Greenhouse Method of Screening for Resistance of Sugar Beet Cultivars to Root Rot caused by *Aphanomyces cochlioides*

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1. Literature review

1.1. Sugar beet (*Beta vulgaris*) and sugar beet seed production

Currently, sugar beet (*Beta vulgaris* L.) accounts for 20% of the world's sugar supply, with global production of about 271 million tons of in 2011 (3). The total acreage for the United States for 2014/2015 was 1.15 million acres (6). The center of origin for sugar beet is the Mediterranean Basin and Middle East (2). The sugar beet predecessor was harvested by ancient Egyptians as a source of food (6). It is said that the genus for sugar beet is named after the Greek letter β, which resembles an overgrown turnip. One of the earliest forms of domesticated beets is chard, *Beta vulgaris* subsp. *cicla* L., which was domesticated around 2,000 B.C. by the Greeks and Romans. The beets were crossed and many variants existed, but red beets were preferential for people of that time (6). In England, beets were imported as food for the poor during times of famine in the 18th century; however, sugar beet was not a good staple food for people, and suited cows better (6). American colonists eventually brought sugar beet to the United States in the 19th century. George Washington experimented with sugar beets on his plantations (6).

Modern sugar beets are a result of selection during the late 18th century from fodder beets in German Silesia, which is located in modern day Poland, based on selections done by

Andreas Marggraf (6, 17). Marggraf isolated sugar from sugar beets and showed it was the same as that from sugar cane. His student Franz Karl Achard began breeding cultivars for best sugar content (6, 17). Sugar beet development took a step forward during the Napoleonic era of the early 19th century, when the military blockade by the British did not allow for the transport of cane sugar from the West Indies or any other British colony to the European continent. As a result of the blockade, Napoleon ordered development of a sugar beet breeding program, and from 1810 to 1815, 79,000 acres were planted in France and 300 sugar beet processing plants were constructed (6). The first successful sugar beet factory in the United States of America (USA) was constructed in California in 1879, which was built upon French sugar beet processing designs (17). Currently, there are about 23 factories that process sugar beets in the United States (17).

Sugar beet is grown as an annual crop that is processed into sugar, but is a biennial seed crop, given the requirement for vernalization to induce flowering (1). This is typical of plant species that are biennial in nature for flowering and seed set (e.g., carrot, onion, many brassicas, etc.) (1). Almost all of the sugar beet seed sold commercially to farmers in the USA is hybrid seed (2). Farmers could, in theory, produce their own seed, but the seed industry provides an option that allows farmers to grow high yielding root crops and not spend time breeding cultivars for their production areas. Sugar beet acres in the USA are limited by the amount of sugar that can be processed by local processing plants (6, 17).

Sugar beet seed production in the USA is limited primarily to private enterprises, and historically has been centered in the coastal Willamette Valley of Oregon State (1). Recent changes in the sugar beet seed industry have led to the roots being produced in two main

locations in the USA for the first year of the biennial season: Yuma, AZ and the Willamette Valley, OR. Roots are grown in beds in these areas from late July to January, with harvest occuring around January when the roots are dug carefully, placed in cold storage for vernalization, and transplanted to other regions for flowering and seed set. Approximately 54% of the seed production stage of the biennial season occurs in the Willamette Valley, and 46% occurs in other regions such as the semi-arid Columbia Basin of central Washington and northcentral Oregon (9). Growing conditions in the Willamette Valley are perfect for sugar beet seed crops as the mild winters are cold enough for vernalization but not too cold to kill plants, and the dry, mild summers are ideal for pollination, seed maturation, and seed harvest (6)

1.2. Root rots of sugar beet

There are a number of soilborne pathogens that can cause root rots of sugar beet (4, 10, 13, 18). Three of the major pathogens associated with root rots include: *Rhizoctonia solani*, which can cause a root and crown rot; *Aphanomyces cochlioides,* which can cause a root rot and damping-off; and *Pythium ultimum*, a damping-off pathogen. Since Aphanomyces root rot presents a significant problem for sugar beet growers and processing plants in the USA (4, 10, 13, 18), this project focused on *A. cochlioides.*

1.2.1. Aphanomyces root rot

1.2.1.1. History, geographic distribution, and importance

Aphanomyces root rot can cause significant problems for sugar beet growers and processing plants (18). The disease was fist observed in the 1930's (16). At that time, research focused on crop rotation, chemical applications, and resistant cultivars to control the disease (16). The disease now is found virtually everywhere sugar beets are grown (10, 18). The

economic impact of Aphanomyces root rot on sugar beet production can be significant. Some farmers have experienced complete loss of stand to the disease (10, 18). An effective, integrated solution to the problem is necessary in order to maintain economically viable sugar beet production (10, 13, 18).

1.2.1.2. Host range and symptoms

A. cochlioides is also known to infect other plants in the Chenopodiaceae besides sugar beet, e.g., spinach (*Spinacia oleracea* L.), as well as plants in the closely related Amaranthaceae, including common weeds such as lambsquarters (*Chenopodium album*) and *Amaranthus* spp. (13). Species of *Aphanomyces* are also pathogens of pea (*Pisum sativum*) and some other legumes (10, 13).

