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RHIZOCTONIA DISEASE IN SUGAR BEET: DISEASE
SCREENING AND
CYTO- HISTO PATHOLOGY OF SUGAR BEET-
RHIZOCTONIA SOLANI
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# RHIZOCTONIA DISEASE IN SUGAR BEET: DISEASE SCREENING AND CYTO- HISTO PATHOLOGY OF SUGAR BEET- *RHIZOCTONIA SOLANI* INTERACTION

By

**Subashini Nagendran**

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# ABSTRACT

## RHIZOCTONIA DISEASE IN SUGAR BEET: DISEASE SCREENING AND CYTO- HISTO PATHOLOGY OF SUGAR BEET- *RHIZOCTONIA SOLANI* INTERACTION

By

**Subashini Nagendran**

*Rhizoctonia solani* AG2-2 attacks sugar beet at the seedling stage causing damping off and, at maturity causing crown and root rot. It is estimated that an average 2% yield is lost to these diseases annually, and it is not uncommon to observe more than 50% losses in individual fields under the disease conducive conditions. The main goals of this research were to develop a robust Rhizoctonia seedling damping off disease screening method and critically observe the sugar beet- *R. solani* interaction during compatible and incompatible disease outcomes. An efficient protocol to screen Rhizoctonia seedling damping- off (RSD) disease in sugar beet was developed and this method was used to study the disease progress pattern. The RSD disease progress curve consisted of three distinct stages – an initial rapid disease progress stage, an intermediate stationary phase and a final resolution phase resulting in death or recovery. *R. solani* AG2-2 R-1 and W22 fungal isolates penetrated the sugar beet seedlings in susceptible and resistant cultivars but death was the uniform outcome only when the R-1 isolate infected a susceptible cultivar, USH20 (compatible interaction). Both the susceptible host infected with the W22 isolate and the resistant cultivar, EL51 infected with either R-1 or W22 isolates survived. Cultivar USH20 was highly susceptible to Rhizoctonia seedling damping off and for the first time resistance to Rhizoctonia seedling damping off was

detected in EL51. In the field when USH20 and EL51 were artificially inoculated with *R. solani* AG2-2 R-1 and W22 fungal isolates, EL51 showed resistance to both isolates but USH20 succumbed to R-1 isolate and recovered from W22 infection. Predicted sugar yield from EL51 was much higher than USH20 in this experiment, primarily due to preservation of plant stand.

The cyto- and histopathology in compatible and incompatible interactions were examined using light, fluorescence, confocal and scanning electron microscopy. *R. solani* AG2-2 R-1 and W22 fungal isolates produced typical infection structures that evidently penetrated the epidermis and were seen in the cortex tissue in both resistant (EL51) and susceptible (USH20) sugar beet seedlings. During the compatible interaction (USH20/R-1) the R-1 isolate ramified the host stele ground tissue During incompatible interactions, resistant plant EL51 limited the growth and penetration of R-1 and W22 isolates to just beneath the endodermis via cork layer formation. When susceptible plant USH20 was inoculated with W22 isolate, the host produced a very thin cork layer that was breached by the fungus and the fungus established in the stele tissue without causing disease. The EL51/R-1 interaction produced higher autofluorescence in the cortex cells compared to interactions EL51/W22, USH20/R-1 and USH20/W22.

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# CHAPTER 1

# OVERVIEW OF RHIZOCTONIA SEEDLING DAMPING OFF IN SUGAR BEETS, THE HOST-PATHOGEN INTERACTION AND RATIONALE OF THE RESEARCH

## HOST: *BETA VULGARIS L*

Sugar beet (Beta *vulgaris* L) belongs to the family Amaranthaceae, which circumscribes approximately 1300 species distributed worldwide with forms ranging from annual herbs to trees. Many species have C4 photosynthesis. The flowers are tiny and inconspicuous, but some species bear showy masses of fruits. Chenopods are common in deserts and especially in saline or alkaline soils. Spinach, sugar beets and common weeds such as goosefoot, pigweed and kochia are members of this family. *B. vulgaris* includes sugar beet, table beet, chard and fodder beets. The wild forms are seacoast plants of Europe, Middle East and Asia and are very variable in habitat. During the Napoleonic wars the supply of sugar cane from the West Indies was restricted and development of an alternative source of sugar was recognized from Marggraf's, and Achard's, demonstration that beets contained the same sweet substance as sugar cane (sucrose). The sugar beet was developed in Europe in the eighteenth century from white Silesian beet, then a fodder crop (Winner 1993). Original forms contained only about 4 to 6 % sugar but selection and breeding have increased this to 15 to 20% in modern hybrids. Sugar beet is grown for their swollen root and harvested at the end of the first year unless being grown for seed. All forms of the species *B. vulgaris* are mainly cross-pollinated.

World sugar production in 2005/2006 was 144,151,000 metric tons, US production was 6,824,000 metric tons. 27% of world sugar for human consumption is produced from sugar beet and is primarily grown in temperate zones of the northern hemisphere particularly in Europe and the USA. Sugar beet production in the USA was about 28,000,000 tons in 2005 (Table 1-1)

Table 1-1: Sugar beet production in USA (year 2001-2005)

| Sugar beet Production (1,000 tons) | | | | | |
|---|---|---|---|---|---|
| Year | 2001 | 2002 | 2003 | 2004 | 2005 |
| Great Lakes | | | | | |
| Michigan | 3,220 | 3,204 | 3,400 | 3,439 | 3,167 |
| Ohio | 12 | 37 | 46 | 37 | 0 |
| Total | 3,232 | 3,241 | 3,446 | 3,476 | 3,167 |
| Upper Midwest | | | | | |
| Minnesota | 7,796 | 8,854 | 10,032 | 9,823 | 9,384 |
| North Dakota | 4,290 | 4,799 | 5,202 | 4,846 | 4,593 |
| Total | 12,086 | 13,653 | 15,234 | 14,669 | 13,977 |
| Great Plains: | | | | | |
| Colorado | 824 | 794 | 644 | 838 | 833 |
| Montana | 1,150 | 1,096 | 1,308 | 1,131 | 1,143 |
| Nebraska | 840 | 760 | 861 | 1,050 | 924 |
| New Mexico | 0 | 0 | 0 | 0 | 0 |
| Texas | 0 | 0 | 0 | 0 | 0 |
| Wyoming | 857 | 659 | 752 | 812 | 801 |
| Total | 3,671 | 3,309 | 3,565 | 3,831 | 3,701 |
| Far West: | | | | | |
| California | 1,596 | 1,960 | 1,959 | 1,995 | 1,707 |
| Idaho | 4,636 | 5,103 | 6,044 | 5,510 | 4,726 |
| Oregon | 290 | 301 | 301 | 396 | 307 |
| Washington | 253 | 140 | 161 | 144 | 69 |
| Total | 6,775 | 7,504 | 8,465 | 8,045 | 6,809 |
| Total U.S. | 25,764 | 27,707 | 30,710 | 30,021 | 27,654 |

Source: Economic research service (ERS) United States Department of Agriculture. Last updated 1/12/2006.

Many nations are actively involved in research and development of renewable energy including developing technologies for bio-energy production from regional agricultural products. Currently energy from cellulose and other lignocellulosic materials are much more economical than sugar crops (sugar beet, sugar cane, sweet sorghum), but the former must be first hydrolyzed to fermentable sugars, increasing both capital investment and operating costs (Ogbonna 2001). About 2% of today's transportation fuels are derived from biomass and blended with fossil fuels. Many nations are projecting that 5% of their road fuels will be bio-derived within the next 5 years (Koonin 2006). This trend may add further demand for sugar beet crop plant.

## FUNGAL PATHOGEN *RHIZOCTONIA SOLANI* KÜHN

Many abiotic and biotic diseases caused by viral, bacterial, fungal pathogens, nematodes and insects affect sugar beet (Whitney and Duffus 1991). *Rhizoctonia solani* Kühn (*Thanatephorus cucumeris* (Frank) Donk) is a facultative parasite that infects wide range of crop plants including sugar beet, rice, corn, turf grass and ornamental plants (Sneh *et al.*, 1996). *R. solani* is a basidiomycete fungus that does not produce any asexual spores (mycelia sterilia) or fleshy sexual basidium (Barnett and Hunter 1999). In nature, *R. solani* exists primarily as vegetative mycelium and/or sclerotia, the undifferentiated aggregation of thick-walled melanized mycelia that can persist in the soil for years (Sherwood 1970).

*Rhizoctonia* species consists of a very diverse collection of teleomorphs that are referred to five different form genera (Moore 1996). The genera with anamorphs referred to *Rhizoctonia* are *Helicobasidium, Thanatephorus, Ceratobasidium, Waitea, Tulasnella* and *Sebacina*. The teleomorph *Thanatephorus* that includes *R. solani* and Ceratobasidium include many destructive pathogens. Anderson and Stalpers (1994) reviewed taxonomy of Rhizoctonia. However genetic variation and systematic relationships within and among subgroups of Rhizoctonia need greater refinement.

*R. solani* is divided into 12 anastomosis groups (AG) based on a somatic compatibility reaction (Anderson 1982). Isolates belonging to the same AG result in hyphal fusion (anastomosis) when co-cultured leading to acceptance (self-pairings). Interpretation of the anastomosis reaction is not always straightforward because the four described hyphal interaction phenotypes (C0 to C3) represent a continuum (Carling *et al.*

1988). Within an AG, two types of hyphal interactions (C2 and C3) are most relevant for the study of population biology. The C2 reaction (also referred as killing reaction), represents a somatic incompatibility response between genetically distinct individuals. The C3 reaction (perfect fusion) between two isolates is indicative of genetic closeness or similarity. Very little is known about the mechanisms controlling this recognition process in *Rhizoctonia*. Biochemical (Jabiji-Hare 1996) and DNA-based studies (Justesen *et al.* 2003, Boysen 1996, Cubeta 1996), support the separation of *R. solani* into genetically distinct groupings, but has revealed considerable genetic diversity within an anastomosis group. Reynolds *et al.* (1983) distinguished different AGs based on their distinct protein patterns. The internal transcribed spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis. Weiland and Sundsbak (2000) differentiated and detected *R. solani* from other sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rRNA. *R. solani* AG 2 isolates were also analyzed based on ITS regions (Salazar *et al.* 1999). Thus, presently identification of Rhizoctonia isolates is based on morphology, pathology and the anastomosis reaction (Ogoshi 1987) in addition to biochemical and molecular approaches. *R. solani* is a ubiquitous soil borne pathogen causing disease on a broad array of host plants (Table 1-2). How does *R. solani* adapt to diverse environment and successfully infect wide range of host plants? There is no clear understanding of underlying mechanism of its adaptability or a clear understanding of genetics of *R. solani.* This organism has been viewed as a functionally non-sexual species (Caten and Jinks 1966) and as a sexual species (Anderson 1982). The genetics of Rhizoctonia and related species is complex and poorly understood and needs further studies to understand the complex, and affinities within and between groups (Adams 1996).

Table 1-2. Host range of different AGs of *Rhizoctonia solani* (Sneh et al., 1996).

| Anastomosis group | Diseases | Host |
|---|---|---|
| AG 1-IA | sheath spot | rice |
| | leaf blight | corn, sorghum, bean, soybean |
| | brown patch | turf grass |
| AG 1-IB | web blight | bean, rice, soybean |
| | rot | cabbage and lettuce |
| | foliar blight | sugar beet |
| AG 1-IC | damping off | buckwheat, soybean, pine |
| | damping off, crown root rot | carrot |
| AG 2-1 | damping off | crucifers |
| | bud rot | strawberry |
| | leaf blight | tulip |
| AG 2-2 IIIB | damping off, crown and root rot | sugar beet |
| | false sheath blight | rice |
| | brown patch | turf grass |
| | crown and brace rot | corn |
| AG 2-2 IV | root rot | sugar beet |
| | large patch | turf grass |
| AG 3 | black scurf stem cankers | potatoes |
| | target spot | tobacco |
| | leaf blight | tomato |
| | brown spot | egg plant |
| AG 4 | fruit rot | tomato |
| | stem rot | pea |
| | damping off, stem canker | potato |
| | damping off, root rots | soybean, cotton, peanuts<br>pea, onions, pine |
| | pod rot | snap beans |
| AG 5 | black scurf | potato |
| | brown patch | turf grass |
| | root rot | beans |
| AG 6 and AG 7 | nonpathogenic group | |
| AG8 | bare patches | cereals |
| AG 9 | weak pathogen | crucifers<br>potatoes |
| AG10 | nonpathogenic group | |
| AG 11 | Culm and stalk disease | wheat |
| AG BI | nonpathogenic | |

## RHIZOCTONIA SEEDLING DAMPING OFF AND CROWN AND ROOT ROT IN SUGAR BEETS AND RATIONALE OF RESEARCH ON SUGAR BEET- *R. SOLANI* INTERACTION

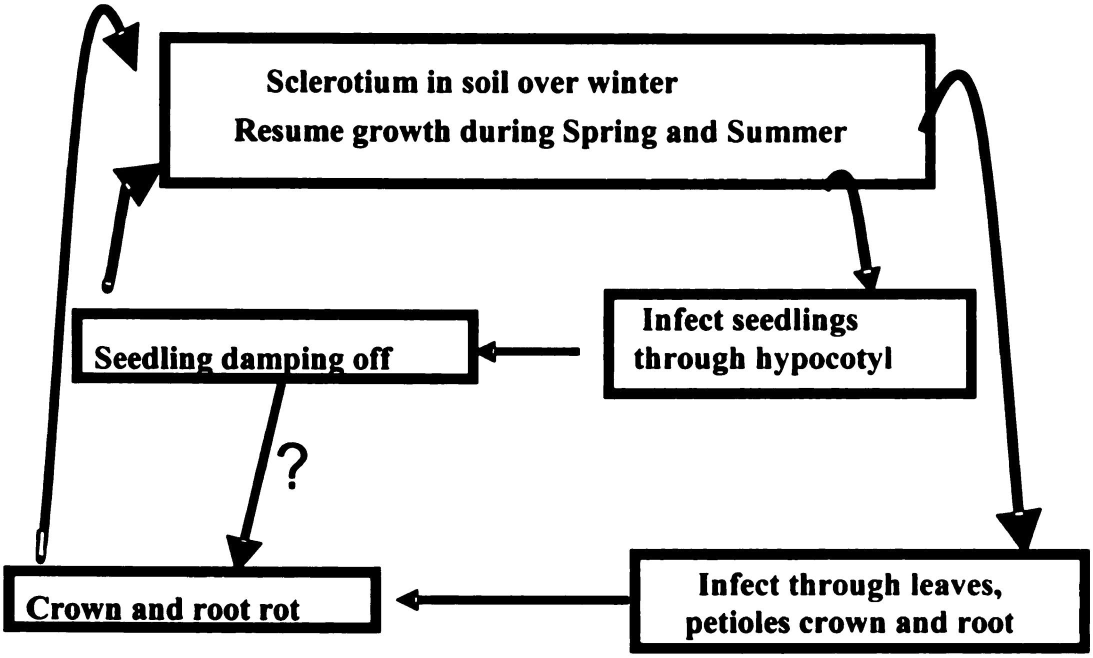
In sugar beets, *R. solani* infects seedlings causing seedling damping off and causes crown and root rot at the adult stage (Figure 1-1). *R. solani* AG2-2 is reported as seedling damping off pathogen and crown and root rot pathogen and AG4 as a virulent seedling damping off pathogen (Herr 1996). Isolates within single AG can vary in virulence and pathogenicity. Typical post-emergence damping off symptoms caused by *R. solani* begin with slight browning of stem just above or at ground level followed by epinasty (downward bending of leaves). As the interaction progresses, the seedlings appear water-soaked, tissue collapses, the seedlings shrivel, turn brown and die.

The first symptoms observed during crown and root rot are sudden and permanent wilting of leaves and black necrosis of petioles at the crown. Wilted plants seldom recover, and after dying often form a dry, dark rosette. Infection begins as discrete, dark elliptical lesions on root surface. These lesions may grow together and eventually cover the entire root surface as disease progresses. Infections may also start in the crown and move downward. Infected roots usually remain firm, and rot seldom penetrates into the interior of the root until advanced stages. A clear margin can often be seen between infected and healthy tissues, and extensively rotted roots will exhibit surface cracks. Economic losses in the sugar beet industry due to *R. solani* were estimated to average 2% in the United States; however, damage can vary greatly (0 to 50%) from field to field depending on cropping history and environment (Schneider and Whitney 1991).

