 17 September, respectively. At three of the four locations, only the center two rows were harvested to reduce the effects of bordering plots, however, due to lower plant populations at the 2001 Hillsboro location, all four rows were harvested for yield analysis. Each sugarbeet root was visually rated for Aphanomyces root rot and recorded on a 0 to 4 scale based on a scale developed by C. Windels, U. of Minnesota-Crookston (personal communication): 0 = Clean root; 1 = Less than 10% of root surface is scurfy; root malformed; 2 = Greater than 10% but less than 25% of root surface is scurfy; root malformed; 3 = Greater than 25% but less than 75% of root surface is scurfy; lower half of root rotted or malformed; 4 = Greater than 75% of root surface scurfy; and/or no root tip.

Root samples were transported to the Minn-Dak Farmers Cooperative Lab (Wahpeton, ND) for yield and quality analysis within 12 hours of harvest. Each individual sample (bag of roots) was rated for root yield, percent tare, sugar content, and impurity level (sugar loss to molasses). Statistical analysis for both locations and for both growing seasons was performed using Agricultural Research Manager (ARM 6.1.12, Gylling Data Management, Inc., Brookings, SD). Assuming a randomized complete block design (Treatments: Biologicals, Blocks: Seed Chemicals), the data collected was analyzed for least significant differences (LSD; Fisher's Exact Test) at the $P = 0.05$ level.

RESULTS

Controlled environment tests with seedlings.

In agreement with previous reports on the efficacy of harpin protein, SA, and riboflavin in the reduction of plant disease symptoms, foliar treatment of sugarbeet seedlings with these compounds reduced seedling root rot resulting from *A. cochlioides* challenge. Disease reduction was most consistent in treatments involving harpin and SA applications and this reduction was observed at several concentrations of A. cochlioides zoospores used for inoculation (Fig. 1). Treatment of seed with B. cepacia, P. fluorescens and hymexazol in growth chamber studies reduced seedling root rot as compared to those of the untreated check after inoculation with A. cochlioides zoospores, but in a variable manner. In these experiments, hymexazol clearly provided the greatest protection against seedling root rot (not shown). The addition of a foliar spray of harpin in conjunction with the seed treatments of hymexazol, B. cepacia, and P. fluorescens decreased the root rot rating in an additive manner, but the differences exhibited high variability and were not statistically significant.

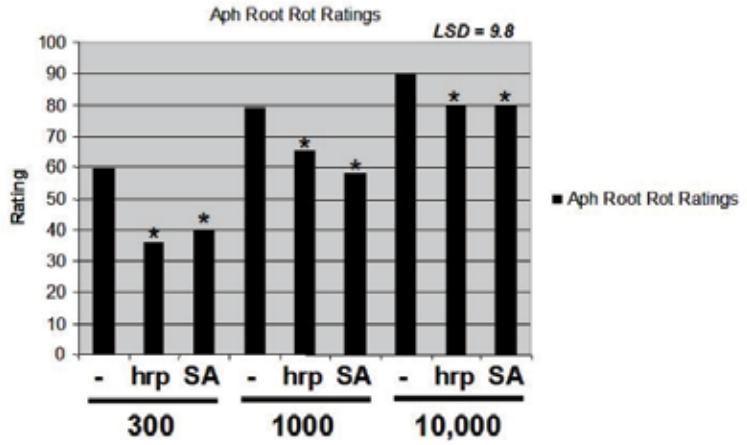

Fig. 1: Seedling root rot from *A. cochlioides* after prior foliar application of harpin and SA in a controlled environment. Check inoculations (-) receiving only water on the foliage were compared to seedlings receiving harpin (hrp) or salicylic acid (SA). The number of *A. cochlioides* zoospores per treatment (300, 1000, 10,000) is indicated below the bars. Asterisks indicate that the foliar treatments resulted in significantly reduced average root rot rating ($LSD_{0.05} = 9.8$) as compared to the respective control inoculation.

Emergence Rate, Stand Establishment and Root Rot Rating.

At both field locations and in both years, weather conditions were favorable for infection of sugarbeet seedlings and adult roots by *A. cochlioides*. Emergence rate was evaluated by recording stand counts on each individual plot at 15, 30 and 45-days post planting. Although significant differences in stand establishment between treatments were observed within a single year, results were not consistent between locations or within locations between years. Worth noting, however, was the lack of significant decrease in stand establishment with the treatments indicating that the biological control agents (BCAs) or harpin treatment were not detrimental to seedling growth.

