IMPROVING MANAGEMENT AND UNDERSTANDING OF MAJOR DISEASES OF SUGAR BEET

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Sugar beet is a sugar-yielding crop, that contributes 25% of the global sucrose production. Economic production of sugar beet is hampered by Cercospora leaf spot (CLS) (*Cercospora beticola*), Rhizoctonia crown and root rot (RCRR) (*Rhizoctonia solani*), Sclerotinia root rot (SRR) (*Sclerotinia sclerotiorum*), and Rhizopus root rot (*Rhizopus arrhizus*) diseases. These diseases can reduce yield by 15 to 40%. On CLS, buildup of fungicide-resistance strains is a major issue due to poor implementation of fungicides and understanding of disease development at early stages. The identification of germplasm resistant to RCRR disease is hindered by the lack of effective inoculation methods. Identification of SRR and RRR pathogens is crucial for their proper management. The objectives of this research were 1. to evaluate the role of adjuvants in improving the efficacy of fungicides on CLS, 2. to characterize the infection process during early stages of infection by *C. beticola*, 3. to identify an effective inoculation method for RCRR, and 4. to identify and characterize the causal organisms of SSR and RRR. The value of adjuvants was evaluated in greenhouse and field conditions. Application of fungicides with or without adjuvants before disease onset reduced disease severity of CLS in greenhouse condition. In field conditions, additions of adjuvants did not improve the effectiveness of fungicides and few of them negatively impacted root yield. The initial stage of infection on CLS susceptible and resistant sugar beet variety were compared using confocal microscopy. *C. beticola* biomass accumulation, percent leaf cell death and disease severity were all significantly greater in the susceptible variety compared to the resistant variety ($P<0.05$). *R. solani* inoculated on the crown and roots were compared in a replicated trial in greenhouse conditions. The root inoculation method provided a more consistent disease rating of the sugar beet variety in the greenhouse for screening of RCRR cultivars in a resistance breeding program. Based on morphological and molecular techniques,

causal organisms of SRR and RRR were characterized and was found to be pathogenic to sugar beet varieties tested *in-vitro* and in the greenhouse conditions.

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DEDICATION

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1. LITERATURE REVIEW

1.1. History of Sugar Beet and Development

Sugar beet (*Beta vulgaris* L.) belongs to the Chenopodiaceae family of angiosperms and is an important source of sugar in many parts of the world. Sugar beet roots contain a rich concentration of sucrose and is thus utilized for commercial sugar production around the world, besides another important sugar crop, sugarcane (*Saccharum officinarum* L.). The sugar beet plants are mainly cultivated as a summer crop in temperate regions of the world whereas sugarcane best grown in the subtropical and tropical zones. Sugar beet harvested usually 5 to 9 months after sowing depending on climatic conditions. The unique capacity of sugar beet to produce a large amount of sucrose that can be extracted and crystallized as well as having a short life cycle led sugar beet to become a popular crop for sugar production in many countries with temperate climates.

Sugar beets are biennial plants which need vernalization to initiate reproductive phase leading stem elongation and flowering. However, early bolting has significant impact on sugar content and sugar extraction (Boudry et al., 1994). Flowering is influenced by temperature, light and physiological mechanisms (Lexander, 1980). The sugar beet plant is divided into several parts, shoot, leaf rosette, and the tap root. The photoassimilates are transported from the leaves to taproot (Schneider et al., 2002). The tap root is fleshy, white and conical shaped and the rosette of leaves grows above the roots. The leaves grow from the crown in a tuft and to a height of 14-inch. The sugar beet contains approximately 20% sugar, 5% pulp, and water content 75%.

Sugar beet mainly produces sugar and the by-products includes pulp and molasses added additional 10% value to the crop, have multiple uses in fish, animal and poultry feed industry. Sugar beet is used in preparing alcoholic beverages, sugary syrup used as a sweetening spread in sandwiches. In North America, anti-icing products used in roads during

winter season prepared from desugared molasses. The uridine and betaine chemicals are produced as by-products of sugar beet. Sugar beet is an important crop for crop rotation cycle. The sugar beet grows best in organic matter rich heavy loams or sandy soil and the optimum rainfall requires 460 mm if there are no irrigation facilities. The minimum temperature for sugar beet seed germination in the presence of adequate moisture is 38^{0} F. Warmer weather is forecasted, starting April 20, with day and night temperatures above 32^{0} F (Khan, 2018; USDA-NIFA, 2018). Sugar beets are grown in the early spring and harvested in late September and October in the Midwest. Sugar beet yields tend to be higher in the Far West, but production costs tend to be higher as well (NASS, 2017).

The journey of sugar beet industry started in 1947 when Andreas S. Marggraf, a German chemist first reported the sugar crystal extraction from beet (Francis, 2006). Franz Carl Achard developed a sugar extraction process, who is considered the father of sugar beet industry (Francis, 2006). In 1811, Napoleon boosted the sugar beet industry through patronizing sugar beet own beet processing factories to reduce the effect of British blockade. Currently, more than 130 countries produce either sugarcane or sugar beet, and ten of these produce sugars from both cane and beet crops (ISO, 2018). In global sugar beet production, the Russian Federation ranked top followed by Germany, USA, France and Turkey (FAOSTAT, 2019). Germany is the leading sugar beet cultivation country in Europe, followed by France, Poland and Great Britain. Sugar beet production gradually expanded to other parts of the world, including Europe, Asia and to the western hemisphere, in the USA, Argentina, Canada, and Chile (Whitney and Duffus, 1986) with much improvement and invention on better technology for cultivation. The very first sugar beet factory in the United States was established in 1838 in Northampton, MA, whereas the first to successfully produce white sugar was built in 1870 in Alvarado, CA (Francis, 2006; Whitney and Duffus, 1986). The world production of sugar is approximately 130 million metric tons where 35% comes from the sugar beet and sugar cane

contributes 65%. In the United States, about 8.4 million metric tons which is 55% of domestic production derived from sugar beet.

Each harvested acre of sugar beets is the source of nearly 4 tons of refined sugar. Total sugar beet production was valued at approximately $1.3 billion in 2015 in the USA. Sugar beets are one of the leading raw materials for the production of manufactured sugar in the United States (ERS, 2017; NASS, 2017). In the U.S., sugar beet is cultivated in the Upper Midwest (Michigan, Minnesota and North Dakota), Great Plains (Colorado, Montana, Nebraska and Wyoming), and the Far West (California, Idaho, Oregon and Washington), the Great Lakes (Michigan; Ohio ceased production in 2005) (Harveson et al., 2002; NASS, 2017). In the Red River Valley of Minnesota and North Dakota, the first sugar beet factory was constructed in 1926 in East Grand Forks (Shoptaugh, 1997). Today there are three sugar beet cooperatives, Minn-Dak Farmers Cooperative, American Crystal Sugar Company, and Southern Minnesota Beet Sugar Cooperative located in Minnesota and North Dakota (Supplementary Fig. 1). Combined, they produced 57% of the US sugar beet crop and estimated up to $5 billion total economic activities in this region (Bangsund et al., 2012).

Production of sugar beet is influenced by many biotic and abiotic (environmental) factors. The most important biotic causes are fungi, bacteria, viruses and nematodes. Among the diseases Cercospora leaf spot, Rhizoctonia crown and root rot, Sclerotinia leaf blight and root rot, Rhizopus root rot, Fusarium yellows, Aphanomyces root rot, Rhizomania are the major problem for economic sugar beet production in the Red River Valley of North Dakota and Minnesota and all sugar beet growing regions in the United States.

1.2. Cercospora Leaf Spot of Sugar Beet

Cercospora Leaf Spot (CLS) is one of the most important destructive foliar fungal disease caused by *Cercospora beticola* Sacc., in all sugar beet growing regions of the world. *Cercospora beticola* Sacc. a filamentous imperfect fungus (Duffus and Ruppel, 1993), but no

sexual stage observed (Lartey et al., 2010; Weiland and Koch, 2004). CLS has become endemic in the Red River Valley sugar beet production area of eastern North Dakota and western Minnesota that accounts for about 60% of the total US crop, producing 13 million metric tons with yields of 42-28 mt/ha (Secor et al., 2013; Windels et al., 1998; Lamey et al., 1996; Ruppel, 1986; McKay and Pool, 1918; Kaffka et al., 2010).

The CLS played a significant role in sugar beet production in the United States of America (Harveson, 2013; Dexter et al., 1998; Gallian and Ocamb, 2013). Warm days and nights with high humidity or free water on the crop canopy are most conducive to serious disease outbreaks (Cattanach et al., 1991; Whitney and Duffus, 1986). Root yield loss estimated up to 40% in humid and warm environment (Bleiholder and Weltzien, 1972; Rossi et al., 2000; Saito, 1996) and also increasing sugar impurities and storage losses resulting higher processing costs (Smith and Ruppel, 1974). Affected sugar beets have to grow new leaves depending on the re-allocation of available sugar from the taproot, which leads to overall loss yield (Holtschulte et al., 2010; Shane and Teng, 1992).

1.2.1. Biology of the Pathogen: *Cercospora beticola*

Conidia are multiseptated and needlelike (2-3×36-107 μm). They are produced on conidiophore that are septate, light brown, and developed from the stroma in cluster form or in tufts (Weiland and Koch, 2004; Skaracis et al., 2010). Conidia starts the infection in the leaves. Conidia produce germ tube and appressoria on stomata to enter the host cell. After penetration, this fungus grows intercellularly. *C. beticola* produces non-host selective toxins like cercosporin and beticolin, and other enzymes which cause the release of soluble nutrients from the cell and results in cell disintegration and death of sugar leaves (Daub and Ehrenshaft, 2000; Fajola, 1978; Goudet et al., 2000). Pseudostromata are the overwintering structures that can survive on plant debris, and acts as a primary source of inoculum for local epidemics (Khan et al., 2008). Pseudostromata can persist on sugar beet or alternative host plant tissue (Knight et

al., 2019a) for over three years if not incorporated into the soil (Khan et al., 2008). A teleomorph has not been found for *C. beticola*, but genetic evidence has supported panmictic populations (Bolton et al., 2012c; Groenewald et al., 2006). Vereijssen et al. (2005) also demonstrated that sugar beet roots could act as a primary infection site for *C. beticola* conidia.

1.2.2. Symptoms of CLS and Epidemiology

Initial disease symptoms are visible with tiny spots which become enlarged. The spots are circular, with dark-brown to reddish-purple borders and light to dark tan centers. Lesions are elliptical which can be found on leaf blades, veins, and petioles. Stomata produces silver to greyish spores which has fuzzy look (Harveson, 2013; Kaffka et al., 2010). In advanced stage of disease development, many spots coalesce and causes blight symptoms which kill larger areas of leaf tissue. Leaves become withered and die in severe disease conditions.

CLS is a polycyclic disease. The primary source of the inoculum is old sugar beet debris of previous years, and infected sugar beet plants in the growing seasons. The other plant hosts are sugar beet related crops (table beet, Swiss chard, spinach), mallow, bindweed, pigweed and wild species of Beta. The CLS disease is favored by warm, humid and rainy weather conditions. Fungal spores spread through wind, rain splash and insects in the growing season. Spore formation occurs at temperatures of 68-79 ^{0}F and in relative humidity of 90 to 100%. Germination of spores and infection occurs at 77-70 ^{0}F (daytime) and at temperature of >60 ^{o}F (night time), and relative humidity >90%. Leaf spots typically occur first on lower, older leaves and progress to younger leaves (Khan, 2018; Kaffka et al., 2010; Harveson, 2013). Leaf spot development varied from 5-21 days after inoculation depending on temperature, duration of wet period, inoculum and host resistance. Affected sugar beet regenerate new leaves through consuming the available sugar from the tap roots resulting overall yield loss (Holtschulte et al., 2010; Rossi et al., 2000; Saito, 1966).