Symptoms of Aphanomyces root rot on sugar beet result from two main phases of infection: an acute phase and a chronic phase. In disease terms, the acute phase refers to a short or sudden phase of symptom development (10). The chronic phase is a long-lasting occurrence of symptom expression (10). The acute phase can be characterized as a damping-off of seedlings resulting from root and/or hypocotyl infection (18, 19). The chronic phase of Aphanomyces root rot occurs during hot days, when sugar beet plants wilt even though adequate water is present, and can be observed as a root rot with plants wilting and developing yellow leaves. In the chronic phase, leaves can become brittle. There usually is no permanent wilting, as the beets tend to recover overnight or during other periods of cool conditions, unless the plants become severely infected, in which case the plants may die (18, 19). The infection appears initially as lesions on the tap root, and sometimes the whole tap root may be destroyed. In some cases, the beet foliage may appear healthy even though the roots are

severely damaged. In rare cases, a healthy root crop can be produced even in the presence of the pathogen, but usually symptoms are present on most of the roots in an infected crop (18, 19).

1.2.1.3. Causal agent and epidemiology

The causal agent of Aphanomyces root rot, *A. cochlioides*, is not a true fungus but an oomycete (19). The pathogen has sexual and asexual stages. Asexual sporangia of the pathogen may develop on infected roots, and produce asexual zoospores. The most important dispersal stage of the pathogen is the asexual zoospore, a soilborne spore that has flagella that enable the spore to move in water in the soil profile to infect root hairs and hypocotyls of sugar beet roots (19). The asexual mycelium of the oomycete produces antheridia, the male sexual structure, and oogonia, the female sexual structure. The antheridium fertilizes the oogonium to produce a thick-walled sexual oospore, a resting structure that can remain viable in the soil for up to 16 years (10, 18, 19). Under favorable temperatures of 22-28^{o}C, an oospore can germinate to produce a sporangium which, in turn, produces zoospores that infect roots or germinate directly to infect seedlings (18, 19).

1.2.1.4. Management

1.2.1.4.1. Cultural control

Limestone (calcium carbonate) amendment to soil can reduce the severity of Aphanomyces root rot by increasing the soil pH, as soil pH has a significant influence on growth of the oomycete (20). Acid soils are more favorable to A. *cochliodes* than alkaline soils, and increasing soil calcium level by limestone amendment improves plant cell defense against this plant pathogen (20). Aphanomyces root rot also is favored by waterlogged soils, so a reduction

in soil water content by implementing practices that improve water infiltration of the soil can reduce severity of the disease (18, 19). Therefore, soil tillage (ripping or deeper plowing) can reduce severity of the disease if the tillage methods are designed to improve soil water permeability (19). Planting cereal cover crops (wheat, rye and barley) has been shown to reduce root rot (23). Planting sugar beet seed early into cool soils can add some protection against the disease as the pathogen does not develop as rapidly in soil temperatures below the optimum range of 22-28^{o}C (19). Crop rotation to non-susceptible plant species (barley, wheat or oat) between sugar beet crops can add significant protection against the disease as the amount of inoculum in the field is reduced during years without a host crop in the field (18, 19, 23). Planting sugar beet seed into severely infested fields should be avoided, and limestone amendment of the soil should be utilized when possible to increase soil pH (above 7.5) and calcium levels (19, 20). Weed control is also important as other plants in the Chenopodiaceae and Amaranthaceae can be infected by *A. cochlioides*, resulting in persistence of inoculum in fields (13, 19).

1.2.1.4.2. Chemical control

The only fungicide registered for use on sugar beet in the US that is effective for managing Aphanomyces root rot is hymexazol (trade name Tachigaren; Mitsui Chemical Co., New York, NY) (11). The fungicide is applied as a seed treatment, and is used in conventional sugar beet production in many countries. However, this treatment only provides a few weeks of protection of sugar beet seedlings against the seedling phase of the disease by reducing post-emergence damping-off and improving plant stands (11). There is currently no other fungicide seed, drench, or other treatment that controls Aphanomyces root rot effectively on sugar beet

(10, 11). Application of 45 to 90 g of Tachigaren (70% active ingredient hymexazol) per 100,000 seeds can achieve protection against Pythium root rot and Aphanomyces root rot in fields with known problems from these two diseases, while 20 to 30 g product/100,000 seeds is recommended in fields with less severe disease pressure (11).

A new fungicide ethaboxam is in the Fungicide Resistance Action Committee (FRAC) group 22 with efficacy against oomycete plant pathogens, including *Aphanomyces* spp., is the fungicide ethaboxam. This fungicide is currently in development and is named INTEGO Solo (Valent USA Corp., Walnut Creek, CA). The product will be registered for use on sugar beet as a seed treatment for control of Aphanomyces root rot. As of October 2016, INTEGO Solo was labeled for use on corn and legumes, but not on sugar beet (18).

1.2.1.4.3. Resistant cultivars

Planting resistant sugar beet cultivars is currently the most efficient strategy for controlling Aphanomyces root rot (18). As noted above, while research has demonstrated that some cultural practices can reduce severity of the disease in fields, e.g., limestone amendments and other forms of calcium addition to soils (14, 20), the use of resistant cultivars to manage the disease is generally most practical and economical for sugar beet growers. There are a number of sugar beet cultivars with resistance to Aphanomyces root rot that are available commercially, e.g., Betaseed, Inc. (Tangent, OR) cultivars such as BTS 18RR4N, BTS 70RR99, and BTS 8337; and the Hilleshog (Glyndon, MN) cultivar 9334RR (9). Resistance to Aphanomyces root rot is not equivalent to immunity, meaning that resistance is not controlled by a single gene or a limited set of genes that confer complete resistance, but is a multi-gene response that results in partial protection of roots against the pathogen, especially in the chronic phase

of infection (10, 18). There are no known races of *A. cochlioides*; however, there are strains that appear to have different degrees of virulence on sugar beet (10, 19).