Due to seasonal summer rainfall and the high incidence of *A. cochlioides* in the chosen test sites, the adult or chronic phase of Aphanomyces root rot was prevalent at both locations and in both years. Characteristic symptoms such as mild foliar chlorosis and severe dwarfing of the roots were observed by early July in the 2001 and 2002 growing seasons, which increased in severity throughout the season. Root symptoms included water-soaked, black discoloration of

the infected area. In plots that were severely affected, many of the roots had a proliferation of the lateral roots. During both seasons, the disease was more prevalent at the Hillsboro site than it was at the Perley location based on pre-plant soil indexing and on the average disease severity of harvested roots.

Yield and Quality Analysis

Yield components were seen to vary at the Perley and Hillsboro experimental locations in both 2001 and 2002. With the high disease pressure present at both locations, little significant improvement was observed in yield with respect to the test treatments, including treatments with hymexazol. An exception in 2001 was the treatment with harpin of plants derived from raw seed resulting in increased yields at the Hillsboro location as compared to the untreated check (Figure 2). Additionally, data from 2002 at the Perley research site showed a significant increase in yield and recoverable sugar (Mg ha^{-1}) where a combined treatment of BCA or harpin treatment with MT+H treatments were compared to treatments with MT (Figure 3). Thus, MT+H treatment of seed induced yields in 2002 of 2.82 Mg ha^{-1} which was not significantly different than treatment with MT alone; addition of either *B. cepacia* on seed or harpin on the foliage, however, onto MT+H pre-treated seed induced yields that were significantly higher than those provided by MT treatment alone ($LSD_{0.05}$ of 0.94 Mg ha^{-1} of recoverable sucrose).

DISCUSSION

Aphanomyces root rot of sugarbeet has been a perennial problem in production in the Central U.S. and Japan and an increasing problem in Europe. The disease impacts both seedlings and adult roots in the field (Papvizas and Ayers, 1974) and pre-disposes harvested beets to storage rot and sucrose loss through increased respiration (Campbell and Klotz, 2006). The control of Aphanomyces root rot has relied on a single applied chemical, hymexazol, that protects germinating seedlings under heavy disease pressure and can maintain an effect through to young plants in fields with moderate disease pressure (Windels, 1990; Windels and Brantner, 2001). From early growth stages through maturation, sugarbeet is dependant upon heritable resistance for protection against root rot disease (Coe and Schneider, 1966).

Results from the present study indicate that the use of the tested BCAs as a solitary preventative agent, or induced resistance as a therapeutic, have poor efficacy in northern Red River Valley, USA fields in reducing chronic root rot caused by *A. cochlioides*. Although *B. cepacia*

and *P. fluorescens* were documented to reduce root rot caused by *A. euteiches* on pea (King and Parke, 1993), it may be that interactions between the BCA, and the host, pathogen, and soil components, either separately or combined, resulted in the poor disease control observed. Interactions between BCAs and host genetics (Smith and Goodman, 1999) and soil types have been documented (Kristek et al., 2006). A lack of any one positive interaction between these BCAs as applied to sugarbeet against the *A. cochlioides* pathogen would explain the lack