1.2.3. Host Ranges

The hosts of *C. beticola* includes Chenopodiaceae and Amaranthacease family (Weiland and Koch, 2004). Besides, the genus of *Beta* and other weed species including *Amaranthus*, *Chenopodum*, *Atriplex*, *Cyclamen*, *Plantago*, *Malva*, *Limonium*, several crop plants can be infected, for example spinach, lattuce and celery (Groenewald et al., 2006; Lartey et al., 2010).

1.2.4. Management of *C. beticola*

Effective management of CLS is a big concern for the sugar beet growers. Integration of all possible methods includes avoiding planting distance (100 yards) between two fields or from the previous year, removal and destruction of crop residues and debris through tillage, rotation of crops with non-hosts crops at least for 3-years, cultivation of tolerant sugar beet varieties, and timely application of fungicides (Cattanach et al., 1991; Khan, 2018; Harveson, 2013; Kaffka et al., 2010; Li et al., 2011; Shane and Teng, 1992). Growers generally use 5-7 fungicides applications in a growing season when disease severity is high.

For control of leaf spot and blight diseases, a wide range of systemic, translaminar, and contact fungicides including benzimidazole, triazole, strobilurin and organotin fungicide groups have been used (Secor et al., 2013). However, the pathogen has a history of developing resistance to many of them, thus it is necessary to mix triazoles with broad spectrum fungicides such as EBDCs (mancozeb), copper and Triphenyltinhydroxide (TPTH) to help preserve the triazole fungicides. Fungicide applications must be made more frequent when disease pressure is high (Franc et al., 2001; Jones and Windels, 1991). Triphenyl tin hydroxide fungicides give the best results for *Cercospora* leaf spot control. Mancozeb and copper fungicides give acceptable *Cercospora* control especially during less severe disease outbreaks (Jones and Windels, 1991). Cercospora leaf spot (CLS) epidemics in sugar beet have been increasing in recent years. Concomitantly, the availability of effective fungicides is at risk because of

resistance development in the fungus, the lack of new active ingredients as well as restrictive approval practices (Vogel et al., 2018). *Bacillus mycoides* (BmJ) and *Trichoderma* species (Bargabus et al., 2002; Lartey et al., 2010) considered promising biocontrol agents and capable of enhancing crop protection in the future. Resistant cultivars inherited quantitative resistance which is controlled by at least 4-5 genes (Smith, 1987) and reduces sporulation of fungi and limits lesion size development in the growing season (Skaracis et al., 2010).

Development of widespread resistance to fungicides with different modes of action has resulted in management failures and significant economic losses (Weiland and Koch, 2004; Karaoglanidis et al., 2000; Secor et al., 2010; Budakov et al., 2014). Besides, effectiveness of chemical control is influenced by many factors, including the type of fungicides, spray volume, rate of application, timing of application and application methods used, frequency of precipitation, and morphology of targeted leaf surface (Cabras et al., 2001; Fife and Nokes, 2002; Schilder, 2010; Hunsche et al., 2007). The efficacy of chemical sprays can be reduced by rainfall and thus enhance its availability from runoff (Reddy et al., 1994). Environmental determinants such as wind, high temperature, photolysis, wash off by rain, volatilization and irrigation water remarkably affect the efficiency of fungicide spray (Schilder, 2010). Among all factors, rain is considered one of the main concerns that can affect the efficacy of fungicides.

Rainfall regulates the potency of fungicides application through washing off, redistribution, and removal of compounds, and thus significantly act on the residual activity of chemicals (Pigati et al., 2010; Inguagiato and Miele, 2016; Stefanello et al., 2016; Rossouw et al., 2018). Success of disease management depends on proper monitoring of frequency of spray which protect fungicides from wash off during the growing seasons. Fungicides wash off into the soil resulting environmental contamination and posing threats to human health.

1.3. Adjuvants

Adjuvants are chemically and biologically active compounds, and they may improve the effectiveness of the pesticides when added to, either by increasing its desired impact and/or decreasing the total amount of formulation needed to achieve the desired impact (Tu and Randall, 2003). Adjuvants play an essential role in increasing the biological efficacy of agrochemicals. The role of adjuvants in pesticide formulations is relatively easy to understand since herbicides must always penetrate the weeds to have an effect. Adjuvant products which increase the surface tension of solutions tend to reduce the atomization of sprays which alters the spectrum of spray droplets formed. A coarser spray can be achieved by increasing the viscosity of the spray mix. Adjuvants, in the form of activators, spreader/sticker, and penetrators/translocator, have been widely used to improve performance of pesticides, especially herbicides. Physiochemical factors (such as spray adhesion, spray retention, wetting and spreading) and systemic factors (such as uptake of the active ingredient) determine the efficacy of herbicide. The most useful classification of adjuvants is by chemical group with the adjuvants divided into the broad categories of surfactants, oils, acidifiers and buffers, fertilizer adjuvants and 'others' and also be classified as spreader and buffer (Underwood, 2000; Valkenburg, 1982; Hazen, 2000; Hess, 1999; Kirkwood, 1994).

Adjuvants have widely been used since 1950s. They were grouped in at least 25 major groups and were mostly added to pesticides to improve spray dispersion, reduce volatility, spray drift, and improve plant penetration, correct issues with the tank water by affecting the pH (McMullan, 2000). Although adjuvants are typically non-phytotoxic compounds, some of them can produce various effects on metabolic and physiological processes within plants, animals, and microorganisms (Norris, 1982; Parr and Norman, 1965; Hull et al., 1982). Certain types of adjuvants may have negative effects on soil properties and aquatic species (Bayer and Foy 1982; Tyler, 1997a and b; Folmar et al., 1979). Combining adjuvants with many fungicides

has the potential to improve disease management by reducing fungicide rates and extending the interval between applications. In soybean rust disease (*Phakopsora pachyrhizi*), Atplus PFA® which is a pourable adjuvant significantly reduced fungal sporulation (CRODA, 2018) and Kinetic® or Latron® AG-98 added to maneb fungicide reduces dry bean rust incidence by 52% (Gent et al., 2003). It has been reported earlier that some penetrants improve disease management in apples (Deford and Beckerman, 2009; Abbott, 2016). Adjuvants added to Captan® consistently reduce disease incidence of scab of apple (*Venturia inaequalis*) even in high-disease pressure environments by increasing coverage and retention of fungicides and reducing the pH of tank water. Ozkan et al. (1992) experimented on 5-drift retardant chemicals for their effect on spray pattern, droplet size and spray drift reduction. They reported that the most effective retardant had 68% less droplets under 100 microns while the least effective had 30% less. Pre-retention and post-retention of adjuvants depends on various physicochemical parameters (Stock and Briggs, 2017). Some adjuvants have ultraviolet (UV) light blockers which reduce UV degradation of pesticides. Adjuvants (surfactants) may suppress zoospores of downy mildew pathogens (Schilder 2014). Fungicides added with adjuvants acts on depositions and improve the activity of active ingredients (Steurbaut, 1993; Zyl and Fourie, 2011; Wagner et al., 2003).

Gent et al. (2003) reported that Organosilicone-based adjuvants improved coverage by 26 to 38% compared with a latex spreader-sticker and water. Captan® fungicides spray with adjuvants significantly reduces disease incidence and increases the coverage and retention period of fungicide for apple disease management (Abbott, 2016). Alternaria leaf blight of muskmelon disease severity reduced by chlorothalonil fungicides (Egel and Harmon, 2001). Sticker-type adjuvants enhance the adhesive quality of chemicals to plant surface and increase resistance against rain. Gaskin and Steele (2009) studied on organosilicone to evaluate the retention and rainfastness of a protectant fungicide on broad bean and cabbage. Addition of

certain adjuvants, *B. cinerea* incidences on Chardonnay grapevine leaves were significantly reduced (incidences of 2.9-17.1% and 10.0-30.8%, respectively) (Zyl et al., 2010). Addition of adjuvants significantly reduced the incidence of *B. cinerea* and when added to azoxystrobin fungicides, improve the disease control of Asian soybean rust (Zyl et al., 2010). Lukach et al. (1999) reported that adjuvants have positive effects in improving fungicide deposition and retention on grain heads and in controlling Fusarium head blight. The success of adjuvants in enhancing the retention and rainfastness of agrochemicals depends on the characteristics of the leaf surface. The ethoxylates significantly enhanced the rainfastness of mancozeb (Hunsche et al., 2007). Metconazole added with surfactant and emulsifiable oil (15:1) rate was found highly active for field testing (Grayson et al., 1995). The rainfastness of the fungicides was found to be inversely related to particle size when added with adjuvants tested in peas and potatoes using rainfall simulator (Kudsk et al., 1991). The droplet size of some nozzle tips is more affected than others by changes in the contents of the spray solution (Klein et al., 2009; Prokop and Kejiklicek, 2002). Sugar beet leaf is easily wettable and leaf surface is composed of thin film and occasional wax mounds (Yao et al., 2014). Placement of water-sensitive spray cards in different positions in the canopy to evaluate spray coverage is very important (Schilder, 2014). Nozzle type and droplet size has a greater effect on dollar spot control during periods of high disease pressure (Fidanza et al., 2009b).

1.4. Rhizoctonia Crown and Root Rot

Rhizoctonia crown and root rot (RCRR) caused by *Rhizoctonia solani* Kühn is one of the major diseases of sugar beet in the United States (Edson, 1915; Franc et al., 2001) and in the Europe (Buddemeyer and Märländer, 2004; Buhre et al., 2009). The fungus is composed of different anastomosis groups or AGs, which attack certain crops and plant parts (Leach, 1986; Sneh et al. 1991) and is further divided into intraspecific groups. AG 2-2IIIB is generally more aggressive in attacking sugarbeet than AG 2-2 IV (Ogoshi, 1987; Windels and Brantner,

2007; Bolton et al., 2010). This disease can cause crop loss estimated 50% or more in severe disease conditions (Allen et al., 1985; Herr, 1996; Windels et al., 2009). In the Red River Valley and Southern Minnesota, RCRR has become more prevalent and severe during the last decade.

1.4.1. Description of the Pathogen: *Rhizoctonia solani* Kühn

Rhizoctonia solani Kühn (teleomorph, *Thanatephorus cucumeris* (Frank) Donk) is a basidiomycete in the family Ceratobasidiaceae (Asher and Hanson, 2006). *R. solani* overwinters in soil as bulbils, or thickened hyphae, monilioid cells, and sclerotia or in plant debris (Whitney and Duffus, 1986; Boosalis and Scharen, 1959). There are fourteen AGs identified and described in *R. solani* (Carling et al., 2002a; González et al., 2006). Based on morphology, nutritional requirement, effect of temperature, host specificity and frequency of hyphal fusion, AGS are sub-grouped into intraspecific groups ISGs (Sneh et al., 1991). Optimum temperature for *R. solani* infection ranges from 18 to 30˚ C but infection can occur at 12^0 C (Bolton et al., 2010). Continuous wet weather and short rotation favors higher disease incidence and severity. *R. solani* germinates as a hyaline sterile mycelium. Mycelia becomes yellowish or light to dark brown in color with age (Agrios, 2005). The teleomorphic stage of various AGs of *R. solani* (*Thanatephorus cucumeris*) occasionally develops during period of high relative humidity (Herr, 1981).

1.4.2. Taxonomic Classification

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Cantharellales

Family: Ceratobasidiaceae

Genus: *Rhizoctonia*

Species: *Rhizoctonia solani* Kühn

1.4.3. Distribution and Host Ranges

Rhizoctonia solani has a broad host range and capable of infecting may crop families in both tropical and temperate regions in the world. *R. solani* isolates of AG 2-2 IIIB and AG IV groups in Europe reported to be non-infective to cereal crops such as wheat, barley, and corn, whereas AG 2-2 IIIB can cause root and stalk rot of corn (Ithurrart et al., 2004; Windels and Brantner, 2008). The most common hosts are rice, ginger, turfgrass, corn, sugarbeet, and *Chrysanthemum* spp. for AG 2-2 IIIB (González et al., 2006). *R. solani* AG 2-2 (IV and IIIB) are distributed throughout the sugar beet-growing areas of Minnesota and North Dakota, while AG 2-2 IV predominates in the Red River Valley and AG 2- 2 IIIB in southern Minnesota (Windels and Brantner, 2007). The AG 4 is responsible for sugar beet damping-off (Nagendran et al., 2009).