2. Greenhouse resistance screening for Aphanomyces root rot

2.1. Objectives

There have been many difficulties at Betaseed, Inc. and in other sugar beet breeding programs at establishing a reliable field nursery to test for resistance to Aphanomyces root rot in sugar beet. Schneider (16) developed a resistance screening nursery in both East Lansing, MI and Waseca, MN, which were established using oospore inoculum. A difficulty at those nurseries was a lack of uniformity (consistency) in disease pressure across the fields. The proprietary nursery at the Betaseed facility in Shakopee, MN has relatively uniform Aphanomyces root rot pressure, but the nursery is prone to flooding. Therefore, a greenhouse resistance screening test for Aphanomyces root rot was developed in an attempt to provide a more effective and efficient way to select for resistant cultivars by reducing variability in disease pressure among experimental units, and to provide a backup to the field nursery screening site.

The primary objective of this project was to identify a method of inoculating *B. vulgaris* cultivars or breeding lines with *A. cochlioides* that consistently yielded a measurable difference in disease severity between resistant and susceptible cultivars in order to improve selection for resistance to Aphanomyces root rot at the Betaseed facility in Shakopee, MN. The second objective was to identify an optimum growth stage of sugar beet plants for inoculation that yielded the greatest difference in disease severity between susceptible and resistant cultivars of

B. vulgaris. Thus, an experiment was carried out to identify: 1) an optimum type of inoculum of *A. cochlioides* to use in resistance screening, and 2) an optimum age of sugar beet seedlings at the time of inoculation for efficient and effective resistance screening in a greenhouse.

The first hypothesis tested was that the use of zoospore inoculum would provide more effective resistance screening than mycelium and oospore types of inoculum of *A. cochlioides*. This hypothesis was based on personal experience of plant pathologists and breeders at the Betaseed, Inc. facility, as well as experience by Schneider (15, 16) in his greenhouse experiments. The second hypothesis was that a more concentrated zoospore inoculum application rate (400,000 zoospores/plant) would provide more effective resistance screening of sugar beet cultivars than a less concentrated zoospore inoculum (200,000 zoospores/plant). The third hypothesis was that inoculation of 2-week-old sugar beet plants would result in greater differentiation of disease severity than inoculating 3- or 4-week-old plants for comparing resistant and susceptible sugar beet cultivars.

2.2. Materials and methods

2.2.1. Treatment and experimental designs

The experiment was set up as split-split plot randomized complete block design (RCBD) with replication (blocks) planted over time. The treatment design was a 3 x 5 x 2 factorial with three plant ages at the time of the inoculation (2-, 3-, 4-weeks after planting), five inoculation treatments (see below), and two sugar beet cultivars (partially resistant and susceptible to Aphanomyces root rot). Plant age treatments were assigned to the main plots in each replication, inoculum treatments were assigned to the split-plots, and cultivars were assigned to the split-split plots.

2.2.2. Pathogen isolates and greenhouse conditions

Inoculum was prepared from a mix of two U.S. isolates of *A. cochlioides*, US-FT-16 and US- FT-21, obtained from sugar beet roots sampled from diseased fields in Crookston, MN by Jason Brantner. Two proprietary sugar beet cultivars were used in this study, one partially resistant and the other highly susceptible to Aphanomyces root rot based on previous research by breeders and plant pathologists at BetaSeed, Inc. Sugar beet seeds of the two cultivars were planted in LC1 potting medium (SunGro Horticulture, Agawam, MA) in 3601 trays. Each tray had 36 cells, and each cell was 5.08 cm x 5.08 cm x 5.08 cm (TO Plastics Inc., Elk River, MN), with 1 seed planted per cell, to which 150 g of 20-20-20 (N-P-K) fertilizer mix was added per ~2.3 kg of potting mix. Eighteen cells/tray were planted with the resistant cultivar, and the other 18 cells with the susceptible cultivar. The plants were randomized within each tray. Seeds were planted at intervals so that the plants were 2-, 3-, and 4- weeks old from the day of sowing at the time of inoculation. The temperature in the greenhouse was set at 20-25^{o}C (15), with a 16 h light/8 h dark daily cycle with supplemental lighting. The experiment was setup in three replications as a result of limited space available in the greenhouse for the study: the first planted in late September and maintained until December 2014, the second planted in January and maintained until March 2015, and the third planted in March and maintained until May 2015. During October to December, the average natural daylight in Minnesota is almost 10 hours, while during January to March the average day length is almost to 11 hours, and from March to May is almost 13 hours. The temperatures during the October to December replication averaged 25^{o}C as a result of greenhouse heating, while for the January to May 2015 replications

the temperature averaged 28^{o}C as a result of sunlight and greenhouse heating. Seedlings were watered twice daily until inoculation. Plants were not fertilized again after adding the initial fertilizer to the soil mix. Great care was taken not to splash or mix inoculum during watering of the plants. Lights were turned off during inoculation and for 24 h after inoculation to help maintain inoculum viability (15).

2.2.3. Inoculation treatments

Each plant was inoculated using one of five inoculum treatments with *A. cochlioides*: 1) zoospores at 400,000 spores/plant, 2) zoospores at 200,000 spores/plant, 3) oospores at 60,000 spores/g soil mix, 4) mycelium at 4 ml suspension/plant, and 5) a non-inoculated control treatment (15, 16). The inoculum application rates were determined based on the work of Schneider (15, 16). Control plants were treated with the same volumes of water as the plants with zoospore suspension, but without any inoculum. Details of the procedures for preparing inoculum are described below, with the rates determined based on the methods used by Windels and Brantner (22).