To control Rhizoctonia diseases in sugar beet, fungicides such as Quadris (azoxystrobin) and cultural practices including crop rotation and field sanitation are followed. Understanding the etiology of Rhizoctonia diseases in sugar beet and finding environmentally friendly methods to control this disease is important.

Scholten *et al.* (2001) developed a greenhouse test to evaluate sugar beets for resistance to Rhizoctonia crown and root rot disease. Here the development of a Rhizoctonia seedling damping off disease screening protocol using growth chambers is described. The sugar beet cultivars that were screened for Rhizoctonia seedling damping off were further tested for their disease susceptibility under both greenhouse and field conditions.

Figure 1-1: Rhizoctonia disease cycle in sugar beet



Field disease evaluation of Rhizoctonia root rot in sugar beets is influenced by environmental conditions (Ruppel *et al.* 1979, Scholten et al.. 2001). The objective of this research was to study the effect of Rhizoctonia disease (seedling damping off and crown and root rot disease) on sugar beet crop and test sugar beet breeding lines for disease resistance and/or different *R. solani* isolates for pathogenicity or aggressiveness under field conditions. Traditionally inocula has been added on the sugar beet crown at the adult stage to screen for Rhizoctonia diseases. In this study inocula was added at the seedling stage adjacent to the plant in the soil to mimic the natural infection process, and also examine the relationship between seedling damping off and crown and root rot disease. Does the seedling damping off harbor inocula for crown and root rot? Does the cultivar's resistance to seedling damping off imply resistance to crown and root rot? Part of the research focused on seedling damping off and final productivity due to Rhizoctonia disease pressure in susceptible and resistant sugar beet cultivars and tested the kinetics between disease resistance and yield. During the Rhizoctonia disease cycle, initially the fungus resumes growth under favorable condition and directly penetrates the seedlings or adult plant. Ruppell (1973) reported that resistance of mature sugar beet roots to AG 2 type 2 isolates is not due to a mechanical barrier, as both resistant and susceptible sugar beet cultivars are penetrated by fungal hyphae. Resistant cultivars restricted the pathogen to the periderm or outer secondary cortex, whereas in susceptible roots several vascular rings were invaded.

Panella (2005) defined *R. solani* isolates that cause mild symptoms on a susceptible sugar beet variety that did not kill the seedlings as "low virulent fungal isolates", while those that caused seedling death as "virulent isolates". Two *R. solani* AG-

2-2 isolates namely W22 and R-1 (kindly provided by Dr. Lee Panella and Dr. Linda Hanson, USDA-ARS, Ft. Collins) were used in this research. *R. solani* AG2-2 W22 isolate was determined to be a low virulent isolate, causing only mild symptoms with no plant death, while isolate R-1 was virulent, causing severe symptoms and seedling death. The objectives of this research were to analyze how the resistant and susceptible sugar beet seedlings interact with *R. solani* AG2-2 R-1 and W22 isolates and study the etiology of the host response under these conditions using light, fluorescence, scanning electron, confocal microscopes. Through this research, an efficient reproducible method to screen Rhizoctonia seedling damping off disease in sugar beet was developed. Sugar beet cultivar EL51 was resistant and USH20 was susceptible to Rhizoctonia seedling damping off disease. Under field conditions EL51 showed resistance to both Rhizoctonia seedling damping off and Rhizoctonia crown and root rot and USH20 was susceptible for both diseases. The disease progress patterns and etiology of the host response when resistant and susceptible sugar beet cultivars were infected with *R. solani* AG2-2 R-1 and W22 isolates were documented. The Rhizoctonia disease resistant germplasm EL51 and knowledge developed on host-pathogen interaction in this research will facilitate a successful breeding program for Rhizoctonia resistance in sugar beets and enhance the knowledge in basic and applied science of host interaction with *R. solani.*

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# CHAPTER 2

# RHIZOCTONIA SEEDLING DAMPING OFF IN SUGAR BEETS – DISEASE SCREENING AND DISEASE PROGRESS PATTERN

## ABSTRACT

This research focused on developing a reliable protocol to screen Rhizoctonia seedling damping- off (RSD) disease in sugar beet and studying RSD disease progress pattern. The bioassay to screen RSD was replicated several times and by different individuals as double-blind experiments in the growth chamber and in the greenhouse. The disease progress curve consisted of three distinct stages – an initial rapid disease progress stage, an intermediate stationary phase and a final resolution phase where death or recovery occurred. Observations indicated that both R. solani AG2-2 R-1 (virulent) and W22 (low virulent) fungal isolates initiated the infection process in susceptible and resistant sugar beet cultivars. Disease progressed rapidly during infection by *R. solani* R-1 (virulent isolate) in the susceptible cultivar and resulted in a compatible interaction (disease). The susceptible host infected with *R. solani* W22 (low virulent isolate) and resistant cultivars infected with either R-1 or W22 isolate recovered. There was no correlation between saprophytic growth rate and pathogenicity of R-1 and W22 isolates.

# INTRODUCTION

Disease resistance of host plants and the estimation and pattern of disease progress are critical inputs in disease management (Jeger 2004). Several factors including fluctuating weather conditions, host susceptibility with age and pathogenicity of infecting agent influence plant disease progress and epidemics (Hau 1990).

This study focused on developing a screening method to evaluate Rhizoctonia seedling damping off (RSD) in sugar beets and document the disease progress pattern. Many pathogenic fungi including *Rhizoctonia solani, Aphanomyces Cochlioides, Pythium ultimum* and *P. aphanidermatum* and several environmental conditions such as excessive or inadequate soil moisture, cool soil temperatures, humid warm temperatures, salinity or compacted soils can cause seedling damping off (Leach 1991). Damping off can occur before seedling emergence (pre-emergence) or after seedling emergence (post-emergence). *Rhizoctonia solani* Kuhn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) is a serious pathogen in sugar beets attacking both seedlings causing damping off and mature plants causing crown and root rot disease ( Herr 1996). Typical post-emergence damping off symptoms caused by *R. solani* begin with slight browning of the stem just above or at ground level followed by epinasty - the downward bending of leaves. As deterioration progresses, the seedlings appear water-soaked, tissue collapses, the seedlings shrivel, turn brown and die.

*R. solani* is a facultative parasite and it may increase its inoculum potential via saprophytic growth. *R. solani* produces sclerotia –the over wintering structure, which is a critical component in the field to ensure disease be completed in continuous succession. *R. solani* is divided into 12 anastomosis groups (AG) based on the somatic compatibility reaction (Anderson 1982). Various AGs of *R. solani* are somewhat morphologically similar but they are isolated genetically. Each AG group is a complex heterogenic collection of isolates displaying diverse characters, including pathogenicity and virulence, which can vary within a single AG (Sneh *et al.* 1991). Schneider *et al.* (2001) showed that Rhizoctonia populations develop in relation to soil temperature and plant development. Plant pathologists have made little progress in breeding for disease resistance or in controlling plant diseases caused by *R. solani.* Understanding how different isolates of the fungus interact among and with host plants, and resolving the complex species concept of *R. solani* will enable the pathologist to develop effective methods to manage Rhizoctonia diseases.

Sugar beet (*Beta vulgaris* ssp.*vulgaris* L.) is a globally important crop, producing a third of world sucrose supplies (Winner 1993). Biotic and abiotic factors challenge germination and survival of seedlings of seed-sowing crop plants like sugar beets (McGrath *et al.* 2000). Field disease evaluation of Rhizoctonia root rot in sugar beets is influenced by environmental conditions (Ruppel *et al.* 1979, Scholten et *al.* 2001). To minimize the weather impact on a disease screening method, we developed a screening protocol in growth chambers. Analyzing the disease progress pattern in growth chambers provided controlled reproducible environments in which uniform experimental plants could be grown at any time of a year. The growth chamber RSD disease screening method on sugar beet seedlings was tested and proven as an effective method to screen for RSD disease. The information obtained from the screening method was extended to greenhouse and field to further study the Rhizoctonia disease in sugar beet. *R. solani*

AG2-2, R-1 (virulent) and W22 (low virulent) isolates on sugar beets (Panella 2005) were used to artificially inoculate resistant and susceptible sugar beet seedlings for studying the compatible and incompatible host-pathogen interaction and begin to elucidate the disease progress pattern.

# 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) (listed in Table 2). For growth chamber and greenhouse experiments, the seeds were soaked in 0.3% hydrogen peroxide (V/V) (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 (Michigan Peat Company, Houston, TX).

## Fungal inocula

*R. solani* AG2-2 R-1 isolate and W22 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) (Criterion, Hardy diagnostics, C5491) 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, and thinned to three plants per pot 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 according to developed criteria (Table 2-1). The mean of the sums of disease score was reported as disease index (DI). Data were subjected to statistical analysis.

**Table 2-1: Scheme of Rhizoctonia seedling damping off disease scoring in sugar beet seedlings**

| Score | Phenotypic symptom |
|---|---|
| 0 | Healthy |
| 1 | Slight penetration scar visible to naked eye |
| 2 | Deep penetration scar very visible, margin of the wound brown to black |
| 3 | Plant showing damping off symptoms, Petioles loosing its turgor and rigidity, hypocotyls (stem) shows water soaked lesions |
| 4 | Plant damping off, leaf blades wilting |
| 5 | Plant dead |

### Rhizoctonia seedling damping off disease progress pattern in the greenhouse

Sugar beet breeding lines USH20 and EL51 were used. Wooden boxes (400cm x 580 cm) were filled to 2 cm below the top with Baccto high porosity soil and were arranged in a randomized complete block design. Thirty seedlings were planted per wooden box and grown in the greenhouse (25°C, 16 hr light and 8 hr dark photoperiod), watered daily, fertilized weekly, and transplanted and maintained thirty plants per box. Four to six leaf stage seedlings were inoculated with single isolate of *R. solani* AG 2-2, R-1 (virulent) isolate or W22 (low virulent) isolate. Each seedling was inoculated by adding 0.1 g of inocula (about 20 fungus –infested millet seeds) on opposite sides of each plant 4 cm away from each seedling. Control plants were mock inoculated with sterile millet. Rhizoctonia seedling damping off disease progress was scored at one-day intervals (Table 2-1). Forty seedlings per treatment were scored and the mean of the sum of disease scores was reported as the disease index (DI). Ten seedlings per treatment- box were randomly selected at each day post inoculation for microscopic observations (Chapter 4) and pathogen isolation. The experiment was repeated several times (>7) and by different individuals as double-blind experiments.

### Analysis of different accessions of sugar beet for Rhizoctonia seedling damping off disease

The sugar beet accessions that have different levels of resistance to various diseases, different agronomic quality (e.g. smooth root) and some wild germplasm were screened for RSD (see Table 2-2). All accessions were screened in the greenhouse. Four seedlings per accession were tested and the test was replicated twice (Appendix B). After DPI 14, the plants that survived and continued growing were classified as healthy (H) and if a seedling failed to survive and grow it was classified as dead (D). A sugar beet accession was classified as "resistant" if 3 or 4 seedlings survived out of 4 tested plants in each trial. An accession was classified as "partially resistant" if 2 plants survived out of 4 tested plants. If all plants were diseased or only one plant survived, that accession was classified as "susceptible".

### Isolation of *R. solani* from diseased sugar beet seedlings

Whole sugar beet seedlings were collected at different post-inoculation time points. Seedlings were washed in running water for 2 hours and leaves, two third of the hypocotyl and the narrow tail of the root was excised. The remaining part of the seedling, about 2 cm of the hypocotyls including the upper portion of the root tissue, were washed in sterile water thrice and blot dried. Subsequent steps were performed under sterile conditions. A razor blade was used to progressively cut small pieces adjacent to diseased tissue, with care to avoid any direct contact with rotted tissue. Tissue pieces were transferred to water agar containing 0.05% lactic acid (V/V) and incubated at 28 °C in the dark for 24 to 72 hours. Tissue pieces were then examined under 40x magnification. If any fungal hyphae were emerging from the tissue, those tissues were mounted on slides, stained with cotton blue and observed using light microscope. *R. solani* was identified by its distinctive mycelial morphological characteristics (Sneh *et al.* 1991).

### Isolation of *R. solani* from infested soil

Soil samples were collected at different post inoculation time points (DPI) from the disease screening experimental units (growth chamber pots and greenhouse wooden boxes). A 3 cm deep layer of top soil from the entire experimental unit was separated and mixed thoroughly. 10 samples each 1 g of soil were collected from this mixed soil. Each soil sample was moistened with sterile distilled water, compacted with spatula and evenly distributed in five clumps (0.2g) on a plate of selective medium (modified from Ko and Hora 1971). Selective medium contained $K_2HPO_4$ (1g), $MgSO_4.7H_2O$ (0.5g), KCl (0.5g), $FeSO_4$.7H2O (0.01g), $NaNO_2$ (0.2g), chloramphenicol (0.05) agar (20g) and distilled water 1000ml. The media was sterilized at 121°C for 20 min, allowed to cool down and added tannic acid (0.4 g), streptomycin (0.05g), metalaxyl (0.0633g) and prochloraz (0.005g) mixed and dispensed (20 ml) into 9-cm diameter petri dishes. Twenty plates were incubated for each type of treatment (*R. solani* AG2-2 R-1 and W22 isolates or sterile millet mock inoculation) at 28°C. The perimeter of the soil clumps were observed at 40x magnification after 24 hours and 48 hours incubation. Fungal hyphae that were emerging from the incubated soil were mounted on slides, stained with cotton blue and were observed under light microscope. *R. solani* was identified by its distinctive mycelial morphology (Sneh *et al.* 1991).

### Statistical analysis

The data from the growth chamber and the greenhouse disease screening experiments were analyzed using mixed model ANOVA with repeated measurement. The data were analyzed by SAS software (version 9.1.2). Tests were adjusted by the Tukey method. Adjusted p-values (<0.05) were considered significant on the response variable (DI) between different treatments (i.e USH20/R-1, USH20/W22, USH20/MOCK, EL51/R-1 EL51/W22 and EL51/MOCK ). Mean Disease Index was plotted against DPI.

### *In vitro* saprophytic growth rate of *R. solani* on different organic media

Saprophytic growth of *R. solani* AG2-2 R-1 and W22 isolates were compared on five different organic media. The organic media were water agar medium (WA) composed of 1.7 % agar, corn meal agar medium (CMA) composed of 1.7 % of Corn Meal Agar (Criterion, Hardy diagnostics, C5491) and Potato Dextrose Agar medium (PDA) composed of 3.9% of Bacto Potato dextrose (DIFCO 213400). Media were sterilized at 121°C for 15 min. Soil extract agar media (SEA) was made by sterilizing 400 g of air-dried soil (with content of organic matter) in 1000 ml tap water for one hour at 121°C and clear soil supernatant was obtained by centrifuging at 2000 rpm for 5 minutes (modified from Rajendran *et al.* 1991). To 1000 ml of clear soil supernatant solution added 15.0 g of agar and sterilized at 121°C for 20 min. Plant extract agar medium (PEA) was made from two weeks old sugar beet seedlings. Five grams of whole plant tissue were crushed in 20 ml sterilized water. The extract was filtered through muslin cloth and centrifuged at 2000 rpm for 5 min. The supernatant was used for media preparation. 17 g of agar was added to the supernatant, made the final volume to 1000 ml with distilled water, and sterilized at 121°C for 20 min. All media were dispensed (20 ml) into 9-cm diameter Petri dishes. Mycelial plugs, 5 mm diameter, cut from the margin of an actively growing colony, were placed on the centre of the dishes. The cultures were incubated at 25°C in dark. Each treatment was tested with four stock sub-isolates per isolate, with three replicates per sub-isolate. The fungal growth was recorded after 4 days. The fungal culture plates were observed under the dissecting microscope and the distance between center of inocula plug and the highest point of the edge of growing colony tip was measured.

