DISCOVERY OF RESISTANCE TO SEEDLING DISEASE CAUSED BY *RHIZOCTONIA SOLANI* AG2-2, DESCRIPTION OF THE HOST-PATHOGEN INTERACTION, AND DEVELOPMENT OF A SEEDLING DISEASE SCREENING NURSERY

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ABSTRACT

Sugarbeet seedling mortality caused by the damping-off pathogen *Rhizoctonia solani* AG2-2 is perhaps the most serious biotic cause of stand failure in the Michigan growing regions, and is likely important worldwide. Resistance to the seedling disease has not been available. Resistance would be beneficial in establishing uniform stands of beets, and the resulting improved harvest quality of similarly sized beets delivered to the factory. An initial set of experiments to examine disease progression in susceptible hosts was conducted with high and low virulence isolates in order to identify targets of opportunity for biotechnology manipulation, and during this work, the crown-and-root-rot (CRR) resistant release EL51 was demonstrated to survive early challenge by the highly virulent isolate R1. Subsequently, the host-pathogen interaction was examined in detail using light and fluorescence microscopy. The resistance reaction was characterized by the failure of the pathogen to ramify the water-conducting stele tissues of the young hypocotyl, with an apparent barrier at the narrow endodermis. Field experiments were initiated to determine if resistance was expressed under agronomic conditions by a simple modification of traditional CRR screening to that of inoculating 3-week-old seedlings. Full stands of EL51 were present at the end of the season, and stands of the susceptible hybrid USH20 were decimated. In 2006, the entire East Lansing CRR nursery was inoculated at the seedling stage, and clear germplasm differences in disease reaction were seen. Interestingly, the disease continued to develop throughout the growing season, suggesting both seedling and CRR resistance can be selected simultaneously with a simple modification in the timing of inoculation.

Abbreviations: CRR – Crown and Root Rot; DI – Disease index; DPI – Days post inoculation; RSD – Rhizoctonia seedling disease; WPI – Weeks post inoculation.

Introduction:

Early season growth (e.g. the first 10 weeks) is a critical phase of the beet's life, not only to have good field stands but also to acquire metabolic capacity for agronomic productivity. Early season development includes acquisition of disease tolerance (from acute symptoms with devastating effects to chronic symptoms that only reduce yield potential), and development of the taproot. This change from seedling to adult vegetative growth coincides, in the field, with warming temperatures (and greater seedling disease), increased growth rate, and increased light interception. Yield of sucrose is directly proportional to the interception of solar irradiation, and maximal interception of sunlight does not occur until the crop canopy is fully developed usually past the summer solstice. Most (if not all) constructive agronomic processes are in place by the

10th week after emergence. Disease losses are a constant concern through the growing season and during post-harvest storage, but are caused by a relatively small number of pathogens for which genetic resistance is generally available, however seedling disease and competition from weeds have the greatest impact on obtaining a profitable crop. The focus of this project has been to evaluate the host-pathogen interaction between sugar beet seedlings and *Rhizoctonia solani* with the aim of discovering mechanisms of resistance, and apply this in breeding for enhanced stand persistence.

Rhizoctonia diseases are increasingly important in the Great Lakes growing region, and elsewhere. Genetic resistance is available for the chronic phase of the disease (crown and root rot), and a number of germplasm lines have been released over the past 20 years, which are now becoming available as resistant hybrids available through seed companies. Seedling resistance to Rhizoctonia blight has only recently been reported. Both crown and root rot and seedling diseases are caused by *Rhizoctonia solani*, a biologically complex species with many sub-types (Anastomosis Groups, AG), of which AG2-2 is the most serious to sugarbeet, and AG4 has been implicated as a pathogen only during seedling growth.

When seedling hypocotyls are infected at or below ground level, diseased seedlings collapse and die (e.g. damp-off). This leads to lack of full stand persistence and is implicated in the range of problems associated with emergence and stand establishment encountered by growers worldwide. Early-season Rhizoctonia disease research has been stimulated with an observation that Quadris and Amistar, fungicides with anti-Rhizoctonia activity, applied early in the season can significantly increase harvest yields. While the precise mechanism is unclear, this observation supports objectives for improving seedling emergence and stand establishment, and suggests that one of the major biotic impediments to improving stand persistence is, indeed, seedling disease caused by Rhizoctonia.