Zoospore inoculum was prepared using the method of Islam et al. (8). A semi-solid agar medium was used that consisted of 17 g cornmeal agar (CMA) + 4 g yeast extract (YE) per liter of 50 mM phosphate buffer (pH 6.8–7.0), sterilized by autoclaving for 30 minutes at 121^{O}C. Six-day-old cultures of *A. cochlioides* grown in the medium were then washed with 50 ml of distilled water (compared to 10 ml in the original paper), and the mycelium left for 24 h in the distilled water in the dark for zoosporogenesis (8). For each of the four types of inoculum, half of the suspension was produced from isolate US-FT-16 and the other half from isolate US-FT-21, with the two isolates mixed just prior to inoculation. For each inoculation type, inoculum

concentration was determined using a hemocytometer and adjusted as necessary by diluting the suspension. An aliquot of the final zoospore suspensions was applied directly to the hypocotyl of the plant using a Pipetteman (Thermo Scientific, Madison, WI) at each of two rates: 200,000 and 400,000 zoospores/plant.

For oospore inoculation at 60,000 oospores/g soil, oospores were grown using the Windels (19) method. A broth was prepared with 5 g rolled oats/liter of water, sterilized by autoclaving. A 1 cm^3 plug of 10% potato dextrose agar (PDA) medium colonized by *A. cochlioides* was placed in each 1,000 ml flask of sterilized oatmeal broth, and the inoculum grown for 30 days at room temperature (25^oC) in a lab in the dark. The oospores were harvested by pouring off the broth and liquefying the remaining contents in a blender for 5 minutes (18). Oospores were then counted using a hemocytometer. The final inoculum concentration was generated by combining half of the suspension of US-FT-16 oospores and half of the US-FT-21 oospore suspension. The mixed oospore suspension was applied directly to the hypocotyl of beet plants using a Pipetteman at 60,000 oospores/g soil mix. Soil mix amount per cell was determined by weighing the total amount of soil that was placed into the tray, and dividing by 36 (for the 36 cells in a tray).

For the mycelial inoculum, one plate of 10% PDA medium colonized by mycelium of *A. cochlioides* for 1 to 2 weeks was used to inoculate sugar beet plants. Colonized PDA from the plates was placed in a blender for 5 minutes. Prior to blending, the weights of the plates were measured to ensure approximately an equal amount of mycelium and agar was used in the inoculation. The suspension of each of the two isolates of *A. cochlioides* was mixed in equal parts, and the final mycelial suspension was then applied directly to the hypocotyl of each plant

with a Pipetteman at 4 ml mycelial suspension/plant. Quantification of the mycelial suspension was not performed directly, only indirectly based on agar plate weight.

For the non-inoculated control treatment, water was applied directly to the hypocotyl of each plant of each age and both cultivars using a pipetteman, and the same amount of water as the aliquot of zoospore suspension applied to each plant.

2.2.4. Plant ages at inoculation

For each of the three ages of the plants at the time of inoculation, one tray of each of the 2-week-old, 3-week-old, and 4-week-old seedlings was placed in a randomized arrangement of the susceptible and partially resistant cultivars for each inoculation treatment.

2.2.5. Disease rating

A scale of 1-to-5 was used to rate the severity of Aphanomyces root rot on each seedling, where: 1 = no symptoms observed, and 5 = the plant died, based on aboveground symptoms of the hypocotyl and foliage (refer to Fig. 1 for details of the rating scale). Ratings were done for each plant 3 days after inoculation, and repeated every 3 days thereafter until 50% of the plants had died, i.e., 48 days after inoculation. No attempt was made to wash the roots in order to rate root rot severity. The disease severity ratings were compared with ratings of the same two cultivars planted in field trials at the BetaSeed, Inc. facility in Shakopee, MN over 10 years. Field ratings were carried out for approximately 90 plants of each cultivar in approximately 210 replicate plots over the 10 years, with rating of Aphanomyces root rot severity based on a 1-to-9 scale, where: 1 = the most resistant response (least amount of disease), and 9 = the most severe Aphanomyces root rot.