# RESULTS

## Growth chamber RSD disease progress pattern

The objective of this research was to develop a reliable protocol to screen Rhizoctonia seedling damping off (RSD) disease in sugar beet to study RSD disease progress patterns. When mean disease index (DI) of treatments was compared ($P<0.05$) up to DPI = 3, no treatments was significantly different. At DPI=4, USH20/R-1 treatment was significantly different from the mock inoculated (sterile millet) plants. Between DPI = 5 to DPI = 12, all treatments were significantly different from mock inoculated plants. From DPI=13 to DPI=15, USH20/R-1 interaction was significantly different from all other treatments including mock inoculation.

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 and reached a plateau by DPI= 9. With the exception of susceptible sugar beet cultivar infected with R-1 isolate (USH20/R-1) all other treatments recovered and showed limited symptoms. For susceptible cultivar USH20 infected with R-1 isolate, this same time frame was characterized by a rapid increase in disease severity (Figure 2-1). 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).

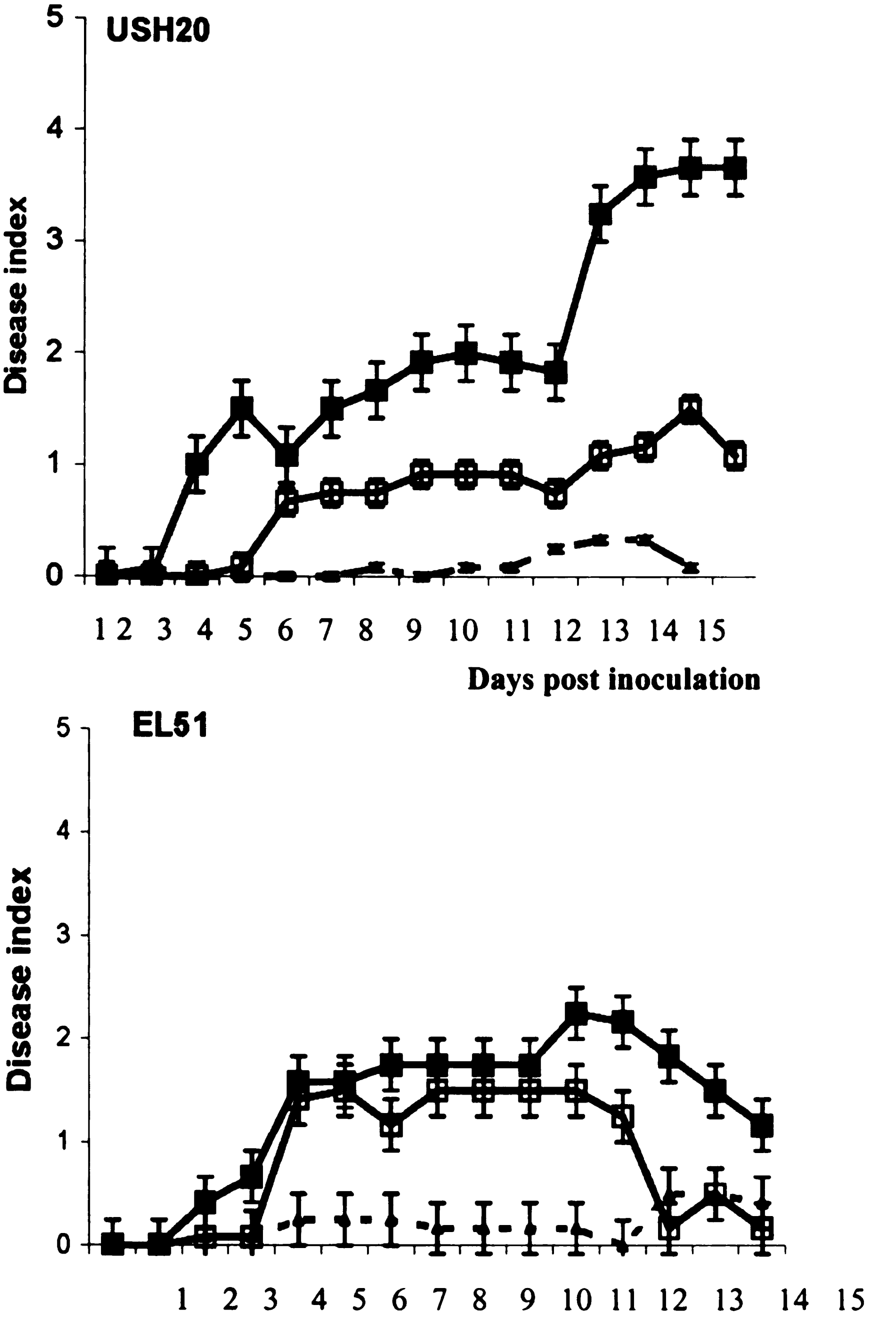

Figure 2-1: Rhizoctonia seedling damping off (RSD) disease progress curve.

RSD disease progress in sugar beets when susceptible cultivar (USH20) (Top panel) and resistant cultivar (EL51) (bottom panel) were inoculated with *R. solani* AG2-2 R-1 (■), W22 (□) and mock inoculation (sterile millet inocula ---). Error bars are std. errors of the DPI measures of DI).

### Disease progress patterns in the greenhouse and a resistance screen for different sugar beet accessions

Under greenhouse conditions (25°C, 16 hr light and 8 hr dark photoperiod), the disease progress pattern of RSD in sugar beet was similar to that of growth chamber disease progress pattern (data not shown). Different sugar beet accessions were screened for resistance to RSD using the growth chamber and the greenhouse disease screening protocols. Only EL51 (PI 598074) showed resistance to Rhizoctonia seedling damping off disease with 75 % to 100 % of the plants recovered from damping off disease and continued to grow in two different sets of experiments. Fifty percent of plants survived in sugar beet accessions PI 558513 and YO3-384-60- self, and were classified as "partial resistant". In all other tested accessions only 0 to 25% of the plants survived and were classified as "susceptible" (Table 2-2).

**Table 2-2: Sugar beet accessions screened for Rhizoctonia seedling damping off**

| Test # | Accession identifier | Rhizoctonia Crown -root rot (1= resist, 9= susceptible) | Comments | RSD score |
|---|---|---|---|---|
| 1 | PI 285590[a] | 3 | | Susceptible |
| 2 | PI 285592[a] | 6, 8 | | Susceptible |
| 3 | PI 285593[a] | 3 | | Susceptible |
| 4 | PI 285594[a] | 3 , 5 | | Susceptible |
| 5 | PI 285595[a] | 3 , 4 | | Susceptible |
| 6 | PI 546539[a] | 3 , 4 | | Susceptible |
| 7 | PI 552532[a] | 6 , 7 | | Susceptible |
| 8 | PI 558505[a] | 3 | | Susceptible |
| 9 | PI 558513[a] | 3 , 6 | | Partially resist |
| 10 | PI 558515[a] | 3 , 6 | | Susceptible |
| 11 | PI 631354[b] (USH20) | 6 | Widely grown hybrid in 1970s | Susceptible |
| 12 | SR96[b] | 6 | Smooth root | Susceptible |
| 13 | PI 598074[a, b] (EL51) | Resistant (not scaled) | hybrid | Resistant |
| 14 | Y03-384-18Self[c] | NA | Aphanomyces resistance | Susceptible |
| 15 | Y03-384-60 Self[c] | NA | Aphanomyces resistance | Partially resist |
| 16 | Y03-384-99 Self[c] | NA | Aphanomyces resistance | Susceptible |
| 17 | Y03-384-70 Self[c] | NA | Aphanomyces resistance | Susceptible |
| 18 | 92RM3mm[b] | 6 | | Susceptible |
| 20 | PI 546537[a] | 3 , 7 | wild | Susceptible |
| 21 | PI 546538[a] | 3 , 7 | wild | Susceptible |
| 22 | PI 546533[a] | 3 | ssp. maritima wild | Susceptible |
| 23 | PI 552532[a] | 3, 6 , 7 | | Susceptible |
| 24 | PI 546510[a] | 3 | ssp. maritima wild | Susceptible |
| 25 | PI 535826[a] | 3 , 5 | | Susceptible |

[a] National Genetic Resources Program (NGRP) http://www.ars-grin.gov

[b] USDA-ARS East Lansing, MI

[c] Yi Yu 2004. PhD dissertation: Genetics of Aphanomyces disease resistance in sugar beet (*Beta vulgaris*), ALFP mapping and QTL analyses.

### Recovering *R. solani* from diseased sugar beet seedlings and soil

USH20 and EL51 inoculated with R-1 or W22 showed typical *R. solani* - induced damping off symptoms up to DPI=9. *R. solani* was recovered on selective medium from roots and lower stems of all sampled seedlings. Other sugar beet seedling pathogens, including Aphanomyces were not recovered. After DPI=9, only susceptible seedlings inoculated with *R. solani* R-1 isolate yielded *R. solani* where as the susceptible (USH20) seedlings inoculated with *R. solani* W22 isolate and resistant cultivar (EL51) did not yield *R. solani* on selective media (Table 2-3). *R. solani* was isolated from the inoculated soil in all the above conditions and all time points.

**Table 2-3: Isolation of *R. solani* from diseased sugar beet seedlings**

| Treatment | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sugar beet | *R. solani* | Days post inoculation (DPI) | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| USH 20 | R-1 | n | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| | W22 | n | Y | Y | Y | Y | y | Y | Y | y | n | n | n |
| EL51 | R-1 | n | Y | Y | y | y | y | y | y | y | n | n | n |
| | W22 | n | Y | Y | Y | Y | n | Y | n | y | n | n | n |

Y=isolated *R. solani*, n= did not isolate *R. solani*, y= *R. solani* isolated from 50% of the tested tissues

### *In vitro* saprophytic growth rate of *R. solani* on different organic media

*R. solani* AG2-2 R-1 grew rapidly and was larger in diameter in WA and CMA compared to W22 ($p < 0.05$). However, in other tested organic media (PDA, SEA and PEA) there were no significant differences in growth between R-1 and W22 isolates (Figure 2-2). On WA media even though the *R. solani* culture grew rapidly and covered a greater diameter, the vegetative mycelia was loose and much less dense compared to other tested organic media. All the tested organic media supported sclerotial formation.

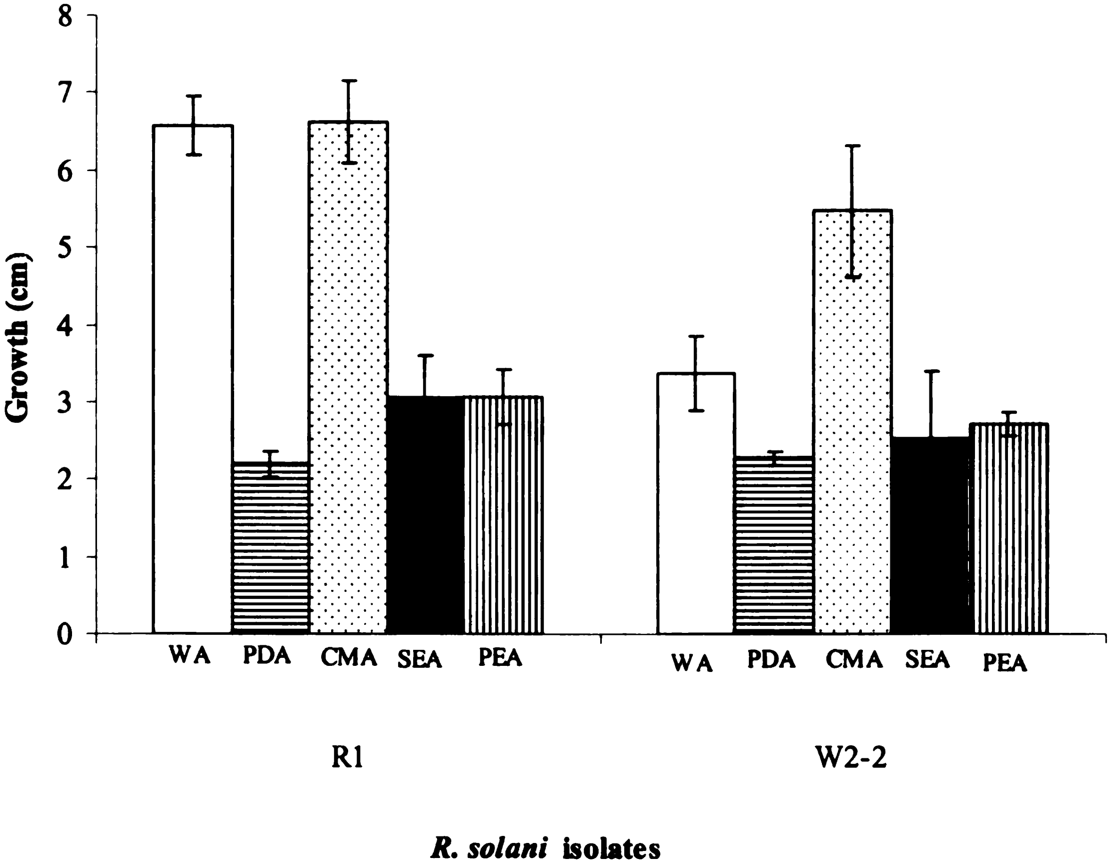

Figure 2-2: *In vitro* saprophytic growth of *R. solani* on different organic media. *In vitro* saprophytic growth (cm) of *R. solani* on WA=Water agar, PDA=Potato dextrose agar, CMA=Corn meal agar, SEA=Soil extract agar and PEA= Plant extract agar. Each value is the mean of fungal colony diameter of four experiments per isolate, with three replicates per isolate.

# DISCUSSION

*R. solani* AG2-2 infects sugar beet seedling causing damping off. This research was carried out to develop an efficient protocol to screen resistance for Rhizoctonia seedling damping off (RSD) disease in sugar beets. The disease screening protocol developed in the growth chamber and in the greenhouse were reproducible when single isolate of fungal inocula were mechanically added to the soil adjacent to the seedlings. Observed stages of infection during RSD disease progress in tested sugar beet cultivars were reproducible. Using this RSD disease screening protocol tissue samples were collected at different disease progression stage for further analysis.

Field based screening methods for Rhizoctonia induced diseases is challenging due to the species complexity of *R. solani* and the influence made by abiotic conditions on disease progress. Infection by the fungus depends on sugar beet cultivar, fungal pathogen isolate, environmental conditions and soil type. The fungus population density and composition are influenced by both biotic and abiotic factors. Therefore RSD disease screening method developed in controlled environments (growth chamber and greenhouse) was used to study the disease progression and the plant-pathogen interaction.

Since the inoculum potential impacts organisms' pathogenicity and virulence, in this study the vegetative growth of different isolates of *R. solani* on different organic media were compared. Data did not show any correlation between pathogenicity and vegetative growth or sclerotia formation. Managing Rhizoctonia diseases through crop rotation is challenging. It is difficult to control both saprophytic growth and sclerotial formation, which are the sources of inocula for subsequent infection. Fungal hyphae were more often found in areas with higher soil porosity, in particular at low soil bulk densities compare to soils with low porosity (Harris *et al.* 2003) .

The genetics of Rhizoctonia is complicated and poorly understood (Adams 1996). Pathogen populations with a high evolutionary potential are more likely to overcome host genetic resistance than pathogen populations with a low evolutionary potential (McDonald and Linde 2002). The evolutionary potential of *R. solani* is not clear. A critical analysis of RSD disease progress pattern and interaction between host and different isolates of *R. solani* will shed light on how host resistance and pathogen virulence operates during the interaction. This knowledge will help in understanding how diseases are caused by many saprophytic fungal pathogens including *R. solani* and assist in breeding for disease resistance. The disease progress curve and statistical analysis of infection by virulent and low virulent isolates of *R. solani* in resistant and susceptible sugar beet cultivars showed that both isolates initiated infection but the virulent isolate succeeded and caused a compatible interaction (disease) in the susceptible cultivar. Low virulent fungal isolate failed to ramify the tissue and resulted in an incompatible interaction (non-disease). The resistant sugar beet cultivar, even though allowed initial infection, prevented the subsequent fungal establishment and recovered from RSD disease. Ruppell (1973) reported that resistance of sugar beet roots to AG 2 type 2 isolates is not due to a mechanical barrier as both resistant and susceptible sugar beet cultivars are penetrated by fungal hyphae. However, resistant cultivars restricted the pathogen to the periderm or outer secondary cortex, whereas in susceptible roots several vascular rings were invaded.