Little has been published on the topic of Rhizoctonia seedling resistance in sugarbeet, although the chronic crown and root rot phase of the disease in sugarbeet has received considerable attention. The primary invasion sites for crown and root rot are lower surfaces of petioles in contact with the soil, natural cracks in the crown, lenticels on the taproot, lateral roots, and opportunistic secondary infections after damage by nematodes or other penetrations. Rhizoctonia seedling diseases of sugar beet differ in pathogenicity and virulence from those causing root rot on older beets. The mode of penetration and the progress of subsequent tissue colonization play important roles in Rhizoctonia causing diseases. There has been no reported resistance to seedling disease caused by Rhizoctonia.

Materials and Methods:

Plant Material: Sugar beet (*Beta vulgaris* L.) consisted of different releases of sugar beet obtained from USDA-ARS, East Lansing, Michigan or the U.S. National Plant Germplasm System (NPGS). For growth chamber and greenhouse experiments, the seeds were soaked in 0.3% hydrogen peroxide (V/V; 88 mM) (J.T.Baker 2186-01) for 24 hours and allowed to germinate on water soaked Whatman filter paper for 48 hours prior to transplanting in the Baccto" high porosity professional planting mix.

Fungal inocula: Rhizoctonia solani AG2-2, R-1 (virulent isolate) and W22 low-virulent isolate; ATCC #18619) were used (provided by Dr. Lee Panella and Dr. Linda Hanson, USDA-ARS Ft. Collins, CO). Fungal isolates were grown on corn meal agar (CMA) in Petri dishes at room temperature. De-hulled seeds of millet, sterilized for three consecutive days at 120°C for 20 minutes each day, were placed as single layer on the actively growing three- day -old CMA

fungal culture and incubated at room temperature for an additional four days. The infested millet seeds were dried and used as inocula.

Growth chamber disease screening protocol: Sugar beet varieties USH20 (PI 631354) (Coe and Hogaboam 1971) and EL51 (PI 598074) (Halloin et al. 2000) and fungal isolates *R. solani* AG2-2 R-1 (virulent isolate) and W22 (low virulent isolate) were used to develop the RSD screening protocol. Pots (9 cm diameter by 8 cm deep) placed on cafeteria trays were filled to 2 cm below the top with "Baccto" high porosity soil and were arranged in a randomized complete block design. Four seedlings were planted per pot and grown in a growth chamber (20°C, 20-hour light and 4-hour dark photoperiod), watered daily, fertilized weekly with water, and thinned to three plants for the test. Four to six leaf stage seedlings were inoculated with single R. solani isolate, with five pots (15 plants total) inoculated per isolate. The amount of inocula to be added to each seedling was optimized taking into consideration that the seedlings should not be killed rapidly and damping off symptoms should progress gradually. Seedlings were inoculated by adding 10 fungus-infected millet inocula around each plant, 2 cm away from the seedling. Control plants were mock inoculated with sterile millet. Post inoculation observations were made at one-day intervals (days post inoculation DPI) and the symptoms were recorded (as per Table 1). Fifteen seedlings per treatment (fungal isolate) were scored and mean of the sum of disease score was reported as disease index (DI). The experiment was repeated several times (>7) and by different individuals as double-blind experiments.

Table 1: Disease Index schema.