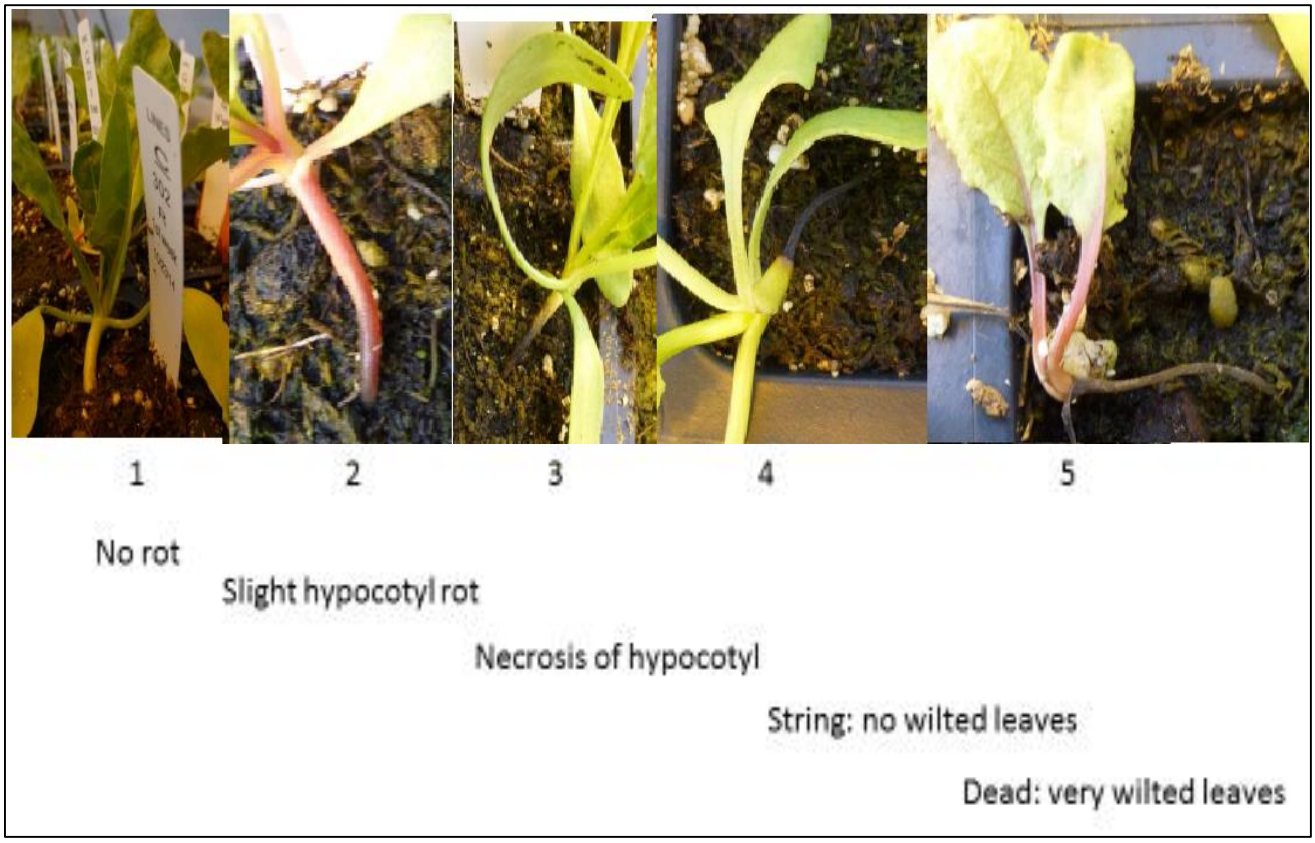

Fig. 1. Rating scale used to assess severity of Aphanomyces root rot of sugar beet seedlings after inoculation with *Aphanomyces cochlioides*.

2.2.6. Statistical analysis

Analysis of variance (ANOVA) was performed using SAS version 9.4 (SAS Institute, Cary, NC) for the 3 x 5 x 2 factorial treatment design as a split-split plot RCBD. Replications were considered a random effect in the model, whereas age of the plant, inoculation treatment, and cultivar were considered fixed terms in the model statement. Means were compared among treatments for each main effect, and for each two-way interaction that was significant using Fisher's protected least significant difference (LSD) at $P < 0.05$, i.e., only if the ANOVA F-test was significant for that factor or interaction.

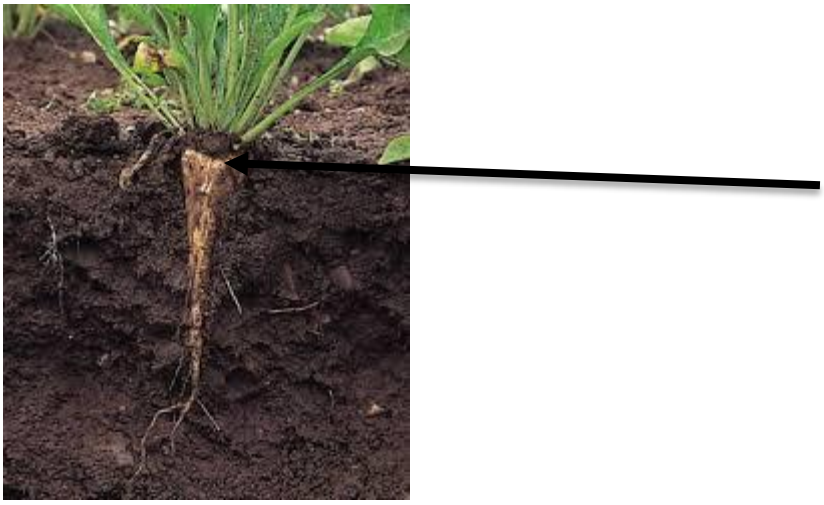

Fig. 2. The development of a sugar beet seedling with natural darkening and secondary growth of the root.

2.3. Results

All of the inoculum treatments resulted in development of symptoms typical of Aphanomyces root rot, whereas none of the non-inoculated control plants developed symptoms of this disease. However, disease ratings completed when sugar beet seedlings started secondary growth, approximately 3 to 4 weeks after planting, were difficult because darkening of the crown at the soil level was easily confused with symptoms of Aphanomyces root rot (Fig. 2).

The main effects of inoculum type ($P<0.0001$) and cultivar ($P<0.0001$) were significant for the final rating of severity of Aphanomyces root rot, whereas the main effect of plant age was not significant ($P=0.1303$) (Table 1). Significant two-way interactions included plant age x inoculum ($P=0.0353$) and inoculum x cultivar ($P<0.0001$) (Table 1). The three-way interaction of plant age x inoculum x cultivar was not significant ($P=0.7241$).