The impact of plant disease and the losses that it causes are a function of disease progress. The RSD disease progress curve have showed three distinct stages-

Initial infection stage, middle static stage and the final decline or recovery stage. Understanding the disease progress pattern will help one to manage the disease and plan appropriate control measures at right time such as spraying a fungicide at a particular disease progress stage. Further investigation is needed to understand the factors that influence disease progress specifically the factors that affect the rate of disease progress which may be used to manage disease impact or forecast the amount of disease or loss. This fundamental knowledge on disease progress can be extended to study the epidemiology of RSD disease. Under the tested conditions the sugar beet variety EL51 (PI 598074) had showed resistance to Rhizoctonia seedling damping off. In many plants, it is reported that yield and disease resistance are two antagonizing characters. Scientific community is in surge of interest to understand the mechanism of the cost of disease resistance (Brown 2002). Interaction between sugar beet variety EL51 and fungal pathogen *R. solani* can be an ideal crop-pathogen model to test this phenomenon.

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# CHAPTER 3

# EFFECT OF HOST RESISTANCE, FUNGAL VIRULENCE AND HOST AGE ON RHIZOCTONIA DISEASE ON SUGAR BEET IN THE FIELD

## ABSTRACT

*R. solani* affects sugar beet at the seedling stage causing seedling damping off and at maturity causing crown and root rot. Field experiments were carried out for three consecutive years to test different sugar beet cultivars for Rhizoctonia seedling damping off (RSD) disease resistance and evaluate disease impact of *R. solani* AG2-2 R-1 (virulent) and W2-2 (low virulent) isolates on sugar beet. Yield at harvest and sugar content under Rhizoctonia disease pressure in resistant and susceptible sugar beet lines were also examined. Data demonstrated that EL51 was resistant and USH20 was susceptible and *R. solani* AG2-2 R-1 was virulent and W22 was low virulent isolates. USH20 inoculated with R-1 treatment caused severe damping off and crown and root rot. USH20 stand count at seedling stage was reduced due to RSD disease and at harvest only a very few beets survived under Rhizoctonia disease pressure. EL51 was resistant to both RSD disease and crown and root rot when inoculated with R-1 (virulent) orW22 (low virulent) isolates. The preliminary data suggest that under Rhizoctonia disease pressure EL51 had high sugar content comparable to that of mock inoculation where as USH20/R-1 treatment caused severe disease and drastic reduction of stands leading to low yield but the sugar content of USH20 was low under mock inoculation treatment.

# INTRODUCTION

Sugar beets (*Beta vulgaris* L.) account for 27% of the world supply of raw sugar (USDA-Economic Research Service -2006). Sugar beet is also a good source for the production of ethanol, which is a combustible fuel that can be blended with conventional fuel. About 2% of today's transportation fuels derived from biomass and blended with fossil fuels. Many nations are expecting that some 5% of their road fuels will be bio-derived within the next 5 years (Koonin 2006). This trend may add further demand for sugar beet crop plant.

Many abiotic and biotic diseases caused by viral, bacterial, fungal pathogens, nematodes and insects affect sugar beet (Leach 1991, Rush 2005). *Rhizoctonia solani* Kühn (*Thanatephorus cucumeris* (Frank) Donk ) is a facultative parasite that infects wide range of crop plants including *Beta vulgaris*, *Gossypium* spp., *Solanum tuberosum*, *Oryza sativa*, *Glycine max*, *Phaseolus vulgaris* and *Lycopersicon esculentum*. *R. solani* is divided into 12 anastomosis groups (AG) based on the somatic compatibility reaction (Anderson 1982). In sugar beets, AG2-2 is reported as crown and root rot and seedling damping off pathogen and AG4 as a virulent seedling damping off pathogen (Herr 1996). Isolates within single AG can vary in virulence and pathogenicity. Schneider *et al.* (2001) showed that Rhizoctonia populations develop in relation to soil temperature and plant development. The fungus produces sclerotia, an over wintering structure that survives and persists in the soil. Chemical control of soil borne fungi like *Rhizoctonia* is difficult and there are several on going investigations to control *Rhizoctonia* such as integrated biocontrol and fungicide applications (Kiewnick 2001), breeding for resistance (Panella 1998) and improving cultural practices such as crop rotation.

It is necessary at the biological level to develop methods to identify the causal organisms rapidly, accurately estimate the disease severity, yield penalty and the virulence mechanisms to effectively manage diseases (Strange and Scott 2005). Analyzing the disease progress, etiology and epidemic processes promises to more effective control practices (Jeger 2004). Field disease evaluation of Rhizoctonia root rot in sugar beets is influenced by environmental conditions (Ruppel *et al.* 1979, Scholten *et al.* 2001).

The objectives of this study were to analyze the Rhizoctonia disease progress pattern and disease impact (seedling damping off and crown and root rot disease) on sugar beet in the field. Sugar beet cultivars EL51 and USH20 that were selected through growth chamber and greenhouse disease-screening methods, as Rhizoctonia seedling damping off (RSD) disease resistant and susceptible cultivars respectively were tested in the field for Rhizoctonia seedling damping off and crown and root rot resistance. Different *R. solani* isolates were screened under field conditions for pathogenicity and virulence. The relationship between Rhizoctonia seedling damping off and final productivity of sugar beets at harvest under Rhizoctonia disease pressure was examined in susceptible and resistant sugar beet germplasm and tested the correlation between disease resistance and yield.

# MATERIALS AND METHODS

## Plant material

Sugar beet varieties USH20 (PI 631354) (Coe and Hogaboam 1971) and EL51 (PI 598074) (Halloin *et al.* 2000) were used in experiments conducted in years 2003, 2004 and 2005. In addition in 2003 sugar beet breeding lines EL –A00 7070 and EL-A007774 and in 2005, EL-A015030 were also tested.

## Fungal inocula

*Rhizoctonia solani* AG2-2, R-1 (virulent) isolate was used in all three years and W22 (low virulent) (ATCC # 18619) was used in 2003 and 2005 (isolate R-1 and W22 were kindly provided by Dr. L. Panella and Dr. L . Hanson of USDA-ARS, Fort Collins, CO). Fungal isolates were grown on corn meal agar (CMA) (Criterion, Hardy diagnostics, C5491) in Petri dishes at room temperature. Eight agar plugs (5 mm diameter) from the freshly growing margins of colonies of *R. solani* were used to inoculate sterile 100 ml of tryptic soy broth (7164A- Acumedia Manufactures, Inc.) in 250 ml Erlenmeyer flasks. The inoculated flasks were incubated as a still culture for two weeks at room temperature. De-hulled millet seeds were soaked in water overnight and sterilized at 121°C for 30 minutes on three successive days in stainless steel trays (32 x 23 x 6 cm). The millet trays were inoculated with 100 ml of *R. solani* (broth culture), incubated at room temperature in the growth chamber, and occasionally mixed to ensure uniform growth. The fungus colonized millet seeds were air-dried for 3 to 4 weeks. Mock inocula was made by following the same procedure omitting the fungus.

### Experimental design

Field experiments were conducted at Michigan State University Botany Farm in East Lansing, Michigan. Sugar beet field plots were rotated annually with sugar beets, corn and beans. In all three experimental years, experimental design was split plot design and precautions were taken not to cross contaminate among plots by restricted agricultural activities.

In year 2003, each treatment (sugar beet variety x *R. solani* isolate) was replicated seven times. Each replicate consisted of a 20' long row (about 30 to 50 plants). In 2004, the treatments were designed to examine adjacent treatment combinations to check plot neighbor effects. Each treatment was replicated fifteen times. In 2005 a split plot design with 10 replications for fungal treatments and 4 replications for mock inoculation treatments was used. Standard crop practices were used. After 2 weeks of planting the fields were manually thinned to create about 4 inches of space between seedlings.

### Field inoculation and disease assessment

### Rhizoctonia seedling damping off

At 6 to 8-leaf stage (about 4 weeks after sowing) each seedling was inoculated by adding 3.3 g of inocula on one side of each plant 4 cm away from each seedling. Seedling damping off was assessed by counting the number of emerged seedlings (stand count) and number of seedlings showing damping off symptoms in each plot at one, two, three and four weeks post inoculation.

### Rhizoctonia crown and root rot

Mature beets were harvested after 20 weeks after planting the seeds.

Harvested roots were rated for Rhizoctonia crown and root rot using a scale of 0 to 3 (0 = healthy, 1= 1% to 24% rot, 2= 25% to 50% rot, 3= >75% to 100% rot).

### Sugar content assessment under Rhizoctonia disease pressure

The harvested beets' narrow apical root tip (tail) was excised and the cut surface was scanned with LabSpec Pro near-infrared spectrometers (Model # LSP1000-1800P, Analytical Spectral Devices, INC). Initially a calibration model was created using known empirical sugar content value derived by AmplexRed method (Trebbi and McGrath 2004). The variation spectrum was examined and optimal number of factors that contained enough vectors to model the sugar and water component without adding much contribution from contaminants was selected. The variation spectra conditions were as follows, PLS 1 program, data preparation –mean centered, path length correct, base line, derivative type none and wavelength of 1000 to 1800 nm. Calibration model and variation spectra were used to create the chemometrics prediction model. Chemometrics prediction model was used to convert the spectra into sugar content values using GRAMS/AI and PLSplus IQ (Thermo Galactic). The weight, percentage sucrose and percentage water of individual beets were recorded.

### Data analysis

Shapiro-Wilk's analysis for normal distribution was performed on seedling damping off data at zero, one, two, three and four weeks post inoculation time. The post inoculation time point where the distribution of data was normal was used for statistical analyses. Standard least square analysis with emphasis on effect advantage was carried out to examine the effects of cultivar, fungal inocula, block and plot.

For seedling damping off, analysis was done on observations made at three weeks post inoculation and results were given as least square mean table to show the combined interactive effect of cultivar and inocula (95% confidence interval).

Total Rhizoctonia disease impact on sugar beet crop was assessed by calculating productivity ratio at the harvest.

$$\text{Productivity ratio} = \frac{\text{Number of healthy beets harvested}}{\text{Seedling stand count before inoculation}}$$

Percent productivity data were given as least square mean table to show the effect of the treatment (95% confidence interval). Mean productivity against each treatment was plotted as a bar chart. The data were analyzed with the JMP software packages (Version 4. SAS Institute Inc., Cary, NC, USA 2000).

# RESULTS

## Rhizoctonia disease progress pattern

Field experiments were carried out to test Rhizoctonia seedling damping off (RSD) disease progress patterns in different sugar beet cultivars when inoculated with *R. solani* AG2-2 R-1 (virulent) and W22 (low virulent) fungal isolates. In the field the fungal inocula was added 4 cm away from the seedlings. Seedlings that were inoculated with R-1 isolate showed damping off symptoms at weeks post inoculation (WPI)= 2 whereas plants inoculated with W22 and mock inoculation showed initial damping off symptoms at WPI= 3 in all tested cultivars. EL51 inoculated with R-1 showed an initial disease phase (mean seedling damping off of 7% at WPI=2 and 14% at WPI=3) and then a recovery phase (mean seedling damping off of 5% at WPI=4). During this time period in other three tested cultivars, USH20, EL-A007070 and EL-A007774 inoculated with R-1 mean seedling damping off had increased from 7 to 10 % to 60 to 70%. At WPI=4 cultivars inoculated with W22 isolate had from 5 to 10 % mean damping off in all tested cultivars. The mock inoculated plants of all four tested cultivars recovered from damping off symptoms at WPI=4 (Figure 3-1 and Table 3-1)

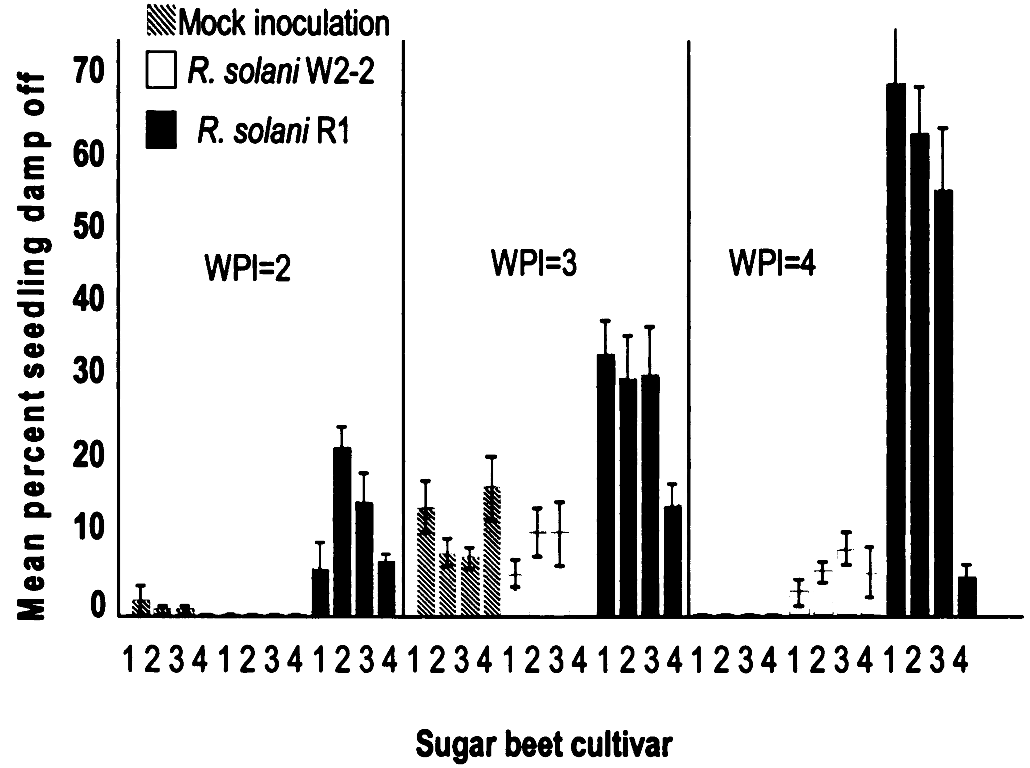

**Figure 3-1**: Mean percent seedling damping off (number of seedlings that were showing damping off symptoms / stand count*100) of sugar beet cultivars 1= EL-A007070, 2=EL-A007774, 3=USH20 and 4=EL51 inoculated with *R. solani* AG2-2 isolates W22 and R-1 and sterile millet as mock inoculation at weeks post inoculation(WPI) 2, 3 and 4.

**Table 3-1 Mean percent seedling damping off of different sugar beet cultivars at weeks post inoculation (WPI) 2, 3 and 4 when inoculated with** *R. solani* AG2-2 R-1 and W22 **fungal isolates**

| WPI | Fungus | Sugar beet germplasm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | USH20 | | EL-A007070 | | EL-A007774 | | EL51 | |
| | | mean | std.dev | mean | std.dev | mean | std.dev | mean | std.dev |
| 2 | Mock | 1.43 | 3.77 | 0.14 | 1.02 | 0.09 | 1.39 | 0 | 0 |
| | R-1 | 5.38 | 8.59 | 20.46 | 6.01 | 13.75 | 9.06 | 5.49 | 1.93 |
| | W22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Mock | 12.66 | 7.79 | 7.41 | 4.24 | 6.79 | 3.07 | 16.86 | 9.69 |
| | R-1 | 30.73 | 10.04 | 27.68 | 12.76 | 28.26 | 14.25 | 12.84 | 6.67 |
| | W22 | 4.66 | 4.79 | 9.7 | 7.51 | 9.58 | 9.9 | 0 | 0 |
| 4 | Mock | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | R-1 | 63.5 | 21.89 | 57.41 | 13.73 | 50.89 | 19.35 | 4.61 | 3.64 |
| | W22 | 2.57 | 4.1 | 4.96 | 3.58 | 7.64 | 5.17 | 4.92 | 7.85 |

**Resistance to seedling damping off**

In the years 2003, 2004 and 2005 field experiments, sugar beet breeding line EL51 showed high level of resistance to seedling damping off compared to USH20. USH20/R-1 had significantly high mean percent seedling damping off (Table 3-2).