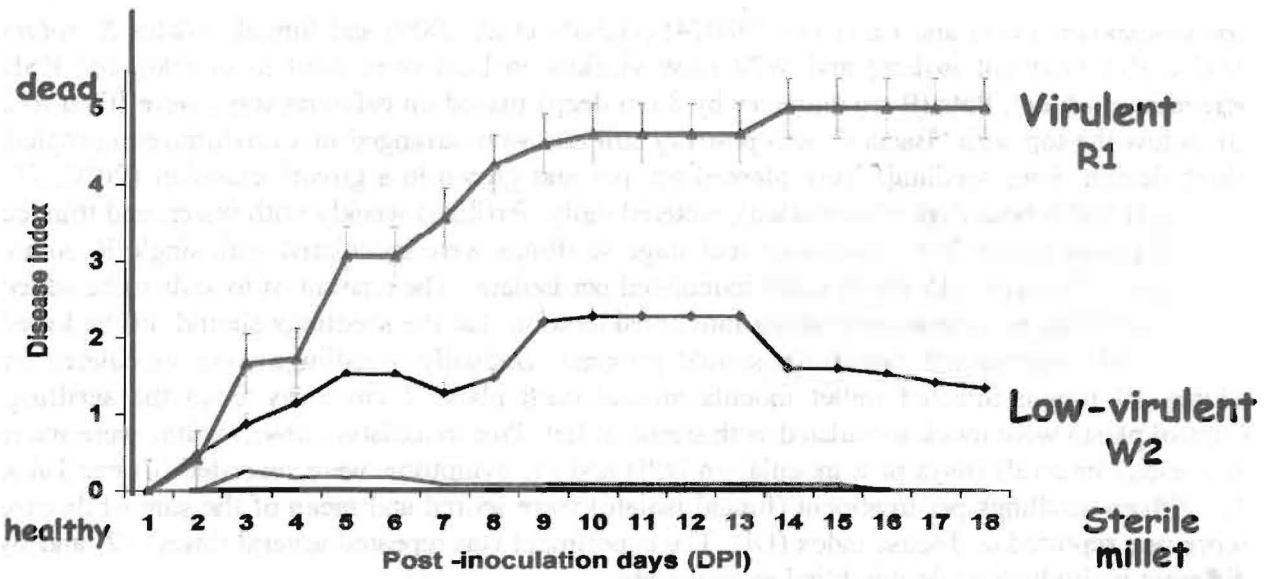
Score	Phenotypic symptoms
0	Healthy
1	Shallow penetration scar, visible to naked eye
2	Deep penetration scar, wound margins brown to black
3	Petioles lack turgor and rigidity, hypocotyl with water soaked lesions
4	Plant damping off, leaf blades wilting
5	Plant dead

Seedling nursery: Trails were conducted at the Michigan State University Plant Pathology Farm on Collins Rd. in East Lansing, MI from 2003 to 2006, and established as routine for a CRR nursery. In contrast to the CRR protocol, plants were inoculated at either the 2-4 leaf stage, or for 2004, the 6-8 leaf stage. Stand counts were taken before and after inoculation at weekly intervals.

Results:

The disease progress curve of RSD disease in sugar beets showed that Rhizoctonia seedling damping off disease was initiated and the disease symptoms progressed in all treatments up to DPI=6 then reached a plateau by DPI=9. USH20 infected with R1 (virulent) eventually died, and USH20 infected with W2 (low-virulent) recovered and showed limited symptoms (Figure 1). The RSD disease progress curve showed three stages. The initial infection stage from DPI 0 to DPI 6 were characterized by rapid appearance of symptoms, the second static phase from DPI 8 to DPI 12 was characterized by little disease progression, and the final resolution phase from DPI 13 to DPI 15 finalized the outcome of the interaction, either acute disease or death (compatible interaction) or recovery (incompatible interaction).

Figure 1: RSD progress curve of susceptible USH20 with either virulent (R1) or low-virulent (W2) *Rhizoctonia solani* isolates as compared to uninoculated control (sterile millet).



A series of 24 germplasm lines with various potentially desirable traits was screened using the RSD assay. 22 of the 24 completely succumbed to the disease, two accessions showed partial survival, and EL51 was identified as resistant. Further experiments contrasted the disease interaction between susceptible USH20 and resistant EL51 plants with virulent R1 and low-virulent W2 isolates.

Table 2: Germplasm screen of CRR characterized germplasm, discovering RSD resistance.