1.4.4. Symptoms and Disease Development

Pre-and post-emergence damping off, crown and root rot are common symptoms caused by the *R. solani* in sugar beet (Edson, 1915). Besides, other soil borne pathogens, *Aphanomyces*, *Pythium*, *Phytophthora* causes similar symptoms (Herr, 1996). Infected seedlings have seedling rot or hypocotyl with deformed and stunted growth (Windels and Jones, 1989). The above ground symptoms of crown and root rot are wilting and chlorosis of leaves and necrotic petiole of crown and wilted leaves gradually die (Asher and Hanson, 2006). These symptoms usually occur on older plants of the developing canopy (Herr, 1996). A defined, circular to irregular sunken lesion develops on tap rot (Whitney and Duffus, 1986). *R. solani* grows intra- and inter-cellularly in sugar beet tissue and the older plants are less susceptible to infection than the younger plants (Ruppel, 1973; Pierson and Gaskill, 1961). RCRR is more severe in moderately wet and water-logged soil and the distribution of the inoculum density is highest in the upper 10-cm soil depth (Agrios, 2005).

1.4.5. Management of *R. solani*

Effective management of this disease requires integrated disease management practices including cultural, chemical, biological, and host resistance. Cultural practices that could be helpful in managing the disease includes using crop rotation, maintaining proper soil fertility by using organic amendments, using different cultivation practices that reduces the inoculum density in soil (Ariena et al., 1996; Ithurrart et al., 2004; Buhre et al., 2009). Biological control is ecofriendly for example, some soil bacteria significantly hamper the formation and survival of sclerotia (Leach and Garber, 1970). *Verticillium biguttatum* significantly reduces the inoculum density of *R. solani* (Velvis et al., 1989).

Rhizobacteria can play a significant role in reducing the inoculum density of *R. solani* and suppress its pathogenic activity (Homma, 1996). *Pseudomonas* CMR12a is a well-known biocontrol strain against *R. solani* AG 2-2 and AG-4 (D'aes et al., 2011). Resistance breeding in sugar beet has been studied for a long time (Panella and Ruppel., 1996). The genetic basis for RCRR resistance in sugarbeet is considerably narrow. Three quantitative loci (QTL) on chromosome 4, 5, and 7 have been identified for RCRR (Lein et al., 2008). Application of fungicides is the most effective method for controlling this disease in sugar beet. Seed treatments with thiram and maneb fungicides provide some control against seed rot and seedling diseases. Azoxystrobin under succinate dehydrogenase inhibitor (SDHI) fungicides group is very effective in controlling *R. solani* (Jacobsen et al., 2004). Azoxystrobin and prothioconazole (Proline 480SC, Bayer Crop Sciences) provide effective control (Brantner and Windels, 2002; Khan et al., 2010). Azoxystrobin is more effective when applied before infection starts and results in delayed infection while enhancing vigorous stands establishment (Karaoglanidis and Karadimos, 2006; Kiewnick et al., 2001). Proper timing of fungicide application is an important key in protecting against RCRR disease of sugar beet (Stump et al, 2004; Windels and Brantner, 2002) but does not ensure complete prevention of infection and

disease development later in the season (Kiewnick et al., 2001). In-furrow, band and foliar application of fungicide effectively reduces the seedling damping off and Azoxystrobin, if applied in furrow application provided better disease control (Brantner and Windels, 2002; Kirk et al., 2008). A mixture of pyraclostrobin and fluxapyroxad applied in a 7-inch band before infection takes place provide effective disease control. Timing of fungicide application is very important. Fungicides to be sprayed when the average daily soil temperature at about 60 to 62 ^{o}F or before the canopy covers the rows at 4-inch soil depth (Khan, 2021). On the other hand, fungicide application before infection starts offered extended protection against the disease (Windels and Brantner, 2002; Stump et al, 2004).

1.5. Sclerotinia Root Rot Disease

1.5.1. Description of the Pathogen: *Sclerotinia sclerotiorum*

S. sclerotiorum (Lib.) de Bary is a soil-borne, cosmopolite plant pathogen from ascomycetes class that can infect more than 500 plant species from dicotyledonous to a number of monocotyledoneous species (Purdy, 1979; Boland and Hall, 1994; Saharan and Mehta, 2008, Sharma et al., 2016a). Ascomycota consist of 3,400 genera and more than 32,000 species (Alexopoulos et al., 1996). The new classification system placed Sclerotinia in the Leotiomycetes class within the Helotiales order (Maddison and Schulz, 2007) and further assigns it to the family of Sclerotiniaceae (Whetzel, 1945). Approximately 33-genera of Sclerotinia has been identified (Willets, 1997) which are based on size of sclerotia, host specificity, ascus and ascospores size and moleculare analyses (Jagger, 1920; Kreitlow, 1949; Ramsey, 1924; Kohn et al., 1988). *S. sclerotiorum* overwinter as sclerotia which is the unique features. Sclerotia produce asci on apothecia (Whetzel, 1945; Holst-Jensen et al., 1997; Kohn, 1979b).

1.5.2. Taxonomic Classification (Maddison and Schulz, 2007)

Kingdom: Fungi

Phylum: Ascomycota

Class: Leotiomycetes

Order: Helotiales

Family: Sclerotiniaceae

Genus: Sclerotinia

Speces: *Sclerotinia sclerotiorum* de Bary

1.5.3. Disease Cycle and Epidemiology

S. sclerotiorum can infect all above ground parts and roots of the plant, including flower petals, leaves, petioles, stems, and pods. This fungus can survive in the soil as sclerotia for many years (Lumsden and Dew 1973; Willetts and Wong 1980) and the sclerotia can germinate carpogenically or myceliogenically depending on environmental conditions. Hyphae growing from myceliogenic germination can infect plant tissue in direct contact (Le Tourneau 1979). Ascospores are the primary source of inoculum for initiating diseases by this pathogen (Abawi and Grogan, 1979). The ascospore starts growing on flower petals followed by germination and colonization in the tissue (Abawi and Grogan 1979; Schwartz and Steadman, 1978; Steadman 1979). Optimum temperature for sclerotia germination is 60-77° F and continuous soil moisture for 10-days (Bardin and Huang, 2001; Wu and Subbarao, 2008). Apothecia formation is favored when soil moisture ranges 23 to 30%. In field conditions, ascospores are released in 5 to 10-days depending on weather conditions (Phillips 1987; Harikrishnan and del Río 2006). Ascospore maturation is influenced by many factors including relative humidity, precipitation, air temperature, canopy development and fungal isolates (Caesar and Pearson 1983).

1.5.4. Management of *Sclerotinia sclerotiorum*

Management of diseases caused by S. sclerotiorum is very complicated. Effective management of this fungus requires integration of all possible techniques including cultural, mechanical, physical, chemical, biological and host resistance to reduce potential yield losses. Crop rotation for more than 3-years with non-host crops including small grains and corns (Rousseau et al. 2007). Cultural practices such as use of clean seeds, recommended plant population densities, judicious use of fertilization can reduce the diseas incidence and severity at certain extend. Cultivation of resistance varieties is an environmentally friendly and cost-effective method. However, complex quantitative host resistance and lacking host specificity in *S. sclerotiorum* make the breeding studies challenging (Mei et al., 2013; Wu et al., 2019; Sharma et al. 2016b). Development of resistant cultivars is difficult as the disease resistance is goverened by multiple genes (Fuller et al., 1984). Biological control is safe and econfriendly which is very effective when synchronized with crop rotation. *Coniothyrium minitans* and *Sporidesmium sclerotivorum* (Adams and Ayers, 1981) are well known mycoparasites, which control *Sclerotinia* populations.

The biological control compounds Serenade and Polyversum are registered for use against Sclerotinia in foliar applications (McMullen and Markell, 2010). Fungicide application is the most effective method for controlling the disease. Foliar application of the fungicides reduces the disease severity and protect significant yield loss (Bradley et al., 2006; del Rio et al., 2002; Spitzer et al., 2017). Timing of fungicide applications are very important. It is recommended that foliar application is required when 20 to 50% of the flowers are open in canola (Thomson et al., 1984). Fungicides used in ND and MN for controlling Sclerotinia diseases includes azoxystrobin, benomyl, boscalid, iprodione, prothioconazole, tebuconazole, thiophanate-methyl, trifloxystrobin and vinclozolin (Bradley et al., 2006).

1.6. Rhizopus Root Rot Disease

1.6.1. Description of the Pathogen: *Rhizopus arrhizus*

Rhizopus arrhizus Fisher is a thermophilic fungus under the family of Mucoraceae. The main feature of this pathogen characterized by the sporangiophores, which arise from the junction of rhizoids and stolon. This fungus commonly found in the rotted plant residues and in most agricultural soils throughout the world. It is considered the most cause of mucormycosis in humans and occasionally infects other animals.

1.6.2. Taxonomic Classification

Kingdom: Fungi

Division: Mucoromycota

Order: Mucorales

Family: Mucoraceae

Genus: Rhizopus

Species: *Rhizopus arrhizus* Fisher

1.6.3. Biology and Disease Epidemiology

In pure culture, *R. arrhizus* appears as creamy white, called mycelium and later becomes gray colored as spore-bearing structures (reproductive structure) called sporangia form. The sporangia are spherically shaped and turn to a blackish color at maturity, appearing like a pinhead and giving the fungal mass a dark appearance. It produces grayish brown spores, which spread through air currents and represents poorly developed rhizoid. *R. arrhizus* spores contains ribosomes as spore ultrstucture. Columellae and apophysis together are globose, subglobose or oval. Metabolism changes from acerobic to fermentation at different stages of its life cycle. *R. arrhizus* is capable of infecting and destroying artificially injured sugar beets most rapidly between 30° and 40 °C, but no growth at 45 ^{0}C, in contrast to a relatively low temperature requirement for the optimum growth of *R. stolonifer* in culture at 24 ^{0}C and with

highest infection capability at 14 ^{0}C to 16 ^{0}C (Hildebrand and Koch, 1943). The *R. arrhizus* Vuillemin (synonym: *R. nigricans* Ehrenberg) is considered weak pathogens that only can damage sugar beet only when the beet is stressed by excessive soil moisture, high temperature, poor surface drainage in the field, mechanical damage to the crown or insect damage (Pitt and Hocking, 1985; Vincelli and Burne, 1989).

1.6.4. Disease Symptoms

Infected plants show characteristic symptoms of wilting of the foliage. The foliage rapidly wilts and becomes dry and brittle, collapsing on the crown and appearing similar in appearance to plants affected by RCRR. Diseased root tissues showed symptoms of deterioration. Gray to brown lesions are seen on the taproot and diseased tissue turned dark and spongy. Infected roots emit acidic odor. A whitish mycelial growth is sometimes evident on the root surface.

1.6.5. Host Ranges and Distribution

R. arrhizus is known as storage pathogen that causes soft rot of sugar beet roots and of other plants, e.g. sweet potatoes (Benada et al., 1984; Szymczak-Nowak, 1992; Takada et al., 1998; Holmes and Stange, 2002). Rhizopus root rot caused by *R. arrhizus* and *R. stolonifer* has been reported to occur in Arizona, California, Wyoming and Colorado in the USA and in Canada (Alberta and Ontario) and Italy, Iran, France and many countries that made up the former USSR (Jacobson, 2006). Soft rot on soursop, longya Lily and potato, and head rot in sunflower caused by *R. arrhizus* have been reported in Mexico, China and other parts of the world (Hahm et al. 2014; Park et al. 2014; Palemon-Alberto et al. 2020).

1.6.6. Management of the Fungus

R. arrhizus is generally considered a weak pathogen that damage only when the host plants that are stressed by excessive soil moisture, high temperature, poor surface drainage in the field, mechanical damage to the crown or insect damage (Pitt and Hocking 1985, Vincelli

and Burne 1989). Integrated management practices provide excellent control against *R. arrhizus*. Removal and destruction of crop residues, avoidance of mechanical damage during intercultural operations, insects damage to sugar beet roots, escape the environmental stresses (high temperature, waterlogging conditions), and avoidance of excess fertilization during the growing seasons provides protection against the fungus (Pitt and Hocking 1985; Gilman 1957, Hanson 2010).