Table 3-2: Analysis of least square mean percent seedling damping off at three weeks post inoculation for three consecutive years.

| Year | Treatment | Mean | Std. error |
|---|---|---|---|
| 2003 | USH20-mock inoculation | 12.66 | 2.94 |
| | USH20- *R. solani* R-1 | 22.27* | 3.07 |
| | EL51-mock inoculation | 14.48 | 3.33 |
| | EL51- *R. solani* R-1 | 12.66 | 2.52 |
| | | | |
| 2004 | USH20-mock inoculation | 8.23 | 8.23 |
| | USH20- *R. solani* R-1 | 73.91* | 22.94 |
| | EL51-mock inoculation | 1.30 | 1.3 |
| | EL51- *R. solani* R-1 | 11.63 | 10.36 |
| | | | |
| 2005 | USH20-mock inoculation | 0 | 0 |
| | USH20- *R. solani* R-1 | 24.92* | 3.41 |
| | EL51-mock inoculation | 1.28 | 1.28 |
| | EL51- *R. solani* R-1 | 4.12 | 0.84 |

* Significantly different at ά=0.05

In year 2004 and 2005 experiments USH20 infected with *R. solani* R-1 isolate showed severe damping off with mean percent damping off of 74% and 25% respectively where as in year 2003, all treatments showed damping off symptom but

USH20 inoculated with R-1 had the highest mean damping off (Figure 3-2).

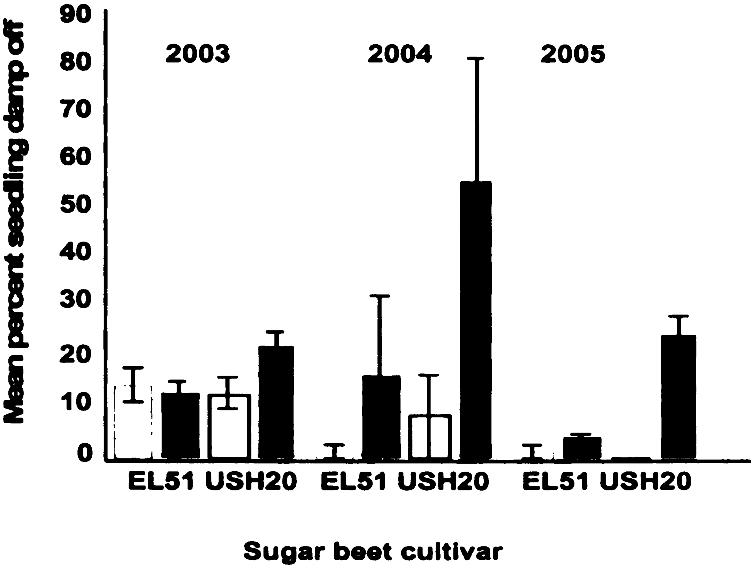

**Figure 3-2.** Mean percent seedling damping off (number of seedlings that were showing damping off symptoms / stand count *100) of sugar beet cultivars EL51 and USH20 inoculated with *R. solani* AG2-2 (R-1) virulent isolate compared to mock inoculation at WPI=3. ☐ Mock inoculation, ■ *R. solani* R-1.

### Mean number of harvested healthy beets under Rhizoctonia disease pressure

The number of harvested beets was significantly different between R-1 and mock inoculation, sugar beet cultivars EL51 and USH20 and among years 2003, 2004 and 2005. EL51 mock inoculation had means 50 (std.dev. 8.2) and 90 (std. dev. 8.3) of number of harvested beets in 2003 and 2005 respectively. USH20 inoculated with R-1 had 15 (std. dev 2.1), 5 (std. dev 11.1 and 5 (std. dev 3.2) mean number of harvested beets in years 2003, 2004 and 2005 respectively (Figure 3-3).

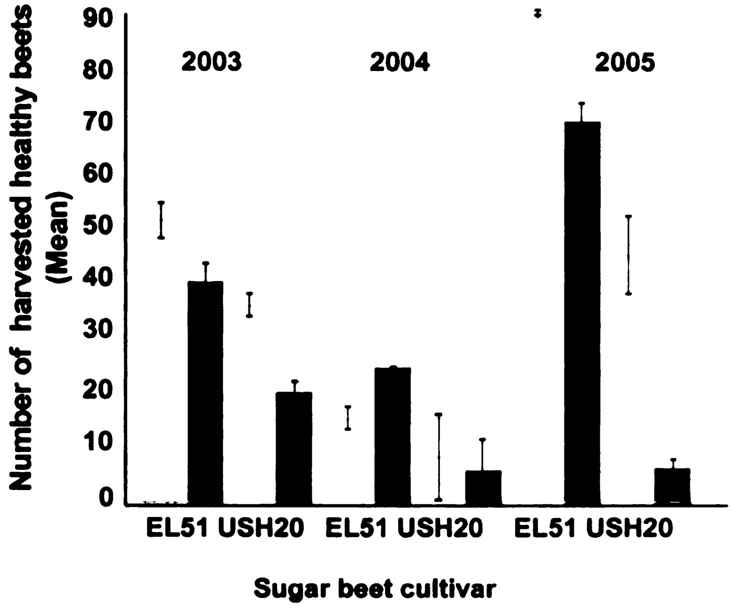

**Figure 3-3.** Number of healthy beets (root rot <50%) harvest in sugar beet cultivars EL51 and USH20 inoculated with *R. solani* AG2-2 (R-1) virulent isolate compared to mock inoculation. ☐ Mock inoculation, ■ *R. solani* R-1

### Productivity under Rhizoctonia disease pressure

Sugar beet productivity under Rhizoctonia disease pressure was measured as a ratio of number of harvested beets to the initial number of seedlings counted. Sugar beet cultivars USH20, EL-A007070 and EL-A007774 inoculated with R-1 had mean productivity less than 0.2 where as EL51 inoculated with R-1 and mock inoculation had mean productivity of 1.2. The mean productivity of USH20 inoculated with W22 and mock inoculation and EL51 inoculated with W22 was 0.6 (Figure 3-4).

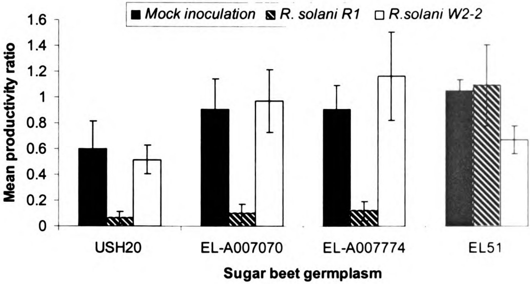

**Figure 3-4.** Mean productivity ratio (ratio of healthy sugar beet roots (rot < 50%) harvested to initial stand count) under Rhizoctonia disease pressure. Sugar beet germplasm USH20, EL-A007070, EL-A007774 and EL51 inoculated with sterile millet as mock inoculation, *R. solani* AG2-2 isolates R-1 and W22.

### Percent sucrose and weight of total harvest under Rhizoctonia disease pressure

EL51 had mean percent sucrose of 12.76 and 12.84 under fungal inoculated and mock inoculation conditions respectively. USH20/mock inoculation had mean percent sucrose of 10.69. USH20 inoculated with R-1 had mean percent sucrose of 12.26 which was similar level of percent sucrose to that of EL51 (Table 3-3).

Table 3-3: Least square mean differences Tukey HSD analysis of percent sucrose of different sugar beet germplasm under Rhizoctonia disease pressure
(ά 0.05, Q= 2.57387)

| Treatment | Percent sucrose Fresh weight | |
|---|---|---|
| | mean | Std. error |
| EL51- *R. solani* R-1 | 12.76 A | 0.09 |
| EL51-mock inoculation | 12.84 A | 0.09 |
| USH20- *R. solani* R-1 | 12.26 A | 0.59 |
| USH20-mock inoculation | 10.69 B | 0.13 |

Levels not connected by same letter are significantly different.

Total fresh weight of sugar beet roots harvested in USH20/R-1 treatment was 17.6 kg (Figure 3- 5) where as EL51-mock inoculation had the highest amount of harvest (614 kg). Under mock inoculation treatment, EL51 had about 422 kg fresh weight and USH20 had 257 kg.

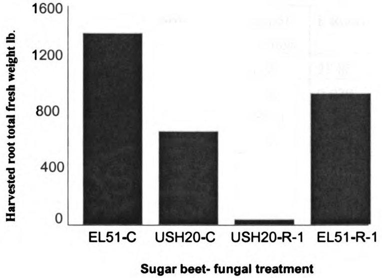

**Figure 3-5:** Total amount (kg) of sugar beet roots harvested in EL51 and USH20 sugar beet plots inoculated with *R. solani* AG2-2 (R-1) fungal isolate compare to mock inoculation (2004). USH20-C and EL51-C=Mock inoculations.

### Neighbor effect

This study was carried out to test if the adjacent plot treatment (germplasm or fungus inocula) had any effect on Rhizoctonia diseases in the test plot. An effect test of experimental plot, cultivar, fungus and adjacent- plot-cultivar and fungus (Fit model analysis) showed that only the interaction between experimental plot, cultivar and fungus had a significant effect on percent seedling damping off where as adjacent-plot- cultivar and adjacent-plot-fungus did not have significant effect on percent damping off (ά=0.05)(Table 3- 4).

Table 3-4: Effect test. Effects of cultivar, fungus adjacent plot cultivar (NC) and adjacent plot fungus (NF) on percent seedling damping off disease of the test plot (Fit model analysis).

| Source | Nparm | DF | Sum of Squares | F Ratio | Prob > F |
|---|---|---|---|---|---|
| Cultivar*Fungus | 1 | 1 | 11.49 | 28.83 | <0.00 |
| Cultivar*Fungus*NF | 1 | 1 | 11.90 | 0.029 | 0.86 |
| Cultivar*Fungus*NC | 1 | 1 | 2423.17 | 6.08 | 0.02 |
| Cultivar*Fungus*NC*NF | 1 | 1 | 30.96 | 0.07 | 0.78 |

## DISCUSSION

The present work showed that sugar beet breeding line EL51 had high level of resistance to *R. solani* AG2-2 at both seedling stage and at mature stages. Adding the inocula adjacent to the plant in the soil mimicked natural field conditions since the fungus that is present in the soil needs to grow towards the plant and infect the plant. In previous methods to screen for Rhizoctonia crown and root rot resistance, the inocula was added to the sugar beet crown (Buttner *et al.* 2004). Data showed that it takes two to three weeks for the plant to show damping off symptoms after adding the inocula to the soil. If plant growth rate is rapid, seedlings may outgrow and escape from seedling damping off. Severity of disease caused by *Rhizoctonia* is related to the rate of seed germination and post-emergence growth in sugar beets (Herr 1996). Some sugar beet varieties including sugar beet variety USH20 have superior emergence potential (McGrath 2000). However USH20 had poor Rhizoctonia seedling damping off resistance.

Poor seedling emergence is a recurrent problem in sugar beet fields that is caused by both biotic and abiotic agents. Biotic agents that cause seedling damping off in sugar beet include *R. solani, Aphanomyces cochlioides, Pythium* species (Leach 1991). In this study, mock inoculation was used to separate seedling damping off caused by inoculated *R. solani* fungal isolate vs. seedling damping off caused by other biotic agents such as *Phythium, Aphanomyces* and indigenous *Rhizoctonia* present in the soil or damping off caused by abiotic conditions. The mock inoculated seedlings of all tested cultivars had a transient damping off which was overcome by the plants within a short time suggesting it could have been caused by an abiotic agent such as water stress. The four tested sugar beet cultivars showed different levels of damping off after inoculation with *R. solani* isolates R-1 and W22. Isolate R-1 generally produced more severe seedling damping off disease than isolate W22, with the exception of the sugar beet cultivar EL51 that showed very high resistance to both isolates. All tested sugar beet cultivars exhibited some level of seedling resistance to isolate W22. Differences in disease response between the two isolates demonstrate that varying levels of virulence, or aggressiveness, exist among *R. solani* AG2-2 isolates which was also reported by Panella (2005). Fungal isolate R-1 is a virulent isolate and W22 is a low virulent isolate on susceptible sugar beet cultivars. The low virulent isolate had caused 5 to10 percent mean damping off and virulent isolate had caused 60 to 70 percent mean damping off in three tested sugar beet cultivars. The ability of EL51 to resist Rhizoctonia infection by both *R. solani* AG2-2 R-1 (virulent) and W22 (low virulent) isolate suggests a genetic potential to resist Rhizoctonia infection.

The accuracy, reproducibility, and efficiency of using this method to screen for Rhizoctonia disease resistance in the field was tested in three consecutive years 2003, 2004 and 2005. Inoculating the plant at seedling stage enabled the researcher to screen for seedling damping off and crown and root rot disease rather than inoculating at maturity to only screen for crown and root rot disease. *R. solani* AG2-2 W22 isolate does not causes significant amount of crown and root rot at maturity whereas the R-1 isolate had caused crown and root rot in three tested susceptible sugar beet cultivars indicating their susceptibility to *R. solani.* The productivity ratio was the ratio between initial stand count before fungal inoculation and number of harvested beets. After the initial stand count was taken different factors were operating on seedlings to reduce or increase the stand count. Stand count reduction agents were such as seedlings that were damping off were withered or seedlings that were weak were removed by wind or new seedlings were added by continuous non-synchronized germination of seeds. Therefore, net number of harvested beets exceeded the initial stand count resulting in productivity ratio being greater than one in some treatments. EL51 had very high mean productivity ratio suggesting that it has very effective defense mechanisms to prevent *R. solani* diseases rather than mere escape or tolerance mechanisms to avoid Rhizoctonia disease.

In this field study the fungus was not incorporated to the soil prior to planting to avoid cross contamination during planting, thinning and other agricultural activities. Neighbor effect experiments demonstrated that under tested field conditions, the cross contamination of test plots with adjacent plot inocula did not occur and adjacent plant-pathogen treatment effect on test plot was not significant compare to test treatment. Thus, this is a robust effective method to screen for Rhizoctonia seedling damping off and crown and root rot disease and might be used by breeders and pathologist for screening Rhizoctonia resistance in the field for multiple isolates and cultivars simultaneously. This method of Rhizoctonia disease screening had showed that USH20 was a highly susceptible and EL51 was a resistant sugar beet breeding line for RSD and Rhizoctonia crown and root rot disease. Resistance to Rhizoctonia in sugar beet is polygenic and genetic resistance remains as the one of the most important means of managing damage by this disease (Panella and Ruppel 1996). Rhizoctonia disease resistance in EL51 is polygenic, race-non-specific and partial in its effect against the disease, which is often durable in contrast, the monogenic, race-specific, complete resistance controlled by gene-for-gene relationships is often short lived. EL51 is a valuable source of genetic material for Rhizoctonia disease resistance; in addition, it has been reported to have moderate resistance to Cercospora leaf spot and blackroot seedling disease (Halloin *et al.* 2000). EL51 had higher level of percent sucrose and stand count compare to USH20 under Rhizoctonia disease pressure.

Disease resistance is assumed to cause a yield penalty and this hypothesis is supported by many studies (Brown 2002). In this study, the sugar beet yield was measured under Rhizoctonia challenged disease pressure condition and mock inoculation condition. The components that were included in the yield measure were fresh weight of the harvested sugar beet root and percent sucrose (fresh weight). Data showed that the sugar beet yield was not affected in EL51 under Rhizoctonia disease pressure and mock inoculation conditions. Only few plants survived in USH20/R-1 treatment resulting in low total weight of harvested beets. Those surviving beets had percent sucrose comparable to EL51. These few surviving beets from USH20/R-1 treatment may be mere escapes from Rhizoctonia infection resulting from late germination or missed inoculation rather than resistant plants. USH20 mock inoculation had less percent sucrose and less total weight of harvested beets than EL51 mock inoculation and EL51/R-1. The basal level of disease pressure that was present in the field could have caused stress in USH20. In crop plants and probably wild species, the basic resistance is maintained by a combination of passive and active defenses (Heath 1991). Is USH20 employing an energy consuming inefficient basal defense mechanism, which is weakening it and making it susceptible to Rhizoctonia whereas EL51 is utilizing efficient, energy serving mechanism to resist basal disease pressure and Rhizoctonia challenge? Understanding the etiology and molecular biochemical analysis of the interaction between resistant and susceptible sugar beet cultivar and *R. solani* AG2-2, R-1 and W22 isolates will provide basic and applied knowledge in breeding for resistance, managing Rhizoctonia diseases in the field and advance the knowledge in plant- *R. solani* fungal interaction.