Mean disease severity for the plants that were 2 weeks old at the time of inoculation was 2.04 ± 0.61 by 48 days after inoculation, and was 1.84 ± 0.60 and 1.61 ± 0.58 for plants that were 3 and 4 weeks old at the time of inoculation, with no significant difference among plant ages. Mean disease severity for plants inoculated with 400,000 zoospores/plant was 2.67 ± 0.97, which was significantly greater than that of plants inoculated with 200,000 zoospores/plant (2.20 ± 0.51). The latter inoculation resulted in more severe root rot than both mycelium inoculation (1.66 ± 0.25) and oospore inoculation (1.59 ± 0.23), with the latter two treatments not differing significantly in the severity of the root rot induced. Non-inoculated control seedling averaged 1.04 ± 0.00, which was significantly less than that all inoculated treatments. Mean disease severity for the susceptible cultivar was 2.25 ± 0.86, which was significantly more severe than 1.42 ± 0.58 for the partially resistant cultivar.

Based on a significant age x inoculum interaction term and inoculum x cultivar interaction term in the ANOVA table (Table 1), means are presented separately for each plant age of each cultivar for all five inoculum treatments (Fig. 3). For the 2-, 3-, and 4-week-old plants of the susceptible cultivar inoculated *with A. cochlioides*, the 400,000 zoospores/plant inoculum treatment resulted in the most severe root rot (Fig. 3A, 3B, and 3C). However, for the partially resistant cultivar, this was only true for the 2- and 3-week-old plants (Fig 3A and 3B), as the 200,000 zoospores/plant inoculum treatment resulted in slightly more severe root rot than the 400,000 zoospores/plant treatment for the 4-week-old plants (Fig. 3C). For the 2- and 3-week-old plants of both cultivars, the next most severe root rot was observed on plants inoculated with the lower zoospore concentration, followed by the mycelial suspension and the oospore inoculum, with no significant difference between the latter two inoculum types; and

the least amount of disease was observed on the non-inoculated plants. This order of disease severity resulting from the inoculation treatments was different for the 4-week-old plants, as there were no significant differences among the five inoculum types for the partially resistant cultivar, including the control treatment; and only non-inoculated, 4-week-old control seedlings of the susceptible cultivar had less severe disease and there were no differences among the four inoculum types of *A. cochlioides*.

The comparison of results of the greenhouse experiment to the field data could not be evaluated statistically because there was a different number of plants in the field plots and different rating systems were used between the two types of trials. A mean root disease severity rating of 6.09 ± 0.61 was calculated for the susceptible cultivar in the field trials, and 2.97 ± 0.34 for the partially resistant cultivar (on a 1-9 scale with 9 being the most severely affected by *A. cochlioides*). In comparison, the susceptible cultivar averaged 2.25 ± 0.86 and the partially resistant cultivar averaged 1.42 ± 0.58 on a 1 to 5 severity scale in this greenhouse study.

Table 1. Analysis of variance (ANOVA) for the effects of replication, sugar beet seedling age, inoculum type of *Aphanomyces cochlioides*, and sugar beet cultivar in an Aphanomyces root rot resistance screening experiment performed from October 2014 to May 2015.[a]

Source of variation	df[b]	Type III SS[b]	Mean square	F value	Pr > F[b]
Replication	2	16.630	8.315	18.44	<0.0001[c]
Plant age	2	16.588	8.294	3.54	0.1303
Rep*Plant age	4	9.372	2.343	5.20	0.0004
Inoculum type	4	167.581	41.895	65.81	<0.0001**
Rep*Inoculum	8	5.092	0.636	1.41	0.1889
Plant age*Inoculum	8	17.567	2.195	4.87	0.0353*
Rep*Plant age*Inoculum	16	12.303	0.768	2.86	0.0353
Cultivar	1	93.056	93.056	206.34	<0.0001**
Plant age*Cultivar	2	2.316	1.158	2.57	0.0777
Inoculum*Cultivar	4	25.416	6.354	14.09	<0.0001**
Plant age*Inoculum*Cultivar	8	2.392	0.299	0.66	0.7241
Total	539	584.798			

[a] The experiment was a split-split plot factorial design with three replications. Sugar beet seedling age treatments were applied to main plots (inoculated 2, 3, and 4 weeks after planting), five inoculum types (two zoospore concentrations, an oospore suspension, a mycelial suspension, and a control treatment) were applied to split plots, and two cultivars (susceptible and partially resistant to *A. cochlioides*) were applied to split-split plots. Refer to the main text for details of each type of inoculum evaluated, as well as the cultivars and ages of plants inoculated.

[b] df = degrees of freedom. Type III SS = type three sums of squares. Pr > F = probability of an F value greater than that calculated, given the relevant numerator df and denominator df.

[c] * and ** = significant at $P = 0.05$ and 0.01, respectively. Significant terms with the replication effect or interaction terms with replications are not noted with asterisks.