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2. ROLE OF ADJUVANTS TO IMPROVE THE EFFICACY OF FUNGICIDES FOR CONTROLLING CERCOSPORA LEAF SPOT OF SUGAR BEET

2.1.Abstract

Cercospora Leaf Spot (CLS) is a destructive foliar disease of sugar beet (*Beta vulgaris* L.), which is caused by the hemi-biotrophic fungus *Cercospora beticola*. This disease results in significant reduction of extractable sucrose. Growers typically use an integrated system of partially resistant cultivars, crop rotation with non-hosts, and timely application of fungicides to manage the disease. However, recent development of resistance to several classes of chemicals by *C. beticola* coupled with untimely applications and/or regular rainfall after fungicide applications have resulted in reduced efficacy of fungicides in controlling CLS. The objective of the study was to evaluate the role of adjuvants on improving the efficacy of fungicides for controlling CLS of sugar beet. In the greenhouse, recommended fungicides were used alone or in mixtures with three adjuvant types before and after inoculation with *C. beticola*. Disease severity was evaluated 14-days post inoculation. In the field, fungicides were used alone or mixed with adjuvants, applied at 14 days intervals and disease severity and recoverable sucrose were evaluated at harvest. Results showed that application of fungicides before disease onset resulted in reduced disease severity in greenhouse condition. Adjuvants did not reduce disease severity when sprayed alone in greenhouse conditions. Inspire XT, a systemic fungicide, typically resulted in less disease severity than Penncozeb and Badge SC which are protectant fungicides. Field experiments showed significant variation on disease severity and yield components ($P<0.001$). Addition of adjuvants did not improve the effectiveness of fungicides in controlling CLS and few of them negatively impacted root yield in field conditions. Adding Complex® to fungicides did not increase yields consistently and combining Transfix® with any fungicide did not increase yield in any year.

It was evident that neither to the adjuvants evaluated reduced disease severity significantly when compared to the fungicide without adjuvant in filed conditions. This study suggested that addition of adjuvants did not improve the effectiveness of fungicides in controlling CLS in the greenhose and field conditions and few of them negatively impacted root yield in field conditions.

2.2. Introduction

Sugar beet is one of the leading economic crops in North Dakota next to Minnesota, combinedly contributing 60% of US Sugar beet production. Cercospora leaf spot (CLS) is a destructive foliar disease of sugar beet (*Beta vulgaris* L.) globally (Holtschulte, 2000; Malandrakis et al., 2006; Skaracis et al., 2010), caused by the fungus *Cercospora beticola* (Fig. 1a and 1b). Root yield loss estimated up to 40% in humid and warm environment (Bleiholder and Weltzien 1972; Shane and Teng, 1992; Rossi et al., 2000; Saito, 1996) and increasing sugar impurities and storage losses resulting in higher processing costs (Weiland and Sundsbak, 2000; Smith and Ruppel, 1973). Affected sugar beets grow new leaves (Fig. 1c) depending on the re-allocation of available sugar from the taproot, which leads to overall loss yield (Holtschulte et al., 2010; Rossi et al., 2000). *C. beticola* reproduce through asexual spores, mycelia or conidia that overwinter in alternate host, plant debris and weeds and are spread by wind or rain splash to infect new leaves or plants (Duffus and Ruppel, 1993; Lartey et al., 2010; Weiland and Koch, 2004). Spots coalesce to extended lesions leading to the complete collapse of leaf tissue. This infection can re-occur several times in a growing season. Ultimately, the disease cycle can result in the destruction of the whole photosynthetically active canopy.

Control efficacy and management towards reducing the chances for the development of pathogen resistance to fungicides largely depends on the proper integration of sustainable techniques (Shane and Teng, 1992; Windels et al., 1998). Currently, disease management practices are focused on integration of cultivation of resistant cultivars, crop rotation and timely

application of fungicides significantly limit disease development and consequent yield loss (Meriggi et al., 2000, Rossi, 1995; Jacobsen and Franc, 2007; Ioanidis and Karaoglanidis 2010; Skaracis et al., 1996; Khan and Khan, 2010; Shane and Teng, 1992; Panella and Frese, 2000; Khan, 2018). Foliar application of *Bacillus mycoides* (BmJ) and *Trichoderma* species (Bargabus et al., 2002; Lartey et al., 2010) is considered a promising biocontrol measurement and capable of enhancing crop protection in the future.

For controlling leaf spot and blight diseases, a wide range of systemic, translaminar, and contact fungicides including benzimidazole, triazole, strobilurin and organotin fungicide groups have been used to control cercospora leaf spots across the sugar beet-producing fields worldwide (Secor et al., 2010). However, development of widespread resistance to different modes of action of fungicides has resulted in management failures and significant economic losses due to repeatedly uses over a long time (Weiland and Koch, 2004; Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974; D'ambra et al., 1974; Pal and Mukhopadhyay, 1985; Weiland and Halloin, 2001; Giannopolitis, 1978; Cerato and Grassi, 1983; Bugbee, 1996; Karaoglanidis et al., 2000; Budakov et al., 2014). Besides, effectiveness of chemical control is influenced by many factors, including the type of fungicides, spray volume, rate of application, timing of application and application methods used, frequency of precipitation, morphology of targeted leaf surface (Bruggen et al., 1987; Evenhuis et al., 1998; Cabras et al., 2001; Fife and Nokes, 2002; Schilder, 2010; Rich, 1954, Hunsche et al., 2006). The efficacy of spray chemicals is reduced by rainfall and thus its availability enhanced for runoff (Reddy et al., 1994).

Environmental determinants such as wind, high temperature, photolysis, wash off by rain, volatilization and irrigation water remarkably affects the efficiency of fungicide spray (Swart et al., 1998; Schilder, 2010). Among all factors, rain is considered one of the main concerns that can affect the efficacy of fungicides. Rainfall regulates the potency of fungicides

application through washing off, redistribution, deposits and removing, and thus significantly act on the residual activity of chemicals (Thacker, 1999; Ramsey et al., 2005; Pigati et al., 2010; Inguagiato and Miele, 2016; Stefanello et al., 2016; Rossouw et al., 2018).

Adjuvants have been widely used since 1950s and grouped in at least 25 major classes. These compounds are added to pesticides to improve spray dispersion, reduce volatility, and spray drift, improve plant penetration, and correct issues with the tank water by affecting the pH (Tu and Randall, 2003). Combining adjuvants with many fungicides has the potential to improve disease management by reducing fungicide rates and extending the interval between applications. In soybean rust disease (*Phakopsora pachyrhizi*), Atplus PFA® which is a pourable adjuvant significantly reduced fungal sporulation (CRODA, 2020) and Kinetic® or Latron® AG-98 added to maneb fungicide reduces dry bean rust incidence by 52% (Gent et al., 2003). It has been reported earlier that some penetrants improve disease management in apples (Deford and Beckerman, 2009; Abbott, 2016). Adjuvants added to Captan® consistently reduce incidence of apple scab (*Venturia inaequalis*) even in high-disease pressure conditions. CLS epidemics in sugar beet have been increasing in recent years causing higher use of fungicides. Concomitantly, the availability of effective fungicides is at risk because of resistance development in the fungus, the lack of new active ingredients as well as restrictive approval practices (Vogel et al., 2018).

C. beticola can become less sensitive to the fungicides used to control them, especially if they are applied frequently over several years (Vaghefi et al., 2016). Populations resistant to QoI fungicides have the G143A mutation (Piszczek et al., 2018, Bolton et al., 2013; Delgado et al., 2012) and are not controlled when these fungicides are applied, which may lead to field failures. *C. beticola* can develop resistance to QoI fungicides very rapidly: for this reason, it is recommended to mix QoI fungicides with a protectant fungicide CLS control (Khan, 2018; Khan and Carlson, 2009). *C. beticola* has developed resistance to thiophanate methyl,

increased in sensitivity to triazoles and triphenyltin hydroxide and acquired cross resistance to fungicides with four major modes of action used for its control. Growers generally use 5-7 spray when disease severity is high. So, it is necessary to mix triazoles with broad spectrum fungicides such as EBDCs (mancozeb), copper and Triphenyltin hydroxide (TPTH) to help preserve the triazole fungicides. Unfortunately, EBDCs and copper fungicides may wash off easily with rain hence not most efficacious in controlling CLS because these give shorter duration of control due to degradation.

However, limited information is available regarding the effect of adjuvants on improving fungicides performance for controlling sugar beet foliar diseases. Therefore, the role of different adjuvants was evaluated on the efficacy of fungicides for controlling CLS of sugar beet.

2.3. Materials and Methods

2.3.1. Plant Material, Greenhouse Condition, Humidity Chamber

Sugar beet (Maribo® seed proprietary materials) plants were (CLS rating: 5.24) grown in 3.5-inch deep square pots containing peat mix (Sunshine mix 1, Sun Gro Horticulture Ltd.; Alberta, Canada) amended with slow release fertilizer (Osmocote 14-14-14; Scotts-Sierra Horticultural Products Company, Marysville, OH). The experiment was conducted in Agricultural Experiment Station (AES) greenhouse at North Dakota State University in Fargo, ND. Greenhouse condition was set to allow for a 16-h photoperiod, and temperature was maintained at 25 ± 2°C (Argus Control Systems Ltd.; British Columbia, Canada). The humidity chamber was set to provide 14/10 hours of photoperiod where light started from 7 am to 9 pm and darkness period started from 9 pm to 7 am. Temperature and relative humidity was set at 28^0C and > 90 %, respectively. Plants were watered daily to maintain adequate moisture for plant growth and disease development.

2.3.2. Inoculum Preparation and Leaf Inoculation in the Greenhouse

C. beticola was grown on CV8 media (100 ml of clarified V8 juice, 1.0 g of CaCo3, 0.01 g of ampicillin, 0.001 g of polymixin B sulfate, and 15 g of agar, volume adjusted to 1 liter with distilled water, pH 7.2) for 5 days under fluorescent light at 25 ± 1°C to induce sporulation. Sporulated plates were then added with sterile water containing 0.01% Tween 20 (Sigma Aldrich, St. Louis, USA) and spores were separated by using autoclaved glass rod. Spore concentration was determined with a hemacytometer and adjusted to 30,000 spores/ml (Vereijssen et al., 2007). Inoculation of *C. beticola* conidial suspension was sprayed with hand sprayer (Preval Spray) on to the upper surface of the leaf and kept the inoculated plants into the humidity chamber for 5-days at temperature of 28 ^{0}C and relative humidity 100%. After that plants were transferred to the greenhouse chamber and the disease severity of CLS was evaluated at 14 days post inoculation (Appendix Fig. 2). There were following treatments for greenhouse experiments (Table 2.1).

2.3.3. Fungicide and Adjuvants

Three different fungicides, Penncozeb, Inspire XT and Badge SC were used in combination with various adjuvants (Table 2.1). Penncozeb (EBDC) is a broad-spectrum, multi-site protectant fungicide (a.i. Mancozeb; FRAC Class M3). Inspire XT (Triazole) is a mixture of two active ingredients, difenconazole and propiconazole from FRAC group 3, 3. Badge SC (FRAC Group M1) is a multi-site contact copper fungicide, composed of Copper Oxychloride and Copper Hydroxide. Adjuvants viz., Complex, Preference, Cerium Elite and Transfix were selected for this experiment. Complex is known as a spreader-sticker, increases contact activity, penetration, adhesion, and it also reduces surface tension of spray droplets. Preference, an activator improves the spray droplets, provides better protection against ultra-violet radiation and reduce surface tension. Transfix is characterized as a sticker that facilitates penetration of active ingredients and enhances leaf coverage.