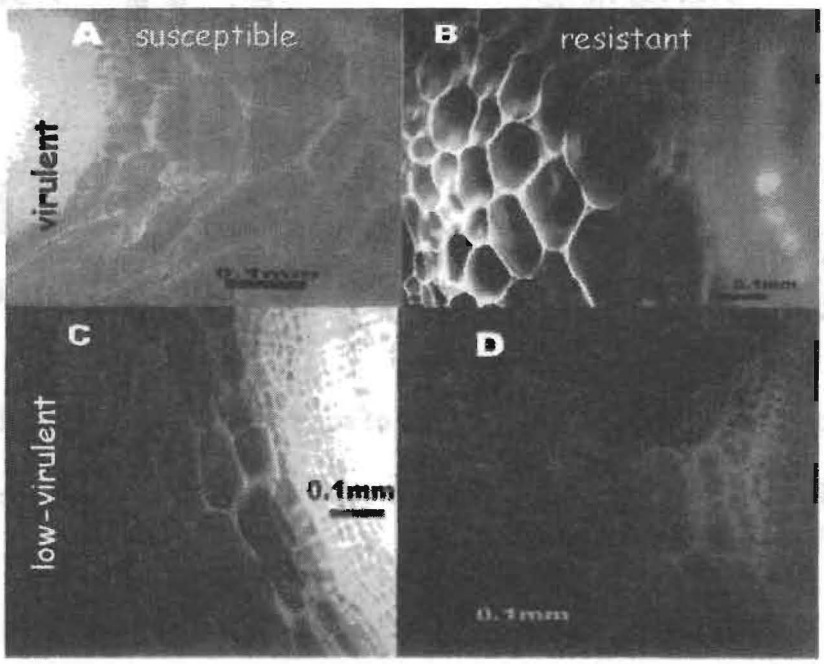
Accession	CRR	Other characters	RSD mean	Reaction
PI 285590	3		5	Susceptible
PI 285592	6		5	Susceptible
PI 285593	3		5	Susceptible
PI 285594	5		5	Susceptible
PI 285595	4		5	Susceptible
PI 546539	4		5	Susceptible
PI 552532	7		5	Susceptible
PI 558505	3		5	Susceptible
PI 558513	6		3	Partially resistant
PI 558515	6		5	Susceptible
USH20	6	Widely grown legacy hybrid	5	Susceptible
SR96	6	Smooth root	5	Susceptible
(EL51)	1	CRR	1	Resistant
Y03-384-18 Self	na	Aphanomyces resistance	5	Susceptible
Y03-384-60 Self	na	Aphanomyces resistance	3	Partially resistant
Y03-384-99 Self	na	Aphanomyces resistance	5	Susceptible
Y03-384-70 Self	na	Aphanomyces resistance	5	Susceptible
92RM3mm	6		5	Susceptible
PI 546537	7	wild	5	Susceptible
PI 546538	7	wild	5	Susceptible
PI 546533	3	ssp. maritima wild	5	Susceptible
PI 552532	7		5	Susceptible
PI 546510	3	ssp. maritima wild	5	Susceptible
PI 535826	5		5	Susceptible

The disease interaction was examined microscopically using 3-week old seedlings at 6-8 DPI. A number of examinations were done, including light microscopy using cotton blue to stain fungal hyphae, auto-fluorescence, and confocal microscopy using FITC-labeled Wheat Germ Agglutinin to differentially stain fungal tissue. Results showed that all interactions resulted in infection and ramification through the cortex (Table 3). Two observations in the incompatible interaction (EL51 resistant with R1 virulent) showed differential results relative to compatible interactions (all other plant-fungal combinations). First was the appearance of an auto-fluorescent material, presumably a phytoalexin, produced in the cortex of the incompatible reaction (Figure 2). The second was the failure of R1 to penetrate through the endodermal layer separating the cortex from the vascular stele tissues (data not shown, color figure is necessary). The implication of these results is that the integrity of the endodermis is required for resistance to be expressed, and this results would maintain the ability of the hypocotyl to conduct water and nutrients.

Table 3: Salient features of the interaction between sugar beet seedlings and Rhizoctonia solani AG2-2. Sus = USH20, Res = EL51, Vir = R1, low-vir = W2.