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## CHAPTER 4

# THE INFECTION PROCESS OF VIRULENT AND LOW VIRULENT *R. SOLANI* IN SUSCEPTIBLE AND RESISTANT SUGAR BEET SEEDLINGS AND ETIOLOGY OF HOST RESPONSE

## ABSTRACT

Plant-microbe interactions are initiated at the level of the cell. Spatial and temporal analysis of events involved during plant-pathogen interaction at the cellular and tissue level become important to understand the mechanism of interaction and final outcome either diseased or non-diseased state. In this research, the interaction among susceptible and resistant sugar beet seedlings with *R. solani* AG2-2 R-1 or W22 fungal isolates were microscopically analyzed. The R-1 and the W22 isolates produced initial infection structures and committed the initial infection process in both resistant and susceptible sugar beet seedlings. Mycelia grew over the plant surface, flattened and attached to the surface, produced T-shape branches and penetrated directly through the epidermis. No major differences in the infection process of both fungal isolates in resistant and susceptible seedlings were observed during the early events of cortex colonization. Fungi penetrated the cortex tissue both inter- and intra-cellularly. The R-1 isolate penetrated the endodermis of a susceptible cultivar causing tissue destruction leading to seedling damping off and death. When R-1 and W22 isolates penetrated the endodermis of the resistant seedlings, the fungal progression was stopped at or just beneath the endodermis by the formation of a cork layer. When the susceptible sugar beet

germplasm USH20, was inoculated with the fungal isolate W22, the host produced a very thin cork layer that was breached by the pathogen and the pathogen established in the stele ground tissue without causing damping off symptoms. Differences were observed in the response of the host plant to infection. Autofluorescence, possibly related to deposition of lignin or lignin-like materials increased more in cortical tissue of the resistant cultivar colonized with *R. solani* R-1 isolate than with the W22 isolate or in the susceptible sugar beer germplasm.

# INTRODUCTION

Disease is the outcome of interaction between the host, the disease agent, and their environment. Plant disease results when a specific agent, such as persistent unfavorable environmental conditions or the activity of a pathogen, disrupts physiological functions causing plants to deviate from normal development. Plant pathogens include fungi, bacteria, viruses, viroids, nematodes, parasitic plants (dodder and mistletoe), mollicutes, and protozoa (Agrios 1997). Many beneficial microorganisms exist in nature and most plant species are resistant to most plant pathogens (Ellis 2006). During disease development, many species of fungi produce additional inocula, which are dispersed by wind, water or by other means resulting in a rapid increase in disease incidence and severity. Some fungi form special resting spores, which permit survival for long periods (several months or years) in soil or plant debris (Coley and Cooke 1971). Plant infection by fungi occurs via a great variety of mechanisms. Some species directly penetrate plant surfaces or enter through natural openings, while others require wounds or injury for infection (Knogge 1996).

*Rhizoctonia solani* Kuhn (anamorph) does not produce asexual spores and is classified as mycelia sterilia (Barnett and Hunter 1998). The teleomorph of *R. solani* is *Thanatephorus cucumeris* (Frank) Donk) that belongs to the kingdom Basidiomycota. In nature, *R. solani* exists primarily as vegetative mycelium and/or sclerotia (undifferentiated aggregation of thick-walled melanized mycelia) that can persist in the soil for years (Sherwood 1970). Once favorable conditions are present, the sclerotia will germinate and infect the plant. When *R. solani* hyphae come in contact with the plant, they start to grow over the plant surface, attach to the plant surface and produce initial infection structures such as directed growth, characteristic T-shape branches and infection cushions. The actual infection process is committed once the fungus attaches to the plant surface. Non-pathogenic isolates do not produce initial infection structures on non-host plants (Keijer, 1996). *R. solani* has different mode of penetrations such as producing infection cushions in potato (Demirci and Doken 1998), beans (Christou, 1962) and in cotton (Armentrout and Downer 1987), by lobate appressoria facilitating penetration to beans (Kenning and Hanchey 1980), radish (Dodman *et al.*1968) and rice plants (Marshall and Rush 1980) or direct penetration as was observed in sugar beet (Ruppel 1973) and barley (Murray 1981). Following the penetration of cuticle and cell walls by infection pegs, fungi penetrate the cortex tissue inter- and intra-cellularly.

*R. solani* host tissue establishment studies on anastomosis groups (AG) 1, 2, 3 and 4 demonstrated that the tissue progression and establishment differ among the different groups (Weinhold and Sinclair 1996). Colonization of potato tissue by AG3 was restricted to few layers beneath the infection cushion (Hofman and Jongebloed 1988). However, AG2-1 (Yang et al 1992) and AG4 (Kenning and Hancheny 1980) colonized the tissue extensively growing deeper into cortex tissue and endodermis and finally penetrating the vascular tissues. Weinhold and Motta (1973) reported that AG4 produces cell wall degrading enzymes prior to penetration. In severely infected hypocotyls, the entire cortex completely disintegrated and the fungi penetrated the endodermis, phloem and xylem parenchyma cells but not the xylem elements suggesting that fungus was not able to colonize the lignified walls (Yang *et al.* 1992). Host resistance to *R. solani* is typically an incremental reduction in disease severity.

All plant-microbe interactions are initiated at the level of the cell. Light microscopy, confocal laser scanning microscopy, electron microscopy and video microscopy, computerized image processing, and an ever-increasing array of fluorescent probes that can be applied to living cells revealed the infection process and microbe-induced changes (Heath 2000). These techniques integrated with molecular genetics and other types of investigations, are likely to play an increasingly important role in studies of plant responses to microbial pathogens and increase our ability to visualize the intimate interaction of fungi and their host plants (Gold *et al.* 2001). In evaluating disease progress patterns not only a comparison of susceptible and resistance forms is of great importance, but also a comparison of the non-disease resistant plant-pathogen interactions due to host resistance or pathogen low virulence with one another is essential to understand the resistance mechanisms. The objectives of this study were to document the infection initiation, progress and establishment of fungi in plant tissue and the host response when the resistant and the susceptible sugar beet cultivars were infected with *R. solani* AG2-2 R-1 (virulent) or W22 (low virulent) isolates using light, fluorescence, scanning electron and confocal laser scanning microscopes.

## MATERIALS AND METHODS

### Fungal inocula

*R. solani* AG2-2 (R-1) virulent isolate and (W22) low virulent isolate (ATCC # 18619) were used (provided by Dr. Lee Panella and Dr. Linda Hansen, USDA-ARS Ft. Collins, CO). Fungal isolates were grown on corn meal agar (CMA) (Criterion, Hardy diagnostics, C5491) 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 a single layer on the actively growing three- day -old fungal culture and were incubated at room temperature for an additional four days. The millet seeds that were completely colonized with the fungi were used as the inocula.

### Plant Material and inoculation

Sugar beet varieties USH20 (Coe and Hogaboam 1971) and EL51 (Halloin *et al.* 2000) that were respectively susceptible and resistant to Rhizoctonia disease were used. The seeds were soaked in 0.3% hydrogen peroxide (V/V) (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 "Baccto" high porosity soil. Wooden boxes (400cm x 580 cm) were filled to 2 cm below the top with "Baccto" high porosity soil and were arranged in a randomized complete block design. Thirty seedlings were planted per wooden box and grown in the greenhouse (25°C, 16 hr light and 8 hr dark photoperiod), watered daily, fertilized weekly, and transplanted and maintained thirty plants per box. Four to six leaf stage seedlings were inoculated with single isolate of *R. solani* AG 2-2 virulent isolate R-1 or low virulent isolate W22. Each seedling was inoculated by adding 0.1 g of inocula (about 20 fungus –infested millet seeds) on 2 sides of each plant 4 cm away from each seedling. Control mock inoculation plants were inoculated with sterile millet. Ten seedlings per treatment-box were randomly collected at days post inoculation (DPI) 0, 3, 5, 7, 9 and 11 for microscopic observations. Three seedlings each were used for scanning electron and autofluorescence studies and two seedlings each were used for light and laser scanning confocal microscopy studies. All experiments were carried out twice.

### Scanning Electron Microscopy (SEM)

Three seedlings per treatment were selected for SEM observation. Seedlings were washed free of soil and epidermal peels were obtained at the presumed fungal entry point and were immediately fixed at 4°C for 2 h in 4% glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.4. After a brief rinse in that buffer, samples were dehydrated in a graded ethanol series (25, 50, and 75%) for 15 min each and with three 10-min changes in 100% ethanol. Samples were dried in a Balzers critical point dryer (Balzers, Liechtenstein) using liquid carbon dioxide as the transitional fluid and then the samples were mounted on aluminum stubs using adhesive tabs. Samples were coated with gold (20 nm thickness) for 5 min in an Emscope Sputter Coater (model SC 500; Ashford, Kent, UK) purged with argon before being examined with a JEOL-35S scanning electron microscope.

### Light Microscopy

#### Fungal initial infection structures and cortex establishment

Seedlings were washed free of soil and a one cm long stem was excised to include the presumptive fungal entry point and immediately fixed in formalin-acidalcohol (FAA) (Ruzin 1999). The samples were free hand sectioned 5 celsl layer thick with a double-edged razor blade and stained with either lactophenol cotton blue or with 0.2% chlorazol black ( Xu *et al.* 2000). 10 to 15 thin sections were observed per treatment under Olympus BX60 fluorescence light microscope and images were captured with Olympus PM-30 automatic photomicrographic system or digital images were made with Optronics MagnaFire SP system.

### Autofluorescence imaging and autofluorescence quantification

Three seedlings per treatment were analyzed for autofluorescence. Seedlings were washed free of soil and a one cm long stem was excised to include the presumed fungal entry point. Immediately each excised-stem was free hand transverse sectioned (TS) and three thin TS per sample were selected and observed under UV fluorescence microscope (Olympus BX60). Autofluorescence images were captured by optimizing the exposure time and magnifications for each sample.

For autofluorescence measurements digital images were captured using Optronics MagnaFire software under UV light with as exposure time of 6.129 seconds with 100x magnification. The total relative intensity (gray scale, white assigned 0 and black assigned 255) was profiled along a line placed across the micrograph of the transverse section of the seedling stem from outer most epidermal cell wall to outer most cell wall of endodermis that included the cortex tissue (Image –Pro Express version 4.0 Media Cybernetics L.P).

## Confocal Microscopy

Seedlings were washed free of soil, a one cm long stem with the potential fungal entry point was excised and fixed in FAA for 24 hours. Tissues were dehydrated in a graded ethanol series (30, 50, 70, 90, 95 and 100%). The tissue samples were free hand transverse sectioned with a double-edged razor blade. The tissue sections were immersed in wheat germ aggulutinin- fluorescein isothiocyanate (WGA-FITC-Sigma) Oregon Green 488 (Sigma W6748) (10ug/ml diluted in Phosphate buffered saline (PBS) pH 7.2) for 30 minutes with 5 minutes vacuum infiltration. The tissues were rinsed in PBS buffer pH=7.2 thrice for three minutes each wash and were mounted on glass slide with double distilled water. Optical sections of the fluorescing tissue samples were collected with a Zeiss 210 laser scanning confocal microscope using 488 nm excitation wavelength and images were collected from 505nm to 530nm band pass filter and 560nm long pass filter. Images were acquired at 512 x 512-pixel resolution.

## RESULTS

To initiate the infection process, the fungus *R. solani* needs to grow over the host plant surface, produce initial infection structures, penetrate the host surface through the epidermis, establish in host cortex tissue, penetrate the endodermis and establish in host stele tissue (see figure 4-1)

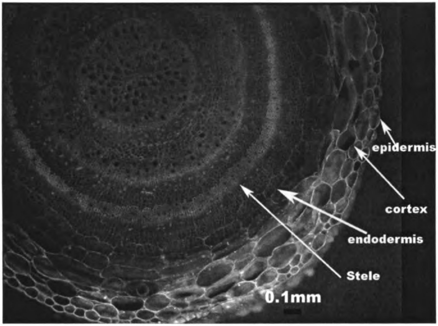

Figure 4-1: Sugar beet seedling stem anatomy. Transverse section of 2 weeks old sugar beet stem.

### Infection initiation

At days post inoculation (DPI) =3 *R. solani* R-1 and W22 isolates had grown over the plant surface as thread-like mycelia strand on resistant (EL51) and susceptible (USH20) sugar beet seedlings (Figure 4-2 to 4-5). Under all four treatments USH20/R-1, USH20/W22, EL51/R-1 and EL51/W22, the mycelia had flattened and closely and firmly attached to the plant surface and produced infection pegs to presumably penetrate the epidermis directly (Figure 4-6). R-1 (virulent) and W22 (low virulent) isolates produced T-shape branches and hyphal aggregates in both resistant and susceptible sugar beet seedlings (Figure 4-6 A and B).

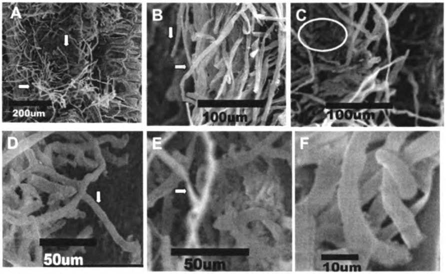

Figure 4-2 Panel A to Panel F: Scanning electron micrographs of initial infection structures of *R. solani* AG2-2 R-1 isolate on stems of susceptible (USH20) sugar beet seedlings at days post inoculation (DPI)=3. Panel A to C are from three different infected seedlings. Panel D to F were obtained at higher magnification to show the infection structures. Round thread like mycelia have grown over the plant surface. White arrows indicate the fungal hyphae and white circle indicates the flatten fungal hyphae.

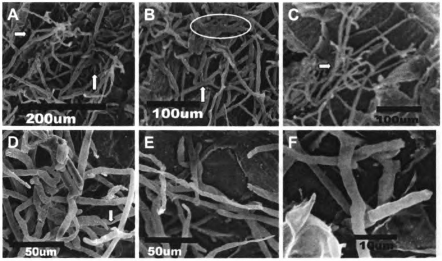

Figure 4-3 Panel A to Panel F: Scanning electron micrographs of initial infection structures of *R. solani* AG2-2 R-1 isolate on stems of resistant (EL51) sugar beet seedlings at days post inoculation (DPI)=3. Panel A to C are from three different infected seedlings. Panel D to F were obtained at higher magnification to show the infection structures. Round thread like mycelia have grown over the plant surface. White arrows indicates the fungal hyphae and white circle indicate the flatten fungal hyphae.

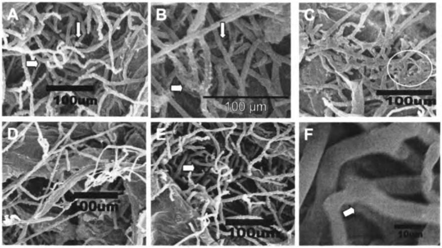

Figure 4-4 Panel A to Panel F: Scanning electron micrographs of initial infection structures of *R. solani* AG2-2 W22 isolate on stems of susceptible (USH20) sugar beet seedlings at days post inoculation (DPI)=3. Panel A to C are from three different infected seedlings. Panel D to F were obtained at higher magnification to show the infection structures. Round thread like mycelia have grown over the plant surface. White arrows indicate the fungal hyphae and white circle indicates the flatten fungal hyphae.