Table 2.1. List of treatments used in the greenhouse experiments

Sl No	Treatments	Rate	Sl No	Treatments	Rate
1.	Penncozeb	2lb/acre	11.	Inspire XT +Cerium Elite	7fl oz/ acre, 2-6 oz/100 gallon
2.	Inspire XT	7fl oz/ acre	12.	Badge SC +Complex	4 pt/acre, 0.5-2 pt/100 gallon
3.	Badge SC	4 pt/acre	13.	Badge SC +Preference	4 pt/acre
4.	Penncozeb +Complex	2lb/acre, 0.5-2 pt/100 gallon	14.	Badge SC +Transfix	4 pt/acre, 4-6 oz/100 gallon
5.	Penncozeb +Preference	2lb/acre, 2 pt/100 gallon	15.	Badge SC + Cerium Elite	4 pt/acre, 2-6 oz/100 gallon
6.	Penncozeb +Transfix	2lb/acre, 4-6 oz/100 gallon	16.	Complex	0.5-2 pt/100 gallon
7.	Penncozeb +Cerium Elite	2lb/acre, 2-6 oz/100 gallon	17.	Preference	2 pt/100 gallon
8.	Inspire XT +Complex	7fl oz/ acre, 0.5-2 pt/100 gallon	18.	Transfix	4-6 oz/100 gallon
9.	Inspire XT +Preference	7fl oz/ acre	19.	Cerium Elite	2-6 oz/100 gallon
10.	Inspire XT +Transfix	7fl oz/ acre, 2-6 oz/100 gallon	20.	Inoculated check	-

Cerium elite is a non-volatile, non-ionic surfactant and a drift control agent (a.i. vegetable oil ethoxylate, oleic acid, alcohol ethoxylate). It is best known for superior spreading and wetting characteristics. Fungicides, Inspire XT, Penncozeb and Badge SC were sprayed at 7 fl oz/ac, 2 lb/ac, 4 pt/ac, respectively. On the other hand, adjuvants, Complex, Preference, Cerium elite and Transfix were sprayed at 2 pt/100-gal, 2 pt/100-gal, 4-6 oz/A, and 6 oz/100-gal, respectively. For both the greenhouse and field experiment, fungicides and adjuvants were sprayed alone or in combination.

2.3.4. Fungicide-Adjuvants Spray

Spraying fungicides and adjuvants were conducted at NDSU Jack Dalrymple Agricultural Research Complex. Treatments were applied using Generation-III Research Sprayer in a spray booth chamber (Appendix Fig. 3). Fungicide treatments were applied before or after plants were inoculated with *C. beticola*. In the former case, after spraying fungicide-adjuvants, plants were kept in the greenhouse for 24 hours before before inoculation with *C. beticola*. For chemical spray, Turbo Twin TeeJet-110-degree flat fan nozzle at 60 PSI and @ 17 GPA (gallon per acre) was used to ensure better spray droplets and wider distribution.

2.3.5. Evaluation of Disease Severity

At 14-days post inoculation (dpi), disease severity of CLS was recorded following Jones and Windels (1991) CLS 1-10 categorical scale where 0= no disease symptoms, 1=0.1% severity, 2=0.35% severity, 3= 0.75% severity, 4=1.5% severity, 5=2.5% severity, 6=3% severity, 7=6% severity, 8=12% severity, 9=25% severity and 10=50% severity, respectively (Appendix Fig. 4).

2.3.6. Data Analysis

Data were analyzed by non-parametric methods using the Rank and GLIMMIX procedures of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Least square mean ranks

of main effect of timing of chemical application was separated using Tukey-Kramer test (*P=0.05*). Median severity of CLS was treatments applied to the least square mean ranks of treatments and their confidence intervals at 95% were estimated and compared (Shah and Madden 2004).

2.3.7. Field Design and Experiment

Field experiments were conducted at Foxhome, Minnesota in 2019, 2020 and 2021. Fungicides were used alone and mixed with adjuvants of different categories following manufacturer recommended dose. Rainfall data were recorded for three years. Recommended dose of fertilizers and other cultural practices such as weeding were done following NDSU Extension sugar beet production guide. The experiments were conducted using a randomized completely block design (RCBD), which consists of total 17-treatments including inoculated check (control) and four replications. The length of each row was approximately 30 feet long and each plot was consisted of six rows. These rows were 0.9 m apart from each other and distance between replications was 4 m. The seeds of a susceptible cultivar (Maribo MA504) were sown in the first week of May in 2019, 2020 and 2021. Treatments evaluated are listed in Table 2.2. Artificial inoculum was used to inoculate sugar beet leaf and each experimental plot was subjected to foliar spray of fungicides treatments where fungicides were sprayed alone, mixed with adjuvants and fungicides mixture (more than one fungicide) in a rotation program. Foliar inoculations of *C. beticola* were made by hand in the early morning when leaves are wet. The untreated plots were sprayed with pure water. Chemicals were sprayed @ 14 days interval using Turbo Twin TeeJet-110-degree flat fan nozzle @ 17 GPA (gallon per acre) tank mix (Appendix Fig. 5-7).

Table 2.2. List of treatments evaluated in field experiments conducted in 2019, 2020 and 2021 in Foxhome, MN.

Sl No	Treatments	Rate	Sl No	Treatments	Rate
1.	Penncozeb	2lb/acre	10.	Badge SC +Complex	4 pt/acre, 0.5-2 pt/100 gallon
2.	Badge SC	4 pt/acre	11.	Inspire XT +Complex	7fl oz/ acre, 0.5-2 pt/100 gallon
3.	Inspire XT	7fl oz/ acre	12.	Fungicides mixtures in rotation +Complex	Recommeded dose*, 0.5-2 pt/100 gallon
4.	Fungicides mixtures in rotation	Recommeded dose*	13.	Penncozeb +Transfix	2lb/acre, 4-6 oz/100 gallon
5.	Penncozeb +Cerium Elite	2lb/acre, 2-6 oz/100 gallon	14.	Badge SC+ Transfix	4 pt/acre, 4-6 oz/100 gallon
6.	Badge SC + Cerium Elite	4 pt/acre, 2-6 oz/100 gallon	15.	Inspire XT+ Transfix	7fl oz/ acre, 4-6 oz/100 gallon
7.	Inspire XT + Cerium Elite	7fl oz/ acre, 2-6 oz/100 gallon	16.	Fungicides mixtures in rotation +Transfix	Recommended dose*, 4-6 oz/100 gallon
8.	Fungicides mixtures in rotation + Cerium Elite	Recommended dose*, 2-6 oz/100 gallon	17.	Check (Inoculated)	
9.	Penncozeb +Complex	2lb/acre, 0.5-2 pt/100 gallon			

******Super Tin= 8 fl oz/a, Badge SC 2 pt/a, Manzate Max 1.6 qt/a, respectively.

CLS was recorded following Jones and Windels (1991) CLS field 1-10 categorical scale where 0= no disease symptoms, 1=0.1% severity, 2=0.35% severity, 3= 0.75% severity, 4=1.5% severity, 5=2.5% severity, 6=3% severity, 7=6% severity, 8=12% severity, 9=25% severity and 10=50% severity, respectively. A rating of 1 indicated the presence of 1 to 5 spots/leaf or 0.1% disease severity and a rating of 10 indicated 50% or higher disease severity. Cercospora leaf spot severity was assessed five times at 14 days interval during the season. The rating performed before harvest on September is reported. Weather data was recorded for each year (NDAWN website) (Appendix Fig. 8). Plots were defoliated mechanically and harvested using a mechanical harvester. The middle two rows of each plot were harvested and weighed for root yield. Twelve to fifteen representative roots from each plot, not including roots on the ends of the plot, were analyzed for quality at the American Crystal Sugar Company Quality Tare Laboratory, East Grand Forks, MN.

2.3.8. Statistical Analysis

Data were analyzed by non-parametric methods using the Rank and Proc GLIMMIX procedures of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Non-parametric and parametric Levene's test for homogeneity of variances were conducted using the generaliuzed linear model procedure (proc glm) of the Statistical Analysis System software v. 9.4 (SAS Institute, Cary, NC) to determine whether severity and yield data, respectively, could be combined for analysis. In a combined analysis, years and replications would be considered random variables while treatments would be considered fixed variables. Least significant mean ranks for disease severity were separated using Tukey's-Karmer test ($P=0.05$). The relative effects of median severity for each treatment and their confidence intervals at 95% were estimated and compared (Shah and Madden 2004). Single degree of freedom contrast analyses were conducted to determine whether the adjuvants improved the protection offered by each fungicide. In addition, the concept of area under the disease progress curve was used to

calculate the area under the rank progression curve (AURPC) analyze the performance of treatments.

2.4. Results

Levene's test for homogeneity of variances for each year was conducted. Since Levene's indicated years were significanlty different from each other ($P<0.001$) lead to analyse the data for each year separately. In greenhouse experiments, spraying method (either after or before) *C. beticola* inoculation was significant ($P<0.001$). The effect of fungicides was significant ($P<0.001$) and the interaction of spraying method and fungicide was also significant ($P<0.001$). When fungicides were sparyed before inoculation of *C. beticola*, CLS severity was lowered whereas higher disease severity was recorded when fungicides were applied after *C. beticola* inoculation. Only eight treatments showed significant interactions ($P=0.05$, Fig. 2a). Median severity was lowest in Inspire XT sprayed plants compared to Penncozeb when fungicide applied before fungus inoculation. The median severity was recorded lowest in Inspire XT sprayed plants compared to Penncozeb treated plants either sprayed before or after *C. beticola* inoculation. On the other hand, when adjuvants mixed with fungicides and sprayed before fungal inoculation, the lowest median severity found in Inspire XT+Complex treatment. No significant differences were found for Penncozeb+Cerium Elite and Inspire XT+Transfix when chemicals sprayed after *C. beticola* inoculation. Similarly, Inspire XT+Complex and Inspire XT+Cerium Elite had identical results but showed comparatively lowered median severity. Median severity was similar in Penncozeb+Complex and Inspire XT+Transfix treated plants when sprayed before fungal inoculation. Similarly, no significant difference on median severity was reorded in Inspire XT and Penncozeb+Cerium Elite treated plants when applied before *C. beticola* inoculation. When chemical sprayed after fungal inoculation, Penncozeb, Penncozeb+Cerium Elite, and Inspire XT+Transfix showed equal level of median severity

(Fig. 2.1). Similary, Inspire XT, Inspire XT+Complex, and Inspire XT+Cerium Elite showed identical disease severity.

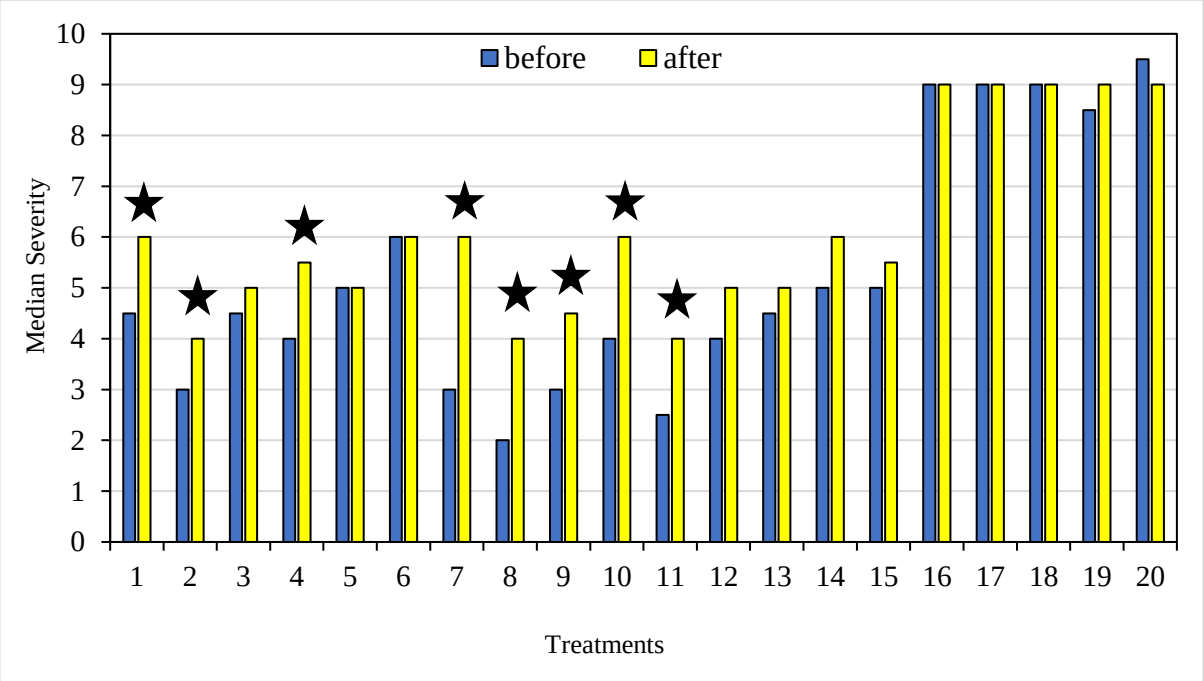

Figure 2.1. Effect of timing of fungicide application on median severity of Cercospora leaf spot on sugar beet in greenhouse conditions (*P=0.05*).