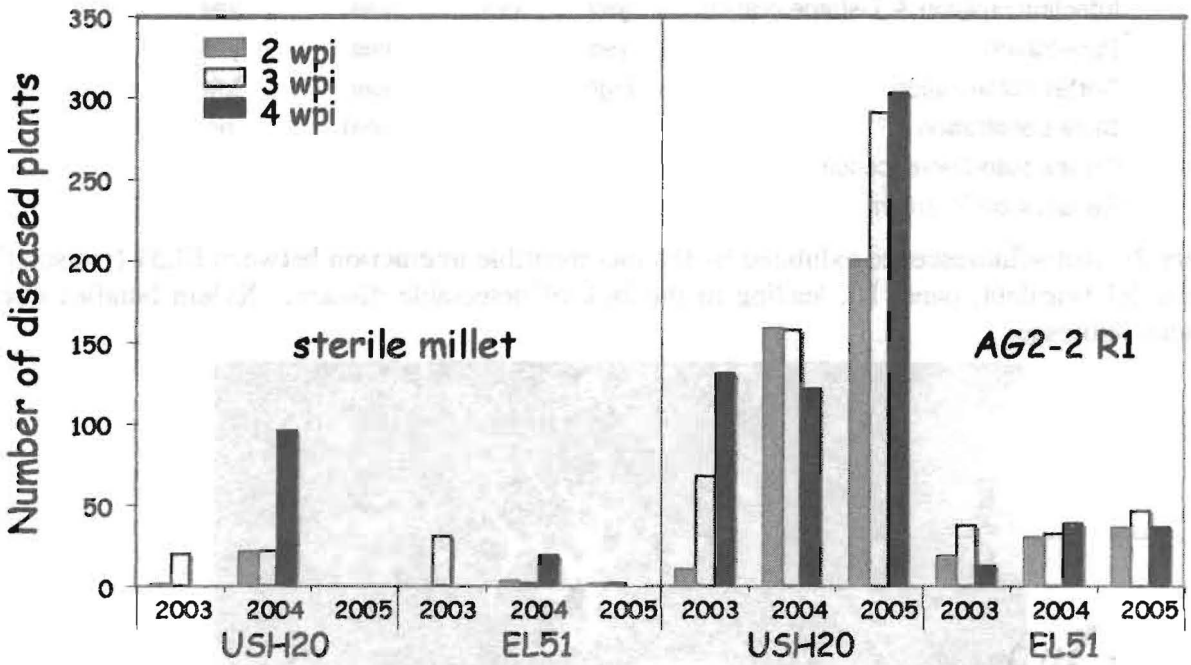
	Sus-Vir	Res-Vir	Sus-low-vir	Res-low-vir
Fungus grows toward host	yes	yes	yes	yes
Attachment	yes	yes	yes	yes
Infection cushion & T-shape branch	yes	yes	yes	yes
Penetration	yes	yes	yes	yes
Cortex colonization	high	low	low	low
Stele penetration	yes	no	(yes)	no
Cortex auto-fluorescence	low	high	low	low
Re-isolation *R. solani*	yes	no	no	no

Figure 2: Auto-fluorescence exhibited by the incompatible interaction between EL51 (resistant) and R1 (virulent, panel B), leading to the lack of detectable disease. Xylem bundles also auto-fluoresce.



Validation of greenhouse / growth chamber / microscopic RSD observations was conducted in the field over three years using USH20 (susceptible) and EL51 (resistant) inoculated with R1 (virulent) or W2 (low-virulent). W2 and sterile millet (control) treatment showed equivalent results, and only sterile millet was used fro comparisons in later years. In 2003, a portion of CRR nursery devoted to seedling disease validation, and included 4 entries, 6 replications, and 4 treatments, inoculated with 4 g inoculum / plant at 2-4 true leaf stage, and stand counts were taken weekly for 4 weeks with a final stand count. In 2004, 2 entries, 15 replications, and 2 treatments were done, Inoculated with 3 g inoculum / plant at 4-6 true leaf stage, with stand counts weekly for 4 weeks, and final stand count, and sugar analyses. In 2005, 3 entries, 10 replications of R1 and 3 replication of controls, inoculated with 3 g inoculum / plant at 2-4 true leaf stage, with stand counts weekly for 4 weeks and a final stand count. Results were consistent with growth chamber and greenhouse assays, with little disease apparent in the sterile inoculants and the EL51 – R1 incompatible interactions, but widespread death and destruction of USH20 – R1 compatible interaction (Figure 3). In the USH20 – R1 interaction, disease consistently progressed throughout the growing season until very few plants remained at harvest (data not shown).

Figure 3: Disease incidence under inoculated field conditions over three years, scored at 2, 3 and 4 weeks post inoculation (wpi).



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