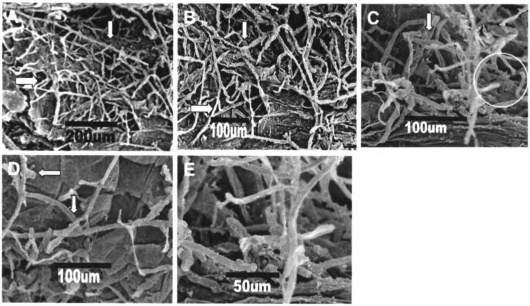

Figure 4-5: Panel A to Panel E: Scanning electron micrographs of initial infection structures of *R. solani* AG2-2 W22 isolate on stems of resistant (EL51) sugar beet seedlings at days post inoculation (DPI)=3. Panel A to C are from three different infected seedlings. Panel D and E were obtained at higher magnification to show the infection structures. Round thread like mycelia have grown over the plant surface. White arrows indicate the fungal hyphae and white circle indicates the flatten fungal hyphae.

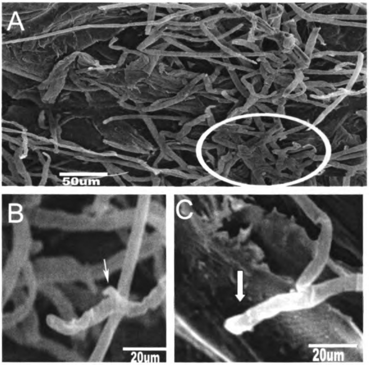

Figure 4-6: Scanning electron micrographs of initial infection structures of *R. solani* AG2-2 isolates. (A) Round thread like mycelia grown over the plant surface and white circle indicate the flattened and firmly attached hyphae (B) White arrow indicates T-shape branch produced by *R. solani* (C) White arrow indicates penetration peg.

## Cortex tissue establishment

By DPI=5 the fungal isolates R-1 and W22 penetrated the epidermis of both resistant and susceptible sugar beet seedlings. Fungi produced characteristic T-shape branches, fungal aggregates and penetration pegs. Presumably the host epidermis was penetrated by the fungal penetration peg directly without producing fungal cushions. After direct penetration R-1 and W22 isolates rapidly invaded the sugar beet seedlings of both resistant and susceptible cultivars. The hyphae extended in all directions in the cortex both inter- and intra- cellular (Figure 4-7C and D) and grew deeper into the cortex and penetrated the endodermis (Figure 4-7E).

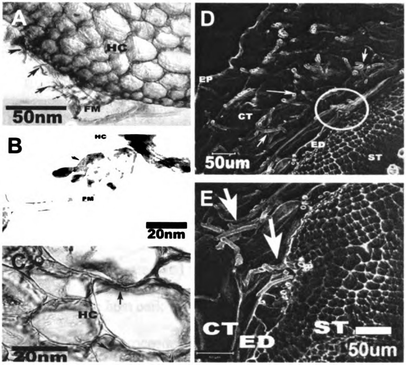

Figure 4-7: Infection initiation and establishment of *R. solani* AG2-2 in sugar beet seedlings. Panel A- T-shaped branches (black arrow). Panel B- hyphal aggregate produced by *R. solani* AG2-2. Panel C - transverse section of the stem of a fungal infected seedling showing fungal establishment in cortex tissue, black arrow indicates the fungus in the cortex tissue. Panel D- inter- and intra- cellular fungal penetration in the cortex parenchyma cells. Panel E- the fungal penetration into endodermis and stele. HC= Host cells , FM= Fungal mycelia, CT=Cortex, ED=Endodermis and ST=Stele

### Endodermis penetration and establishment

By DPI=9 both R-1 and W22 penetrated the endodermis in USH20 and EL51. In EL51/R-1 and EL51/W22, cork layer that was produced in the host stele tissue restricted fungal penetration just beneath endodermis in the interstitial parenchyma tissue (Figure 4-8). Fungal mycelia were not observed beyond the cork layer. In the USH20/R-1 interaction the fungus penetrated the endodermis and ramified through the stele ground tissue. Stele tissues disintegrated and the seedling collapsed (Figure 4-9 A1 and A2). In USH20/W22, thin loose host cork layer was produced. The fungus was not restricted at this barrier layer instead it proceeded through the barrier cork layer and established in stele ground tissue (Figure 4-9 B1 and B2). The fungal mycelia were observed in the stele ground tissue between vascular rings. Xylem penetration by the fungus was not observed.

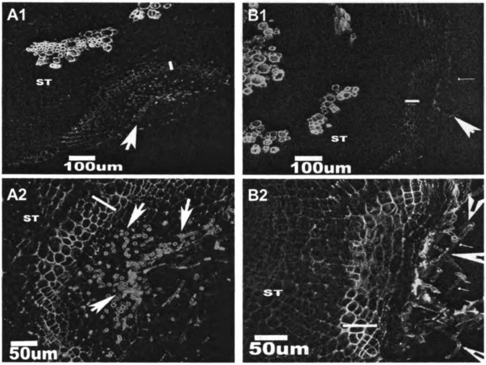

Figure 4-8: Transverse sections (TS) of stele tissue after the seedlings were inoculated with *R. solani*. Fungal mycelia indicated by white arrows. Fungal progression was restricted at the host cork layer indicated by white line. Panel (A1) and (A2) - EL51/R-1 and (B1) and (B2) EL51/W22. ST=Stele

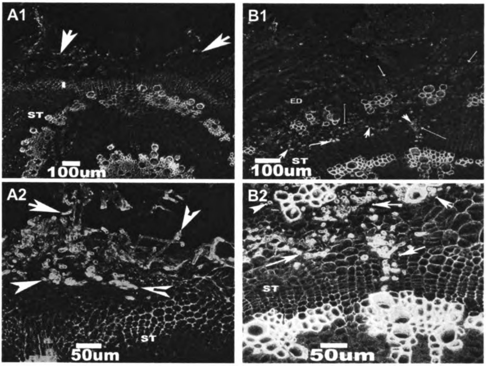

Figure 4-9: Transverse sections (TS) of stele tissue after the seedlings were inoculated with *R. solani*. Fungal mycelia indicated by white arrows. Panel (A1) and (A2) USH20/R-1 where hyphae had ramified through the stele ground tissue. Panel (B1) and (B2) USH20/W22 where thin host barrier layer was present which the hyphae had penetrated established in inner stele ground tissue. ST=Stele

## Host response

### Cortex autofluorescence

When fresh transverse sections of stems of the *R. solani* infected seedlings were observed under UV light, the EL51/R-1 interaction showed high level of autofluorescence whereas the other three interactions showed low levels of autofluorescence (Figure 4-10).

The relative quantity of autofluorescence measurements, (in the gray scale, white was assigned 0 and black was assigned 255) under given constant exposure time and UV, showed that EL51/R-1 had significantly high level of relative autofluorescence (value= 29) and mock inoculated tissues had the least amount of autofluorescence (value=16) (Table 4-1). EL51-W22 had a value=26 and USH20-W22 had a value=18.

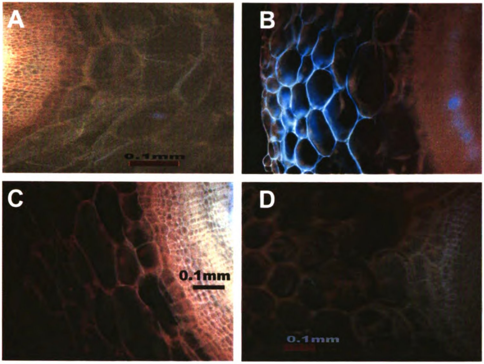

Figure 4-10: Cortex autofluorescence of transverse section of the stems of infected sugar beet seedlings. Panel (A) USH20/R-1 (B) EL51/R-1 (C) USH20/W22 and (D) EL51/W22. Each transverse section was observed under UV light and captured under optimal exposure time for each sample. Images in this dissertation are presented in color.

**Table 4-1: Least square means differences Tukey HSD analysis of autofluorescence of the *R. solani* infected cortex tissue.**

| Treatment | Mean | Std. error |
|---|---|---|
| EL51/Mock | 16.47 | 0.73 |
| EL51/R-1 | 29.96 | 0.76 |
| EL51/W22 | 26.43 | 0.73 |
| USH20/Mock | 15.93 | 0.74 |
| USH20/R-1 | 22.26 | 0.99 |
| USH20/W22 | 18.20 | 0.85 |

### Cork layer formation

The cork layer cells accumulated suberin in their cell wall which exhibited autofluorescence when observed under UV light (Figure 4-11). When the fungal isolate R-1 penetrated the endodermis of USH20, a very thin cork layer was observed and the pathogen was present beyond the cork layer in the stele ground tissue causing host tissue destruction and death (Figure 4-11 A1 and A2). When EL51 was infected with R-1 or W22, the fungal progression was restricted by the formation of cork layer, which prevented further invasion by the pathogen beyond the initial lesion (Figure 4-11 B1 and B2 and 4-8 B1 and B2). USH20/W22 interaction had produced less dense cork layer that was breached by the fungus (Figure 4-12 A1 and A2).

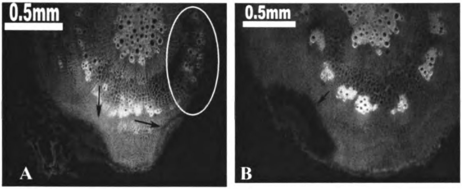

Figure 4-11: Transverse sections of the stems of sugar beet seedling inoculated with *R. solani*. Fungal progression was restricted at the host cork layer indicated by black arrow. (A) USH20/R-1 where fungi had ramified through the stele tissue and tissue integrity was damaged (affected tissue indicated by white circle), (B) EL51-R-1 fungal progression was stopped by host cork layer.

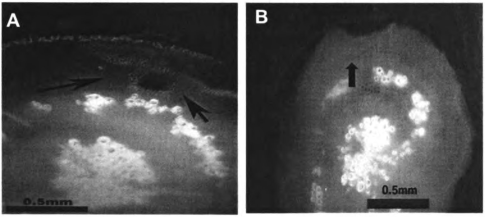

Figure 4-12: Transverse sections of the stems of sugar beet seedling inoculated with *R. solani*. Fungal progression was restricted at the host cork layer showed by black arrow. (A) USH20/W22 fungus was present beyond the thin host cork layer. (B) EL51 R-1 fungal progression was stopped by host cork layer.

# DISCUSSION

The infection process of *R. solani* AG2-2 (R-1) and (W22) isolates were studied in resistant (EL51) and susceptible (USH20) sugar beet seedlings. The inocula were added to the soil 4 cm from the seedlings. At DPI= 3, hyphae of *R. solani* R-1 and W22 isolates grew over the plant surface and produced initial infection structures in both resistant and susceptible sugar beet seedlings. The mycelia grew indiscriminately over the plant surface and the round thread-like mycelia flattened and attached to the plant surface at different locations. Mycelia produced T-shape branches, which are determining characteristics of *R. solani* infection (Keijer 1996). *R. solani* R-1 and W22 isolates produced similar initial infection structures in both resistant and susceptible sugar beet seedlings. It is reported that *R. solani* may penetrate the plant by producing an infection cushion (Demirci and Doken 1997), appressoria or via direct penetration (Keijer 1996). In this study, direct penetration by the fungus was observed similar to that reported by Ruppel (1973). Subsequently, the hyphae of *R. solani* R-1 and W22 isolates rapidly invaded the cortex tissue inter and intra-cellularly in both resistant and susceptible sugar beet seedlings. There were no apparent necrosis were present preceding the fungal penetration and establishment in cortex tissue.

The hyphae directly penetrated the endodermis. In susceptible seedlings, the R-1 isolate penetrated the endodermis causing cell destruction, tissue collapse and death. When R-1 and W22 isolates penetrated the endodermis of the resistant seedlings, the fungal progression was stopped by the formation of a cork layer. Infection by pathogens induces plants to form several layers of cork cells beyond the point of infection (Agrios 1997). Ruppell (1973) reported that resistance of mature sugar beet roots to AG 2 type 2 isolates is not due to a mechanical barrier as both resistant and susceptible sugar beet cultivars are penetrated by fungal hyphae. However, resistant cultivars restricted the pathogen to the periderm or outer secondary cortex, whereas susceptible roots had several vascular rings invaded. Invasion of endodermis by pathogens was restricted during many resistant plant- pathogen interactions such as Benzo-(1,2,3)-thiadiazole-7-carbothioicacidS-methylester (BTH) treated tomato plants that were resistant to *Fusarium oxysporum* f.sp. radicis-lycopersici, the fungal growth was usually restricted to the outermost root tissues (Benhamou and Belanger 1998). *Piriformospora indica* is an endophetic fungus and when barley seedlings were grown in the fungus inoculated substrate, the hyphae were not detected in the central part of the roots beyond endodermis (Waller *et al.* 2005). Cork layer production and restriction of fungal progression beyond endodermis by EL51 against both R-1 and W22 isolates suggest that resistance mechanism in EL 51 is non-isolate specific.

Did the resistant sugar beet germplasm restricted the fungus penetration into endodermis and prevented the onset of RSD disease or the host had multiple layers of defense strategies that accumulated and prevented the on set of the disease? It will be useful to determine the mode of EL51 disease resistance to other pathogens to understand the defense mechanisms in the host. During infection process in plants, pathogens undergo different developmental process (Sesma 2004). When USH20 was infected with W22 isolate, the host produced a very thin cork layer that was breached by the pathogen and thus the pathogen established in the stele ground tissue with out causing damping off symptoms. USH20 succumbed to R-1 but tolerated W22. *R. solani* is a facultative parasite that can preferably feed on dead host tissue but can survive in living tissue. By penetrating the endodermis and establishing in living stele tissue, does the fungal isolate W22 benefit and the fungus stimulated the strategy? The host could have suppressed W22 to prevent plant cell death but failed to suppress R-1. Alternatively, some earlier events in the infection process that failed to induce proper cork layer formation or a late response and the fungus sneaked in with out notice? Further study on spatial and temporal aspect of cork layer formation and analyzing the molecular biochemical aspects of sugar beet- *R. solani* interaction could answer some of these questions.

The EL51/R-1 interaction had the highest relative measurements of cortex autofluorescence. Autofluorescence is correlated to disease resistance mechanism in rice (Ono *et al.* 2001), Arabidopsis (Yu *et al.* 1998), and sorghum (Wharton *et al.* 2001). The observation of autofluorescence in resistant lines may be due to a phytoalexin. Phytoalexins prevent fungal growth or change the host cell impermeability that would prevent pathogen invasion and protect host protoplasm from toxins (Hammerschmidt and Nicholson 2000). When EL51 was infected with W22 the autofluorescence was about 4 units less than that of EL51/R-1 but 10 units higher than USH20 infected with R-1 or W22 suggesting that the defense mechanism by EL51 against *R. solani* was modulated by the pathogen virulence or aggressiveness. The resistance mechanism against *R. solani* W22 isolate by EL51 is different from USH20 since USH20 allowed fungal stele establishment where as EL51 restricted the fungus at periphery of endodermis. The interactions among the resistant (EL51) susceptible (USH20) sugar beet seedlings and *R. solani* R-1 and W22 isolates have unique underlying interactions and mechanisms, which will be excellent model systems to study the plant-nectrotrophic fungal interaction and understand the different layers of defense mechanisms.

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# CONCLUSIONS AND FUTURE DIRECTIONS

Efficient and reproducible Rhizoctonia seedling damping off (RSD) disease screening protocol was developed to screen different sugar beet accessions and *R. solani* isolates in the growth chamber and in the greenhouse. Sugar beet breeding line EL51 was resistant to *R. solani* AG2-2 R-1 and W22 isolates and USH20 was susceptible to R-1 isolate (virulent) but recovered from infection by W22 isolate (low virulent). The RSD disease progress curve consisted of three distinct stages – an initial rapid disease progress stage, an intermediate stationary phase and a final resolution phase resulting in death or recovery.

In the field, sugar beet breeding line EL51 showed resistance to both RSD disease and Rhizoctonia crown and root rot disease. For the first time resistance for Rhizoctonia diseases in sugar beet was found in EL51. Under Rhizoctonia disease pressure EL51 had high stand count, sugar content and final harvest weight compare to that of susceptible sugar beet variety USH20.