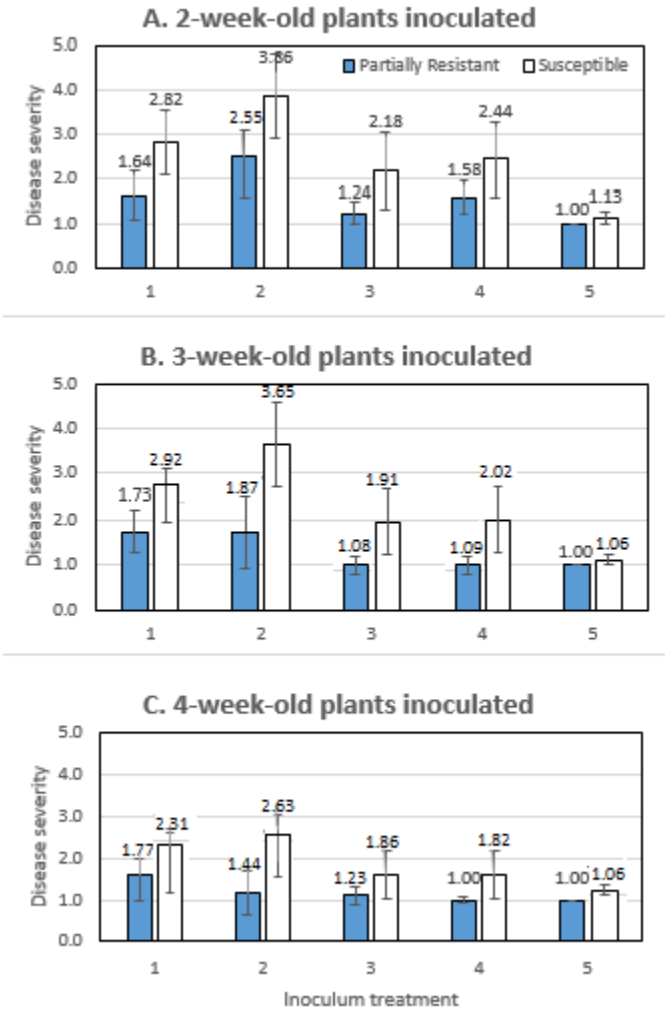

Fig. 3. Comparison of average severity of Aphanomyces root rot on sugar beet seedlings in a greenhouse experiment evaluating the effects of inoculum treatment of *Aphanomyces cochlioides* on 2-week-old (A), 3-week-old (B), and 4-week-old (C) sugar beet seedlings at the time of inoculation of a susceptible cultivar or a partially resistant cultivar to Aphanomyces root rot. Each bar represents the mean and standard deviation of the disease rating for 18 seedlings measured 48 days after inoculation. Each plant was rated on a 1-to-5 scale (see Fig. 1). Inoculum treatments evaluated were: 1) 200,000 zoospores drenched/plant, 2) 400,000 zoospores drenched/plant, 3) 60,000 oospores drenched/g soil, 4) 4 ml mycelial suspension drenched/plant, and 5) non-inoculated control treatment. The proprietary sugar

beet cultivars were partially resistant or highly susceptible to Aphanomyces root rot based on prior resistance screening.

2.4. Discussion

The main objective of developing a method for screening sugar beet cultivars and breeding lines for resistance to Aphanomyces root rot in greenhouse conditions available at the Betaseed, Inc. facility in Shakopee, MN was developed as part of this experiment. Based on the results, an effective method for screening sugar beet lines was identified. Protocols for the staff working in the facility were developed as a result of carrying out the experiment. The method identified for further screening is the application of zoospore inoculum at 400,000 zoospores/plant, as a result of this project as well as previous research (15). The more concentrated zoospore inoculation rate (400,000 zoospores/plant) resulted in more severe Aphanomyces root rot than the 200,000 zoospore/plant rate, which made it more efficient to differentiate the responses of the partially resistant and susceptible cultivars evaluated in this study. This made the task of the raters simpler.

The age of the sugar beet plants at the time of inoculation was not significant as a main effect in this study, but did significantly affect the responses of the two sugar beet cultivars to the pathogen (significant inoculum x plant age interaction). Aphanomyces root rot was most severe on the susceptible sugar beet cultivar for plants that were 2-weeks-old and 3-weeks-old at the time of inoculation with zoospores. For the partially resistant cultivar, a similar pattern was observed with a zoospore inoculum rate of 200,000 zoospores/plant. Therefore, in the interest of developing a rapid resistance selection protocol, it is best to inoculate plants 2 to 3 weeks after sowing, as supported by the results of Schneider (15).

The experiment in this study raised some interesting questions. A better experimental design could have been implemented for the experiment, e.g., a completely randomized block design with each tray having all of the inoculum types, but it would have been difficult to prevent inoculum spread between adjacent plants. Another variation of the test performed both by Schneider (15,16) and Windels (19) is planting a greater number of seeds into a square pot and counting the number of emerged plants that died, or utilizing different inoculation methods. It would be beneficial to have a highly resistant cultivar included in the study as a control treatment, but there are no completely resistant cultivars known (10,19). A study performed by Brantner and Windels (22) is very similar to what was done in this research in terms of conclusions reached, although they used a disease scale of 0-to-7 with 0 for the healthiest seedlings. They focused on evaluating 4-week-old plants at the time of inoculation, which in their opinion is more typical of plants in field conditions at the time of initial disease onset. The results of their experiment also pointed to large differences in root rot severity depending on the *A.cochlioides* isolate (in this study we acquired virulent isolates from J. Brantner). Therefore, we could evaluate testing older plants, using different isolates of the pathogen, and testing a broader range of inoculum rates, as well as setting up the experiment for more effective statistical analysis.

Comparison of results between the field trials and greenhouse test is vital for the successful interpretation and adoption of the greenhouse test. The greenhouse results were not compared statistically with the field trials because of the different rating scales used in the field plots and greenhouse trial. As the next step, it would be important to correlate the field plot data for these two cultivars to the greenhouse test results. This would entail establishing

inoculum levels in the field trial plots to try and ensure similar disease pressure as in the greenhouse, in order to test cultivars under the same conditions, and using a disease rating scale that can be adopted in both the greenhouse and field trials. This should lead to an effective comparison (24).