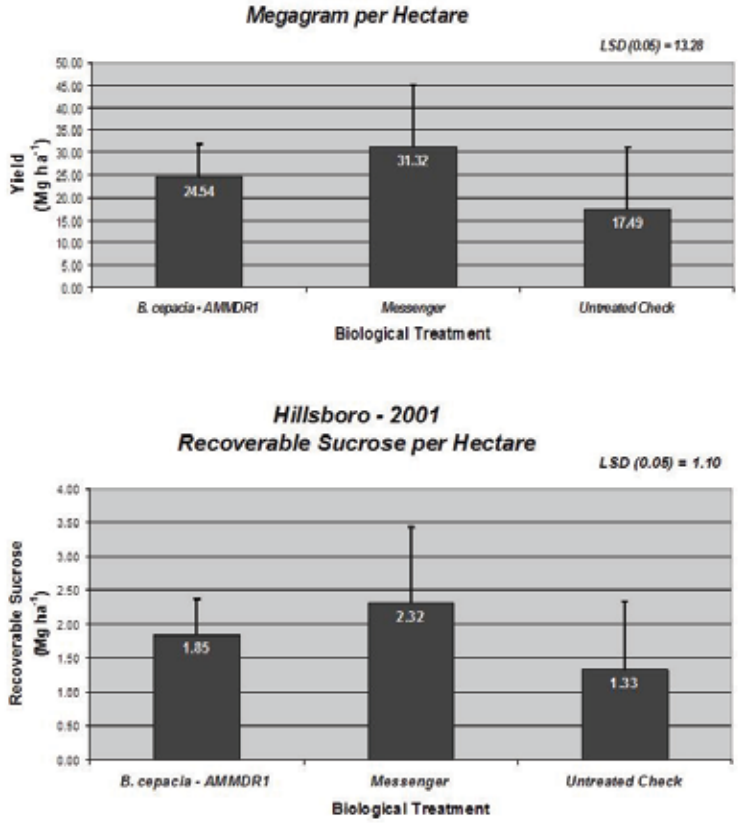

Fig. 2: Root yield (top graph) and recoverable sucrose yield (bottom graph) in 2001 at Hillsboro, ND after seed treatment with *B. cepacia* and foliar treatment with harpin. All seed, including the untreated check, lacked chemical fungicide. The significantly higher root yield ($LSD_{0.05}$ = 13.28 Mg ha^{-1}) with the harpin treatment resulted in a higher recoverable sucrose per hectare ($LSD_{0.05}$ = 1.1 Mg ha^{-1}).

of significant control in these tests (Handelsmann and Stabb, 1996; Lugtenberg et al., 2001). The observed trend towards improved yield in treatments involving the BCAs in fields with moderate disease pressure, however, is compelling and warrants further investigation. BCAs previously have been shown to be effective at reducing *A. cochlioides* damage to sugarbeet in field studies outside of the Red River Valley (Jacobsen et al., 2000; Williams and Asher, 1996; Kristek et al., 2006)

The induction of systemic resistance in plants by compounds has been known for decades (reviewed by Hammerschmidt and Kuc, 1995) and harpin originally was investigated for these properties (Wei et al., 1992). Recent data are more consistent with harpin's role as a growth promoter (Dong et al., 2004), although a species-specific role in disease protection probably exists. In agreement with this, harpin exhibited only a moderate ability to protect sugarbeet seedlings in a growth chamber from the effects of *A. cochlioides* when infection was initiated with zoospores after harpin treatment at the recommended rate. Poor disease control also was observed in the field, although this is likely compounded by colonization of seedlings by the pathogen before the

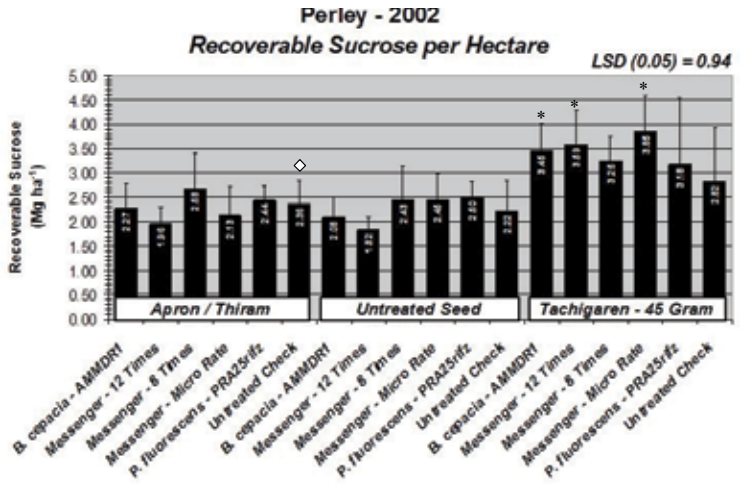

Fig. 3: Recoverable sucrose (Mg ha^{-1}) in 2002 at Perley, MN after seed treatment with *B. cepacia* and foliar treatment with harpin. Untreated seed was compare to that possessing MT or MT+H. Additional check, biological seed coating, or foliar inducer treatments are indicated below the bars. The open diamond (◇) indicates the check against which the significant increase in RSH (denoted by *; $LSD_{0.05} = 0.94$) is noted.

first application of harpin. Nevertheless, the results illustrate the efficacy of harpin in increasing yields even when root rot ratings were not improved and constitutes the first report to our knowledge of improving yields in *Aphanomyces* infested soils using a foliar-applied resistance inducer. The high pressure of *A. cochlioides* at the two locations best explains the reduced control afforded by the industry-standard MT+H treatment in 2002.

Biocontrol strategies continue to offer promise for reducing costs and yield losses to producers with an associated reduction in environmental degradation (Cook and Baker, 1983; Becker and Schwinn, 1993; Jacobsen et al., 2000; Kristek et al., 2006). Yet in few crop industry paradigms has the disease control offered by BCAs proved to be as effective as those afforded by exogenous chemicals or host genetics. The results presented here point to approaches combining chemical, BCA, and induced resistance concepts for the formulation of new strategies to protect sugarbeet from yield losses due to *A. cochlioides* infection. It further is anticipated that these concepts could be extended to the control of other sugarbeet diseases.

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