Microscopic studies have demonstrated that *R. solani* AG2-2 R-1 and W22 isolates produced the initial infection structure, penetrated the epidermis and established in the cortex tissue. When R-1 and W22 isolated penetrated the endodermis of the EL51 seedling the host produced cork layer and restricted the fungi at or just beneath the endodermis. *R. solani* AG2-2 R-1 isolate penetrated the endodermis of USH20 and established in the stele causing tissue destruction and cell death leading to seedling damping off and death. *R. solani* AG2-2 W22 isolate was present in the susceptible sugar beet breeding line USH20 with out causing any phenotypic symptoms. EL51/R-1 interaction caused high cortex autofluorescence compared to other treatments.

Sugar beet- *R. solani* interaction that produced the final out come as disease (compatible, USH20/R-1) and the interactions that resulted in non-diseased state (incompatible, USH20/W22, EL51/R-1 and EL51/W22) are excellent model systems to study sugar beet- *R. solani* interactions and analyze the resistant and susceptible host response to virulent and low virulent fungal isolates. Molecular analysis of these plant-fungal systems will elucidate novel host resistant genes and fungal pathogenicity genes. In USH20/W22 interaction, the fungi was present in the stele without causing damping off symptoms resulting in incompatible interaction where as in EL51/R-1 and EL51-W22 the fungi was restricted from stele by the cork layer formation and resulted in incompatible interaction. Biochemical analysis of the different incompatible interactions will elucidate the underlying mechanisms involved in non-disease state.

Sugar beet breeding line EL51 is a good genetic source to breed for resistance for Rhizoctonia in sugar beets. An efficient breeding program to breed for Rhizoctonia resistance in sugar beets can be developed by utilizing the Rhizoctonia disease screening methods developed through this research (RSD disease screening protocols in the growth chamber and greenhouse and the Rhizoctonia disease (RSD and crown and root rot) screening method in the field) and the EL51 genetic material.

# APPENDIX A

# DIFFERENTIAL GENE EXPRESSION DURING COMPATIBLE AND INCOMPATIBLE SUGAR BEET- *R. SOLANI* INTERACTIONS

## INTRODUCTION

*R. solani* causes Rhizoctonia seedling damping off in sugar beets. In this plant-fungal system, fungal isolates and plant germplasm were selected to include both compatible (disease) and incompatible (Non-disease) interactions. When susceptible sugar beet seedling USH20 was infected with *R. solani* AG2-2 R-1isolate, the outcome was disease. However, when resistant cultivar EL51 was inoculated with *R. solani* R-1 or W22 isolates or when susceptible seedling USH20 was inoculated with W22 isolate, host plants recovered from RSD disease. The RSD disease scoring in sugar beet seedlings showed that R-1 and W22 fungal isolated initiated the disease both in susceptible and resistant sugar beet cultivars. The RSD disease progressed in three characteristic stages- the initial infection stage is characterized by rapid appearance of symptoms, the second static phases characterized by little disease progression, and the final phase finalized the outcome of the interaction, either acute disease or death (compatible interaction) or recovery (incompatible interaction). The objective of this study was to identify the host defense genes and fungal genes that were involved during compatible and incompatible interaction between sugar beet- *R. solani* systems. cDNA-AFLP (complimentary DNA-Amplified fragment length polymorphism) technique (Bachem *et al.* 1998) was used to identifying host defense genes and fungal pathogenicity and virulence genes. cDNA-AFLP does not need any pre-existing sequence information, which makes it an excellent tool to identify novel genes.

## MATERIALS AND METHODS

### Rhizoctonia seedling damping off disease in Greenhouse and tissue collection

Sugar beet breeding lines USH20 (PI 631354)) and EL51 (PI 598074) were used. Wooden boxes (400cm * 580 cm) were filled to 2 cm below the top with "Baccto" high porosity soil and were arranged in a randomized complete block design. Thirty germinated seeds were planted per wooden box and grown in the green house (25°C, 16 hr light and 8 hr dark photoperiod), watered daily, fertilized weekly, and transplanted and maintained thirty plants per box. Four to 6 leaf stage seedlings were inoculated with single isolate of *R. solani* AG 2-2 R-1 (Virulent) isolate or W2-2 (low virulent ) isolate. Each seedling was inoculated by adding 0.1 g of inocula (about 20 fungus –infested millet seeds.) on 2 opposite sides of each plant 4 cm away from each seedling. Control plants were mock inoculated with sterile millet. The seedlings were harvested at post inoculation day four (DPI 4) and DPI 8. Washed the seedlings free of soil, excised the leaves and about two third of the root. Hypocotyl and upper part of root was flash-frozen in liquid nitrogen and immediately stored at -80C until use.

### Total RNA extraction cDNA synthesis

Total RNA was extracted using the plant tissue protocol from Qiagen's RNeasy Mini Extraction Kit (Cat. No. 74904). On average 100 mg of frozen sugar beet hypocotyl tissue yielded 15 µg of total RNA. Several rounds of total RNA extractions were done to obtain minimum of 100 µg of total RNA. 80 µg of total RNA was used to isolate the mRNA (Promega Poly ATtract mRNA isolation kit Z5310). The cDNA was synthesized using Promega Universal RiboClone cDNA synthesis system following manufacture's guidelines. cDNA samples were size fractionated using Sephacryl S-400 resin and spin columns in the universal RiboClone system to remove small-sized (<400bp) cDNA.

### cDNA amplified fragment length polymorphism (cDNA-AFLP)

cDNA-AFLP analysis of the double stranded cDNA was done using LI-COR AFLP Expression Analysis Kit (LI-COR Biosciences). The cDNA was digested with *Tag*I and *Mse*I followed by adaptor ligation. Performed pre-amplification according to the manufacture's instruction. After 50 fold dilution of the pre-amplicon, a selective amplification was done to create subsets of pre-amplified templates. Selective amplification primers consist of two additional nucleotides at the 3' end of the per-amplification primers. PCR product from Selective amplification was gel electrophoresised on a LI-COR DNA sequencer (LI-COR Biosciences) on 6 % gel. The image data was viewed using Adobe Photoshop software. Once the desired bands were detected, the selective amplification samples containing the desired bands were loaded onto a new 8% gel for DNA recovery. The electrophoresis was terminated after the smallest desired band passes the laser detector. Removed the gel from sequencer and scanned on Infrared imaging system (Odyssey LI-COR Biosciences). Loading-wells were stained with bromo phenol blue and few characteristic blue shapes were placed on the gel to help orient the gel on its scanned image for the removal of selected bands. The selected bands were excised for DNA recovery. The cut band was placed in 50µl TE buffer and was freeze-thawed thrice to elute the DNA. Re-amplified the eluted DNA, according to manufacture's instruction. The re-amplified fragments were checked on a 1% agarose gel and cloned subsequently into pCR2.1-TOPO vector (TA cloning Kit version V. Invitrogen life technologies) and were sequenced using M13 Forward primer (Genomics Technology Support Facility Michigan State University). Data base search was done with BLAST service at the National Center for Biotechnology Information (NCBI). The sequences were deposited at NCBI.

**Table 5.1. Summary of differentially expressed sequence tags (ESTs) isolated from the *R. solani* infected sugar beet seedlings.**

| GenBank id | Select amp – lification primer set | Clone id | Treatment | Blast X e value | Blast X hit detail |
|---|---|---|---|---|---|
| CX788979 | MACTAC | MM13-F07 | Mock USH20 | 3.00E-15 | Hypothetical protein (yeast) |
| CX788980 | MACTAC | MM13-G07 | Mock USH20 | E-106 | Xyloglucan transglucosylase/ hydrolase protein |
| CX788981 | MACTAC | MM13-H07 | Mock EL51 | 9.00E-11 | Hypothetical protein (Yeast) |
| CX788982 | MACTAC | MM13-B08 | EL51/R-1/DPI4 | 1.00E-19 | Beta-galactosidase (Lactase). |
| CX788983 | MACTAC | MM13-C08 | EL51/R-1/DPI4 | 3.00E-10 | Mannose/glucose-specific lectin. |
| CX788984 | MACTAC | MM13-D08 | EL51/R-1/DPI4 | 1.00E-36 | Drought Medicago truncatula cDNA clone |
| CX788985 | MACTAC | MM13-E08 | EL51/R-1/DPI4 | 0.007 | Hypothetical serine-rich protein |
| CX788986 | MACTAC | MM13-F08 | EL51/R-1/DPI4 | 0.02 | 60 kDa jasmonate-induced protein(rRNA N-glycosidase). |
| CX788987 | MACTAC | MM13-G08 | EL51/R-1/DPI4 | 1.00E-30 | Glycine-rich RNA-binding protein 2, |
| CX788988 | MAGTAG | MM15-C07 | USH20/W22/DPI4 | E-48 | polyprotein (Zea mays) |
| CX788989 | MAGTAG | MM15-D04 | EL51/W22/DPI4 | 2.00E-17 | putative pentatricopeptide (PPR) repeat-containing protein |
| CX788990 | MAGTAG | MM15-F01 | EL51/R-1/DPI4 |  | No significant similarity found |
| CX788991 | MAGTAG | MM15-F02 | EL51/W22/DPI4 |  | No significant similarity found |
| CX788992 | MCATCA | MM14-C01 | Mock USH20 | 8.00E-07 | Hypothetical protein (Oryza sativa) |
| CX788993 | MGTTGT | MM14-C06 | Mock EL51 | 1.00E-05 | hypothetical protein (Neurospora crassa) |
| CX788994 | MGTTGT | MM14-B07 | EL51/W22/DPI4 | 0.033 | reverse transcriptase (Gossypium barbadense) |
| CX788995 | MAGTAG | MM14-C11 | Mock USH20 | 1.00E-17 | hypothetical protein (Corynebacterium efficiens) |
| CX788996 | MCATCA | MM14-D01 | Mock USH20 | 6.8 | exo polygalacturonase [Sclerotinia sclerotiorum]. |
|  |  |  |  |  |  |

| GenBank id | Select amp – lification primer set | Clone id | Treatment | Blast X e value | Blast X hit detail |
|---|---|---|---|---|---|
| CX788997 | MGTTGT | MM14-D06 | Mock EL51 | 2.00E-05 | hypothetical protein (*Neurospora crassa*). |
| CX788998 | MGTTGT | MM14-D07 | EL51/W22/DPI4 | 1.8 | Cytochrome b561 homolog 2 |
| CX788999 | MAGTAG | MM14-D11 | Mock USH20 | 1.00E-29 | aconitase (*Propionibacterium acnes)* |
| CX789000 | MGTTGT | MM14-E06 | Mock EL51 | 1.00E-06 | structural molecule, putative (*Cryptococcus neoformans*) |
| CX789001 | MCATCA | MM14-E01 | Mock USH20 | 6.8 | exo polygalacturonase (*Sclerotinia sclerotiorum*) |
| CX789002 | MGTTGT | MM14-F06 | EL51/R-1/DPI4 | 0.002 | hypothetical protein (*Xanthomonas axonopodis*) |
| CX789003 | MGTTGT | MM14-F08 | EL51/W22/DPI4 | 1.8 | Cytochrome b561 homolog |
| CX789004 | MGTTGT | MM14-F09 | USH20/W22/DPI4 | 3.00E-07 | ABC-type multidrug transport system, permease component |
| CX789005 | MCATCA | MM14-G01 | Mock EL51 |  | No significant similarity found. |
| CX789006 | MGTTGT | MM14-G06 | EL51/R-1/DPI4 | 1.00E-06 | structural molecule, putative (*Cryptococcus neoformans*) |
| CX789007 | MGTTGT | MM14-G08 | EL51/W22/DPI4 | 1.8 | Cytochrome b561 homolog |
| CX789008 | MGTTGT | MM14-G09 | USH20/W22/DPI4 | 3.00E-07 | ABC-type multidrug transport system, permease component |
| CX789009 | MCATCA | MM14-H01 | Mock EL51 |  | No significant similarity found. |
| CX789010 | MGTTGT | MM14-H09 | USH20/W22/DPI4 | 3.00E-07 | ABC-type multidrug transport system, permease component |
| CX789011 | MGTTGT | MM14-A07 | EL51/R-1/DPI4 | 0.002 | hypothetical protein (*Xanthomonas axonopodis*) |
| CX789012 | MGTTGT | MM14-A08 | EL51/W22/DPI4 | 1.00E-06 | structural molecule, putative (*Cryptococcus neoformans*) |
| CX789013 | MGTTGT | MM14-A09 | EL51/W22/DPI4 | 1.00E-06 | structural molecule, putative (*C. neoformans*) |

| **GenBank id** | **Select amp – lification primer set** | **Clone id** | **Treatment** | **Blast X e value** | **Blast X hit detail** |
|---|---|---|---|---|---|
| CX789014 | MGTTGT | MM14-A10 | USH20/W22/PID4 | 3.00E-07 | ABC-type multidrug transport system, permease component |
| CX789015 | MCATCA | MM14-B01 | Control USH20 | 6.8 | hypothetical protein (*Thermoproteus tenax spherical* virus) |

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# APPENDIX B

# DISEASE SCORE OF RHIZOCTONIA SEEDLINGS DAMPING OFF IN DIFFERENT SUGAR BEET GERMPLASM

**Notes:** Four to six leaf stage sugar beet seedlings were inoculated with *R. solani* AG2-2 R-1 isolate and the disease was scored on the days post inoculation 14 (DPI 14). If a seedling survived at DPI=14, it was classified as healthy (H) and if the seedling was dead it was classified as D (dead). In both trails, if 75% or more of the seedlings were classified as H that sugar beet germplasm was classified as "Resistant". If in each trial only 50% to 75% of the seedlings were scored as H, that germplasm was classified as "Partial resistant". If 25% or less seedlings survived (H) at DPI 14, then that germplasm was classified as "Susceptible".

**Table 6-1: Sugar beet germplasm screened for Rhizoctonia seedling damping off resistance**

| Test # | Accession identifier | Trail 1 Disease score | | | | Trail 1 Disease score | | | | conclusion (Resistant or susceptible |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | PI 285590 | D | D | D | D | D | D | D | D | Susceptible |
| 2 | PI 285592 | D | D | D | D | D | D | D | D | Susceptible |
| 3 | PI 285593 | D | D | D | D | D | D | D | D | Susceptible |
| 4 | PI 285594 | D | D | D | D | D | D | D | D | Susceptible |
| 5 | PI 285595 | D | D | D | D | D | D | D | D | Susceptible |
| 6 | PI 546539 | D | D | D | D | D | D | D | D | Susceptible |
| 7 | PI 552532 | D | D | D | D | D | D | D | D | Susceptible |
| 8 | PI 558505 | D | D | D | D | D | D | D | D | Susceptible |
| 9 | PI 558513 | H | H | D | D | H | H | D | D | Partially resistant |
| 10 | PI 558515 | D | D | D | D | D | D | D | D | Susceptible |
| 11 | PI 631354 (USH20) | D | D | D | D | D | D | D | D | Susceptible |
| 12 | SR96 | D | D | D | D | D | D | D | D | Susceptible |
| 13 | PI 598074 (EL51) | D | H | H | H | H | H | H | H | Resistant |
| 14 | Y03-384-18Self | D | D | D | D | D | D | D | D | Susceptible |
| 15 | Y03-384-60 Self | H | H | D | D | H | D | D | D | Partially resistant |
| 16 | Y03-384-99 Self | D | D | D | D | D | D | D | D | Susceptible |
| 17 | Y03-384-70 Self | D | D | D | D | D | D | D | D | Susceptible |
| 18 | 92RM3mm | D | D | D | H | D | D | D | H | Susceptible |
| 20 | PI 546537 | D | D | D | D | D | D | D | D | Susceptible |
| 21 | PI 546538 | D | D | D | D | D | D | D | D | Susceptible |
| 22 | PI 546539 | D | D | D | D | D | D | D | D | Susceptible |
| 23 | PI 552532 | D | D | D | D | D | D | D | D | Susceptible |
| 24 | PI 546510 | D | D | D | D | D | D | D | D | Susceptible |
| 25 | PI 535826 | D | D | H | H | D | D | H | H | Susceptible |
| 26 | USH20 (H2O2) | D | D | D | D | D | D | D | D | Susceptible |
| 27 | YO3-384-18H2O2 | D | D | D | D | D | D | D | D | Susceptible |
| 28 | EL51 (H2O2) | H | H | H | H | H | H | H | H | Resistant |