3. Literature cited

1. Campbell, S. C., and Mast, A. A. 1971. Seed Production. In Advances in Sugarbeet Production: Principles and Practices. R. T. Johnson, J. T. Alexander, G. E. Rush, and G. R. Hawkes, Editors. Iowa State Univ. Press, Ames, IA.
2. Center for Environmental Risk Assessment. Determination of the safety of Monsanto's sugar beet H7-1 (glyphosate-herbicide tolerant sugar beet) for direct use as food, feed and for processing. http://www.cera-gmc.org/files/cera/GmCropDatabase/docs/decdocs/09-131-016.pdf [Accessed 26 Jul. 2015]
3. Food and Agriculture Organisation. http://faostat.fao.org/site/565/default.aspx
4. Harveson, R. M. 2006. Identifying and distinguishing seedling and root rot diseases of sugar beets. Online. Plant Health Progress doi:10.1094/PHP-2006-0915-01-DG.
5. Harveson, R. M., and Rush, C. M. 2002. The influence of irrigation management and cultivar blends on the severity of multiple root diseases in sugar beets. Plant Dis. 86:901-908.
6. Harveson, R. M. 2014. History of Sugarbeet Production and Use [Online]. University of Nebraska–Lincoln https://cropwatch.unl.edu/sugarbeets/sugarbeet_history.

http://www.the-aps.org/mm/Publications/Info-For-Authors/Composition/Example-References.html [Accessed 26 Jul. 2015]

7. Heyman F., Lindahl, B., Persson, L., Wikstro, M., and Stenlid, J. 2007. Calcium concentrations of soil affect suppressiveness against Aphanomyces root rot of pea. Soil Biol. Biochem. 39:2222–2229.
8. Islam, Md. T., Sakasai, M., Hashidoko, Y., Deora, A., Sakihama, Y., and Tahara, S. 2007. Composition of culture medium influences zoosporogenesis and differentiation of *Aphanomyces cochlioides*. J. General Plant Path. 73:324-329.
9. Klein, D. Betaseed Inc., Shakopee, MN. *Personal communication*, 26 Jul. 2015.
10. Leach, L. D. 1986. Seedling diseases. Pages 4-8 in: Compendium of Beet Diseases and Insects. E. D. Whitney and J. E. Duffus, Editors. The American Phytopathological Society, St. Paul, MN.
11. Mitsui Chemicals Agro. 2015. Tachigaren 70 WP label. Retrieved from: http://www.kellysolutions.com/erenewals/documentsubmit/KellyData%5CND%5Cpesticide%5CProduct%20Label%5C48210%5C48210-1%5C48210-1_TACHIGAREN_70_WP_FUNGICIDE_1_25_2012_2_11_50_PM.pdf. [Accessed 26 Jul. 2015]
12. Olsson, A., Persson, L., and Olsson, S. 2011. Variations in soil characteristics affecting the occurrence of Aphanomyces root rot of sugar beet – Risk evaluation and disease control. Soil Biol. Biochem. 43:316-323.
13. Papavizas, G. C., and Ayers, W. A. 1974. *Aphanomyces* species and their root diseases in pea and sugarbeet. U.S. Dep. Agric. Tech. Bull. No. 1485.

14. Persson, L., and Olsson, S. 2000. Abiotic characteristics of soils suppressive to Aphanomyces root rot. Soil Biol. Biochem. 32:1141-1150.

15. Schneider, C. L. 1954. Methods of inoculating sugar beets with *Aphanomyces cochlioides* Drechs. Proc. Am. Soc. Sugar Beet Technol. 8:247-251.

16. Schneider, C. L. 1978. Use of oospore inoculum of *Aphanomyces cochlioides* to initiate blackroot disease in sugarbeet seedlings. J. Am. Soc. Sugar Beet Technol. 20:55-62.

17. Sugerbeet History [Online]. American Sugarbeet Growers Association. https://www.americansugarbeet.org/who-we-are/sugarbeet-history.html [Accessed 26 Jul. 2015]

18. INTEGO new fungicide treatment, Valent Biosciences. http://devcanada.valent.com/products/intego-solo/

19. Windels, C. E. 2000. Aphanomyces root rot on sugar beet. Online. Plant Health Progress doi:10.1094/PHP-2000-0720-01-DG.

20. Windels, C. E., and Harveson, R. M. 2009. Aphanomyces root rot. Pages 24-27 in: Compendium of Beet Diseases and Pests, 2nd Edition. R. M. Harveson, L. E. Hanson, and G. L. Hein, Editors. American Phytopathological Society, St. Paul, MN.

21. Windels, C. E., Brantner, J. R., Sims, A. L., and Bradley, C. A. 2009. Five-year effect of a single field application of various rates of spent lime on *Aphanomyces*, sugar beet and rotation crops. 2008 Sugar Beet Res. Ext. Rept. 39:237–249.

22. Windels, C. E., Brantner, J. R. 2000. Variability of spore production and aggressiveness of *Aphanomyces cochlioides* on sugarbeet. Sugarbeet Res. Ext. Rept. 31:241-246.

23. Windels, C. E., Nabben-Schindler D. J. 1990. Effect of soil incorporated green crop residues on Aphanomyces root rot of sugar beet seedlings. Sugarbeet Res. Ext. Rept. 21:164-175.

24. Windels, C. E., Beale, J. W., and Kinkel, L. L. 2002. Spatial distribution of *Aphanomyces cochlioides* and root rot in sugar beet fields. Plant Dis. 86:547-551.