The effect of Inspire XT alone or added to Preference was similar when sprayed before inoculation of fungus. On the other hand, Badge SC alone or mixed with Complex, Preference, Transfix and Cerium Elite showed no interaction either being sprayed before or after inoculation of *C. beticola* (Fig. 2.1). When Penncozeb added to Preference and Transfix showed significant interaction on CLS control irrespective of spraying methods (*P=0.05*). In the case of adjuvats, Complex, Transfix, Preference, and Cerium Elite treated plants showed similar levels of median severity either sprayed before or after fungal inoculation. Inoculated check (control) plants had the highest median severity (Fig. 2.1).

Field experiments conducted in 2019, 2020 and 2021 showed significant variation on disease severity and yield components (*P<0.001*). We conducted contrast analyses of the individual treatments for each year for disease severity and yield (Supplementary Table 1). In

2020, there were no significant effect found among the treatments (*P=0.3387*) (Fig. 2.2a and Table 2.3) but the effect of treatments was significant in 2019 and 2021 (*P<0.001*). In 2019, only Badge SC and Badge+Complex showed significant interaction which resulted increased yield compared other treatments. Adding Cerium Elite to any of the fungicides did not increase yield in any of the year. Adding Complex to fungicides did not increase yields consistently. Badge+Complex increased yields in 2019 but not in other years. Combining Inspire XT with Complex increased yields in 2020 (unprotected trial) but not in other years. Combining Transfix with any fungicide did not increase yield in any year. Rotation consistently produced good yields that were significantly different than some fungicides and the non-protected control in two of the three years. Combined analysis of 2019 and 2021 indicated that fungicides Rotation (with or without adjuvants), penncozeb with adjuvants, and Inspire with or without adjuvants significantly reduced severity compared to non-protected control other treatments did not (Fig. 2.2 b and Fig. 2.3).

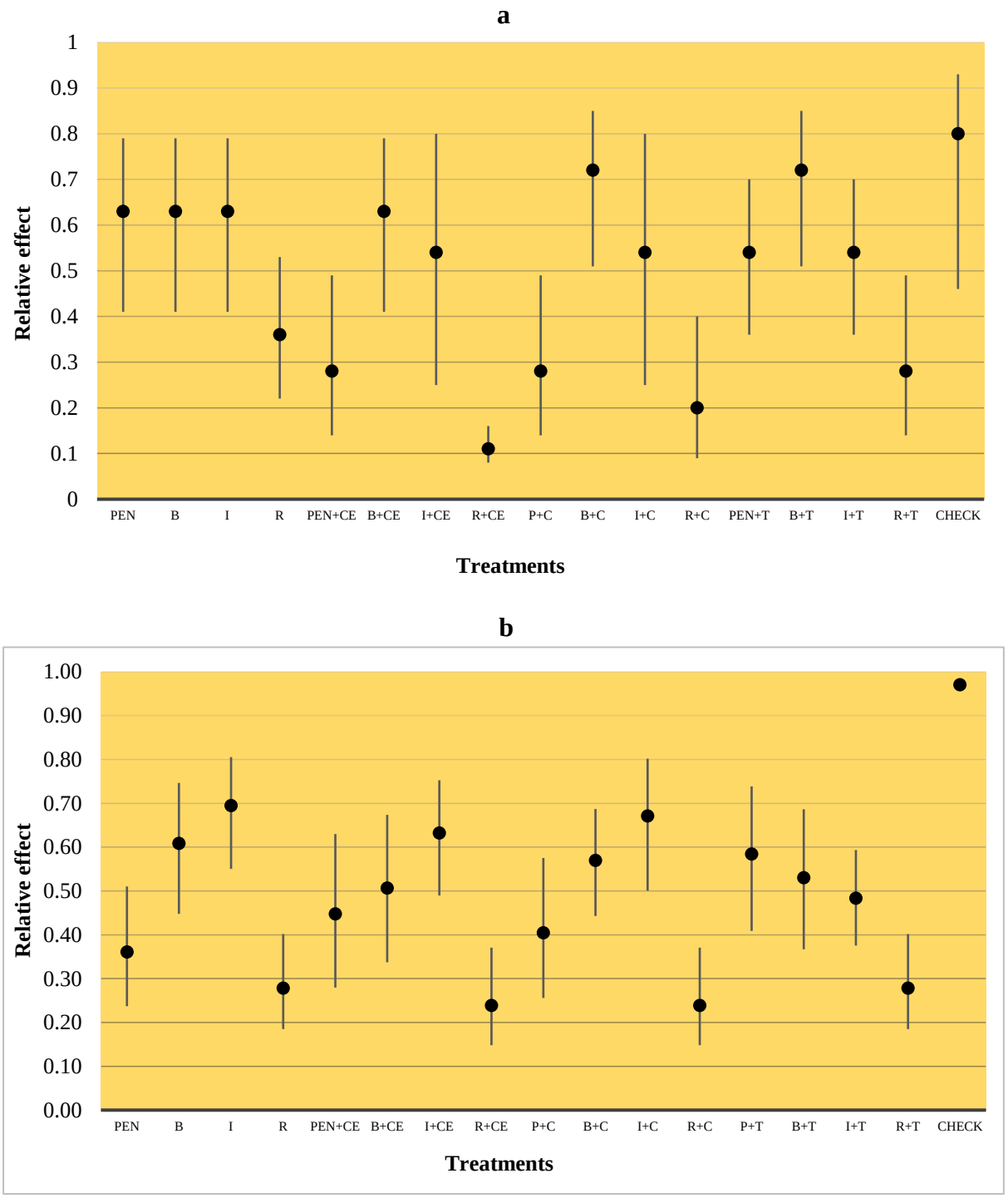

Figure 2.2. (a) CLS disease severity in 2020 and (b) CLS disease severity in 2019 and 2021 (combined) was measured by the relative effect (solid circle). The bar reperesent 95% confidence interval of the estimated relative effect.

Table 2.3. Recoverable sucrose (yield) in pounds sugar/Acre-2020

Treatment	None	Cerium Elite	Complex	Transfix
Check	2187	2187	2187	2187
Penncozeb (EBDC)	2572	2229	2411	2576
Badge SC (Copper)	2511	2625	2602	2396
Inspire XT (Triozole)	3204	2774	2042	2550
F/cides Rotation	2515	2295	3022	2416
Statistical significance	NS*	NS*	NS*	NS*

* NS= non-significant according to yy test with *P=0.05*.

The only exception was adjuvant complex increased protection with penncozeb and Penncozeb+Complex (*P=0.0266*). When the use of adjuvants was evaluated using single degree of freedom contrast analysis, it was evident that neither to the adjuvants evaluated reduced disease severity significantly when compared to the fungicide without adjuvant.

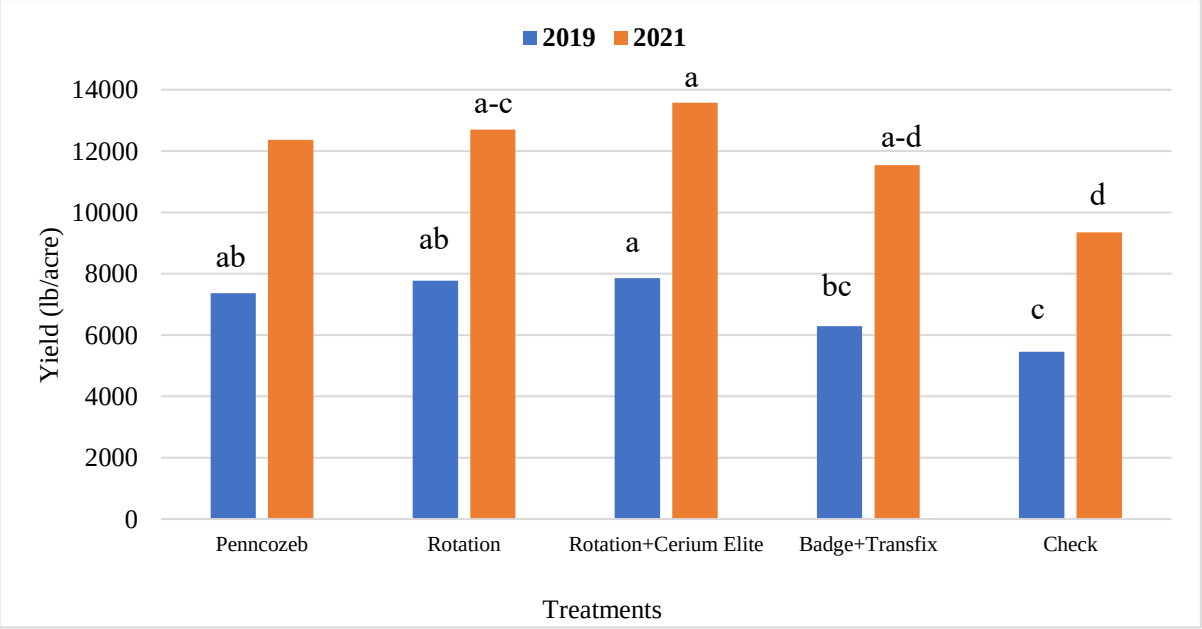

Figure 2.3. Recoverable sucrose (yield) in pounds sugar/acre calculated for 2019 and 2021, separately.

2.5. Discussions

In greenhouse conditions, Inspire XT, a systemic fungicide provided better control CLS contrl than the contact fungicides Penncozeb and Badge SC. The addition of the adjuvants Complex and Cerium Elite to Inspire XT lowered disease severity when applied before pathogen inoculation. Adjuvants applied alone did not have any effect on CLS severity in the greenhouse or the field. Previously, several researchers investigating the role of adjuvants and reported a positive impact of adjuvants on disease control (Deford and Beckerman, 2009; Egel and Harmon, 2001; Gent et al., 2003; Grayson et al., 1995; Zyl et al., 2010; Lukach et al., 1999; Mullin et al., 2016).

Schilder (2010 and 2014) reported that systemic fungicides were quickly absorbed and redistributed by the plants when applied in mixture with adjuvants, hence providing effective disease control. Suheri and Latin (1991) found that contact fungicides (viz. mancozeb) showed reduced efficacy in wet conditions. Surfactant (spreader: Regulaid®) was found to be effective for Phythophthora collar rot disease of apple when mixed with penetrants adjuvants (Deford and Beckerman, 2009) whereas Captan fungicides added to any oil and other adjuvants resulted phytotoxic to apple (Abbott and Beckerman, 2018; Beckerman, 2016). Sticker adjuvants enhance adhesion of pesticide sprays to plant surfaces and increase their resistance to rain to control broad bean and cabbage disease (Gaskin and Steele, 2009). Aksoy and Katicioglu (1998) investigated the effect of diniconazole mixed with spreader-sticker (Citowett®) to control rose rust and found that it reduced the dose and number of applications and improved disease control. In this study, the adjuvants did not have a positive effect on diseae control, except in eight of 20 cases.

In field trials, Complex mixed with the copper fungicide Badge SC improved disease control and resulted increased yield 2020. Similarly, Complex in greenhouse conditions provided better disease control when mixed with contact (Penncozeb) and systemic (Inspire

XT) fungicides. Complex® is a spreader which helps improve the efficacy of pesticide spray mixtures and thus improve contact activity, and adhesion to plant surface. Besides, Complex® reduces surface tension due to its surfactant properties, which promotes uniform coverage of spray surface. Irish et al. (2002) showed that adjuvants are with non-ionic surfactant properties found to be effective and ensure comparable control to the QoI (Azoxystrobin®) fungicides to reduce white rust on spinach. In field experiements, only few combinations (3 out of 45 treatments) had a significant effect on CLS control when adjuvants were added to fungicides since the role of adjuvants were inconsistent for the years. We found that adjuvants, Transfix®, Cerium® Elite and Complex® added to any fungicide did not increase yield for any year except 2020 in field conditions.

It has been reported earlier that adjuvants have phytotoxic effects, decreasing the potential of the pesticides or increasing harmful effects to non-target plants and animals (Norris, 1982; Mesnage and Antonuiou, 2018). Few of these chemicals interfere on biochemical process within plants (Parr and Norman, 1965). Spreader-sticker type adjuvants are toxic to tender annuals and herbs (Foy and Pritchard, 1996). Some adjuvants have stimulatory or inhibitory effects on the growth and metabolic processes (Parr, 1982) and toxic at high level (Norris, 1982; Czarnota and Thomas, 2013). Khan et al. (2007) showed that adjuvants (Agri-Dex®, Activator®-90, Silwet® L-77) added to Pyraclostrobin fungicide (Headline® 2.09 EC) did not improve the CLS control and in some instances were phytotoxic. Selection of adjuvants and use of proper rate is extreamly critical to reduce the phytotoxicity because of the sensitivity of crops. Grayson et al. (1996b) also found the similar results of phytotoxicity due of alkylamine, nonylphenol, and silicone-based adjuvants to dimethomorph for controlling downy mildew of grapes although increased therapeutic control of potato late blight and Pythium root rot was improved in cucumber due to addition of adjuvants to dimethomorph and with stand application non-ionic surfactant adjuvants (Grayson et al., 1995;

Grayson et al., 1996a; Stanghellini et al., 1996). The success and effectiveness of adjuvants depends on the host morphology, host-pathogen relationship, cultivars, age (Gent et al., 2003; Steurbaut, 1993). The greenhouse and field study suggested that addition of adjuvants did not improve the effectiveness of fungicides in controlling CLS and few of them negatively impacted root yield in field conditions.

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3. HISTOPATHOLOGICAL INVESTIGATION OF VARIETAL RESPONSES TO *Cercospora beticola* INFECTION PROCESS ON SGUAR BEET LEAVES

3.1. Abstract

Cercospora leaf spot (CLS) is the most destructive foliar disease in sugar beet (*Beta vulgaris* L.). It is caused by *Cercospora beticola* Sacc., the pathogen that produces toxins and enzymes which regulate membrane permeability and cell death during infection. Fungal hyphae penetrate through stomata, grow intercellularly, and colonize parenchymatous cells resulting in degenerative changes around the infection sites. However, little information is available regarding the time of penetration and development of this pathogen on the host tissues. Therefore, we investigated the temporal development of CLS on leaf tissues of susceptible and resistant sugar beet varieties using confocal microscopy. Inoculated leaf samples were collected at 12-h intervals between 0 and 120-hours post inoculation (hpi), and stored in DAB (3,3'-Diaminobenzidine) solution until processed. Samples were stained with Alexa Fluor 488 dye to visualize fungal structures with a confocal laser scanning microscope. Fungal biomass accumulation, percent cell death, and the area under disease progress curve for both susceptible and resistant varieties were evaluated to compare the host response to *C. beticola* inoculation. Upon DAB staining, susceptible and resistant cultivars showed remarkable differences in cell death. No cell death was found on any cultivar until 36-hpi; after that, the susceptible cultivar showed significantly greater ($P=0.05$) disease severity at every time point evaluated. Hyphae from germinating spores penetrated susceptible and resistant cultivar tissues directly or producing appressoria. The appressorium was observed in cases where conidia were situated over a stomatal opening. Conidia of *C. beticola* penetrated directly through stomata at 48-hpi in susceptible cultivar while it took 60-hpi for conidia to enter stomata of the resistant variety. Lesion development was visible after 120-hpi in the inoculated leaves in the susceptible variety. Appressoria from secondary hyphae were observed on

stomatal guard cells starting at 60-hpi in susceptible plants and 72-hpi in resistant plants. Penetration of hyphae inside the parenchymatous tissues varied in accordance with time post-inoculation and varietal genotypes. The pathogen produced lower amounts of fungal mycelia in the leaf tissues of resistant plants than in susceptible plants. *C. beticola* grew 1.6 times faster and accumulated 1.7 times greater biomass in susceptible plants compared to the resistant variety. The percent cell death was 3-times higher in the susceptible plants than in the resistant plants. Overall, this study provided evidence of the events that occur during the early stages of infection of sugar beet tissues by *C. beticola*. It also demonstrated that genetic resistance to CLS is expressed through slower penetration and reduced rate of colonization of leaf tissues.

3.2. Introduction

Cercospora beticola Sacc. is a destructive foliar fungal pathogen that causes Cercospora leaf spot (CLS) on sugar beets. This disease is a major constraint for sugar beet production worldwide (Byford, 1996; Holtschulte, 2010; Malandrakis et al., 2006; Skaracis et al., 2010; Lartey et al., 2010). *C. beticola* is a hemi-biotroph that produces non-host selective toxins, e.g., cercosporin and beticolin, and cellulase and pectinase enzymes during its necrotrophic stage (Daub and Ehrenshaft, 2000; Goudet et al., 2000). Due to its release of cellulase and pectinase enzymes, plant cells become necrotic, which interferes with normal plant cell functions (Staerkel et al., 2013; Assante et al., 1977; Moser et al., 1990; Mike`s et al. 1994). This disease reduces photosynthetic areas and negatively affects taproot yield, causes storage losses and increases sugar impurities (Weiland and Sundsbak, 2000; Staerkel et al., 2013). Severe CLS outbreaks may result in crop losses that reach up to 50% or more (Skaracis et al., 2010).

Pool and McKay (1916) first studied *C. beticola* infection mechanisms and concluded that open stomata exerted stimulus (either chemo or hydrophobic) to germ tubes for penetration. In 1928,

Schmidt provided experimental evidence that *C. beticola* penetration is a hydrophobic response. But his findings were refuted by Vestral (1933) and Darpoux et al. (1953) who reported that *C. beticola* penetration to the host occurs purely by accident. No further detailed studies were done on C. beticola infection strategies until 1960s. Canova (1959c) supported the findings of Schmidt (1928).

A breakthrough came into the scientific world on *C. beticola* infection mechanisms in late 1970. Rathaiah (1977) proposed that this fungus enters the host tissue through open stomata at 3 to 4 days after inoculation (DAI). More recently, other researchers have reported that hyphae grow intercellularly, and colonize in parenchymal tissue within 7-days, and start the degeneration of cells at the point of infection (Feindth et al., 1981; Steinkamp et. al., 1979). Fiendth et al. (1981) reported that leaf age and stomatal opening influence conidial penetration and disease severity. Histological studies in the late 1970s and early 1980s concluded that reorientation of hyphal growth to stomata was non-thigmotrophic. Hyphae grow densely and form pseudostromata below stomata (Weltmeier et al., 2011). The production of conidia on the plant surface often depends on environmental triggers viz., relative humidity, and droplets of free water. Kim et al. (2011) reported that light significantly regulated stomatal tropism and infection in *Cercospora zeae-maydis*. Light induces stomatal opening for appressorium to enter stomata in rust fungi also (Yirgou and Caldwell, 1968). Certain fungi find their way to enter through natural openings, for example, stomata to infect leaves (Agrios, 20045) but the molecular mechanism is still unclear. Although, penetration of thigmotrophic fungi, like the causal agents of rusts and powdery mildews through stomata is well documented (Maheshwari and Hidebrandt, 1967; Rowell, 1984; Hoch et al., 1987; Hückelhoven, 2005; Lorrain et al., 2019; Solanki et al., 2019), information regarding the penetration pathway non-thigmotrophic fungi viz., *Cercospora beticola* is inadequate. It is apparent that natural openings have a

significant effect on foliar pathogens ingress but how plants respond to these attacks are still unclear (Kortekamp, 2003; Melotto et al., 2006).

Observation of fungal structures within plant tissues has been limited by the efficacy of staining techniques. Conventional techniques of fungal staining are not effective enough because it does not get adequate penetration of fluorescent dye beyond the cuticle of a leaf. The waxy layers on the leaf surface exert a major barrier to visualizing pathogenic structures developing inside the host cell. Intermediate staining steps may include heat treatments (Aylife et al., 2011) of leaf samples but the heat facilitates tissue disruptions during slide preparation and staining. Dyes like Uvitex 2B and Calcofluor have been used in histological studies; but Uvitex 2B reduces fluorescence intensity (Diagne et al., 2011; Dugyala et al., 2015) and Calcofluor is less efficacious after counterstaining, and it fades quickly (Bonifaz et al., 2013). Advanced microscopy techniques offer exciting potential for investigating fungal morphology in the host cells. In the recent past, fluorescence laser microscopes have been widely used for cellular exploration (Dunst and Tomancak, 2019; Solanki et al., 2019; Shetty et al., 2019). Some researchers have studied the *C. beticola* infection on sugar beet using staining and microscopy but, in most cases, sampling was done randomly for investigation of fungal development on the hosts. An extensive histopathological study is important for a better understanding of the spatial and temporal circumstances of the pathogen in host plants.

This study reports findings obtained through a systematic observation of the penetration and colonization of sugar beet leaf tissues by *C. beticola* that occurs in the first 120-hpi using advanced staining and confocal laser scanning microscopy.

3.3. Materials and Methods

Two types of studies were conducted to compare the reactions of a susceptible and a resistant sugar beet variety to CLS. The first study was conducted in greenhouse environment and compared symptom development of both varieties during the first eight days after

inoculation with *C. beticola*. The second study was conducted in the laboratory and explored the temporal progress of the disease in leaf tissues of both cultivars using confocal microscopy. Each study was conducted twice, each time using a replicated completely randomized design.

3.3.1. Greenhouse Study: Plant Materials and Pathogen Inoculation

This study was conducted at the Darlymple Greenhouse facility of North Dakota State University, Fargo, ND, USA. In the greenhouse, plants were grown in plastic pots measuring 10x7x12 cm (T.O. Plastics Inc., Clearwater, MN, U.S.A.), filled with peat mix (Sunshine mix 1, Sun Gro Horticulture Ltd.; Alberta, Canada). At planting time, 20 g of slow-release fertilizer 15-9-12 (N-P-K) (Osmocote, Scotts-Sierra Horticultural Products Company, Marysville, OH) was added to each pot. Plants were regularly watered and monitored until they were subjected to inoculation. The greenhouse room was set to maintain 12 h photoperiod and temperature at 25 ± 2°C (Argus Control Systems Ltd.; British Columbia, Canada). The humidity chamber was set to provide 14/10 hours of photoperiod where light started from 7 am to 9 pm and darkness period started from 9 pm to 7 am. Temperature and relative humidity was set at 28^0C and > 90 %, respectively. One *C. beticola*-susceptible (Maribo® seed proprietary materials) and one-resistant (Beta® seed proprietary materials-D) sugar beet varieties were used for this study (Fig. 3.1 a-b). The American Crystal Sugar company rates these cultivar's reaction to CLS at 4.8 and 2.0, respectively (ACS 2020). *C. beticola* was grown on clarified V8 media plates at 25±2 ^{0}C under constant fluorescent light. Spores were harvested from 5-days old sporulated plates by flooding the plates with sterile water containing 0.03% v/v Tween 20 (Sigma Aldrich, St. Luis, MO) and rubbing the colony surface with a sterile glass rod. Conidial suspensions were strained through two layers of cheesecloth to remove mycelial threads. Conidial concentrations were determined with a haemocytometer and adjusted to $3x10^4$ spores/ml (Vereijssen *et al*., 2003). The 6-leaf growth stage plant leaves were sprayed with the spore suspension using a

hand sprayer (Preval Sprayer System, 27x13x10 cm). Inoculated plants were incubated in humidity chamber at 28 ^{o}C for 5 days to stimulate infection.

a b

Figure 3.1. (a) Susceptible (a) and the (b) resistant varieties were used for leaf inoculation in the greenhouse.

3.3.2. Evaluation of Disease Severity on Plants

CLS severity on susceptible and resistant varieties was recorded at two-day intervals between the seventh and 15th day after inoculation. Plant reaction to CLS was evaluated using the 0-10 CLS rating scale of Jones and Windels (1991) where, 0=no disease symptoms, 1=0.1% severity, 2=0.35% severity, 3=0.75% severity, 4=1.5% severity, 5=2.5% severity, 6=3% severity, 7=6% severity, 8=12% severity, 9=25% severity and 10=50% severity, respectively.

3.3.3. DAB Staining, Fixation and Clearing of Leaf Samples

For this study, leaf samples were taken from the varieties evaluated in the greenhouse study immediately after inoculation and then at 12 h intervals until 120 h post inoculation (hpi). Each time, a 1 x 3 cm sample was cut from the middle part of leaves and was taken immediately to the lab for processing. Leaf samples were immersed for six hours in a 3, 3'-diaminobenzidine (DAB) solution prepared according to manufacturer protocols (Sigma Aldrich, Germany) and incubated on an orbital shaker (VWR®, USA) set for 120 rpm at 25 ^{o}C temperature. After incubation, the samples were washed twice with anhydrous Farmer's fixative (FF) (3-ethanol:1-glacial acetic acid) and transferred to 50 ml tubes containing 30 ml of fresh FF and incubated for 12 hours at 25 ^{o}C for clearing and fixing. The FF was changed after 12 hours,

and samples were cleared for an additional 3 hours. Cleared samples were stored in 45 ml FF of fresh solution in dark until processed for microscopy (Fig. 3.2 a-b).

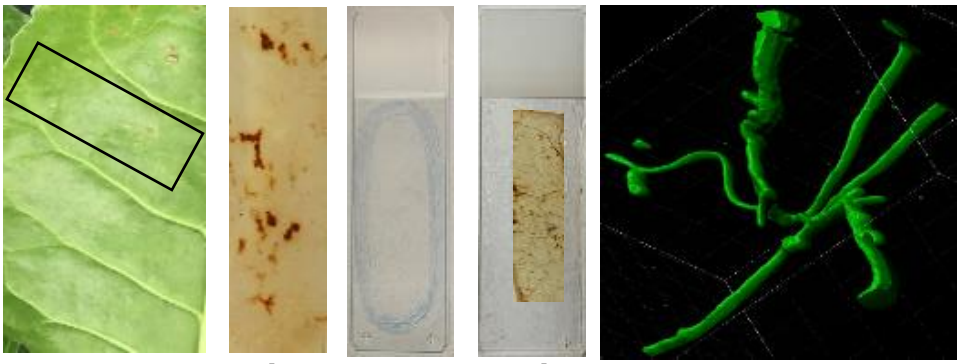

Figure 3.2. Sugar beet leaf samples collection, processing and fixing on glass slide for visualization under confocal microscopy (a-e).

3.3.4. Heat Treatment for Sample Processing

Cleared samples stored in FF were washed twice in 30 ml of 1x PBS (phosphate buffer saline, pH: 7.5) supplemented with 0.05% v/v Tween-20 for 5-minutes by centrifuging them at 60 rpm in 50 ml polypropylene tubes (VWR®, USA). The samples were then rinsed twice with Tris HCl (pH 7.4) supplemented with 0.05% Tween-20 for 5-minutes at 60-rpm. ImmeEdge hydrophobic barrier PAP pen (Vector Laboratories) was used to draw two concentric hydrophobic oval-shaped boundaries on the Histobond™ microscope glass slide (Fisher Scientific). A leaf sample was placed inside the circle keeping the adaxial surface of the leaf sample facing up. The slides were then transferred to an autoclaved rack where 350 µl 1M KOH + 0.05% Tween-20 solution was carefully pipetted into the hydrophobic circle of each slide to cover the leaf samples. The hydrophobic barrier ensures the leaf samples remain hydrated and submersed in the liquid solution. The rack with the slides was kept on a plate shaker at 50 rpm (rotation per minute) for three minutes. After that, the liquid solution was replaced with fresh 1M KOH + 0.05% Tween-20 solution, and the samples were covered with aluminum foil to prevent drying slides and autoclaved for 15 minutes at 121 ^{0}C and 103.4 kPa.

After autoclaving, the remaining KOH buffer on samples was pipetted off and the slides were cleaned with a Kim Wipe to remove the distorted hydrophobic boundary without disturbing the leaf samples. Then again, a new hydrophobic boundary was drawn encircling the sample. Samples were then washed twice with 1x PBS + 0.05% Tween-20 solution for 5 minutes at 50 rpm and the buffer solution was pipetted off (Fig. 3.2 c-d).

3.3.5. Sample Staining and Mounting

Heat-treated samples were stained with WGA-Alexa Fluor 488 dye (20 µg/ml) that was dissolved in 1x PBS buffer supplemented with 0.05% v/v Tween-20. For each sample, 350 µl of dye was pipetted on the cleared samples and incubated at 25 ^{o}C for one hour on a shaker at 50 rpm. After staining, samples were washed thrice with Tris HCl pH 7.4 (without Tween-20) for 5 minutes at 60 rpm to remove the excess dye from the leaf surface. To prepare slides for confocal microscopy, the hydrophobic circle was wiped off using Kim-Wipe tissue. The Ecomount mounting media (Biocare Medical) was pipetted on the leaf samples in sufficient volume to cover it completely and a cover slip was successfully placed on top avoiding formation of air bubbles. The slides were dried at room temperatures overnight in the dark and stored in 4 ^{o}C temperature until visualization under microscope.

3.3.6. Quantification of Fungal Biomass and Cell Death

Disease progress on cleared leaves was evaluated in two ways, measuring fungal biomass and quantifying cell death. Samples were observed using an LSM 700 laser scanning confocal microscope (Zeiss Thornwood, NY) with a Plan-Apochromat 40x/1.3 oil immersion lens and 10x objective lens. Red light excitation (555/580 nm wavelength) was used for autofluorescence detection and the green light (488/520 nm wavelength) was set for visualization of WGA-Alexa Fluor stained tissues. The Z-stack images were taken based on the depth of *C. beticola* structures developed in the parenchyma tissue (100-300 images per infection site up to 120 µm deep). ZEN (Zeiss Thornwood, NY) software was used to perform

computations to obtain collapsed pictures from the Z stack and the Imaris (9.0.1) (Bitplane, South Windsor, CT) software was used to obtain 3D image reconstructions for hyphal volume analysis (Fig. 3.2e). Imported images were put through channel correction for the red and green channels. For the green channel, attenuation correction was done maintaining intensity front 256 and intensity back 128 values with median filter was 5x5x5. Contrast changes of images were done using normalized layers function to remove the excess background. This helps in the visualization of pathogen volume growth inside the plant cells. Once the volume of pathogen growth was determined through the surface creation function, the surface area of pathogen growth was created specifically for the green channel. All nonspecific signals were removed using volume filtering.

3.3.7 Statistical Analysis

Data on CLS severity on plants, fungal biomass, and percentage of cell death were analyzed using various procedures of the statistical Analysis System software version 9.4 (SAS Institute Inc., Cary, NC, USA). For each data set, the variances of both trials were compared using Levene's test for homogeneity of variances to determine whether a combined analysis could be conducted. When combined analyses were allowed, trials were considered random variables. The percentage severity equivalencies in the severity scale (e.g., 0.1% for 1, 0.35% for 2, and so on) were used to calculate the area under the disease progress curve (AUDPC). The AUDPC was analyzed using the generalized linear model procedure (GLM) and the means for cultivars were compared using the least significant difference with $P = 0.05$. In addition, the exponential, logistic, and linear regression models were fit to CLS severity expressed as percentages of leaf covered to estimate the temporal rate of disease progress. Data on biomass accumulated 120 h post inoculation was analyzed using the mixed procedure and the lsmeans of cultivars were compared using the Tukey-Kramer post-hoc test at $P = 0.05$. A linear regression model was used to describe the temporal increase of biomass in each cultivar. The

AUDPC concept was used to calculate the area under the mortality progress curve (AUMPC) using cell death data. The AUMPC data was analyzed using the GLM procedure. Treatment means were compared using Fisher's protected least significant difference (LSD) test at *P=0.05.*

3.4. Results

3.4.1. Greenhouse Evaluation of CLS Severity

Significant differences in CLS severity were observed between the susceptible and resistant sugar beet varieties. Lesions on the susceptible variety Maribo® MA504 were observed 5 days after inoculation; two days later, they were observed on the resistant variety Beta®. Ten days later, CLS had increased to an average severity of 11% in the susceptible variety but remained at 1% in the resistant variety Beta (Fig. 3.3). CLS temporal development was explained equally well by the exponential and logistic models (Table 3.1). Both models indicated the disease progressed 1.3 times faster on the susceptible than in the resistant variety.

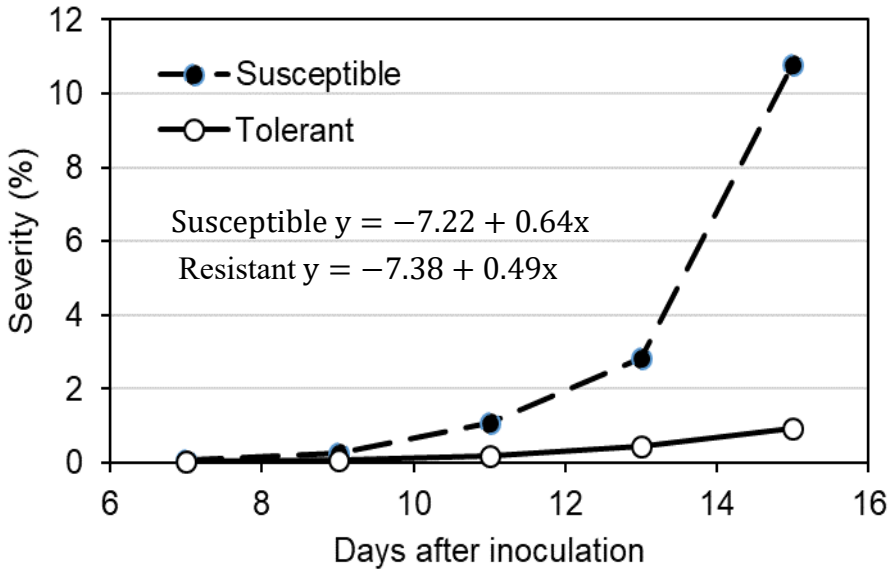

Figure 3.3. Temporal disease progress of Cercospora leaf spot inoculated on sugar beet varieties, Maribo (susceptible) and Beta (resistant) in the greenhouse conditions.

Table 3.1. Fitness of temporal disease progress models for Cercospora leaf spot inoculated with *Cercospora beticola* on susceptible and resistant sugar beet varieties in greenhouse conditions.

Model	Cultivar	Intercept	Slope	R^2	P	MSE
Linear	Susceptible	-10.19	1.12	0.49	<0.001	12.09
	Tolerant	-0.842	0.11	0.68	<0.001	0.046
Exponential	Susceptible	-7.22	0.64	0.89	<0.001	0.39
	Tolerant	-7.38	0.49	0.80	<0.001	0.51
Logistic	Susceptible	-11.94	0.65	0.90	<0.001	0.49
	Tolerant	-11.99	0.49	0.80	<0.001	0.51

3.4.2. Fungal Penetration into Host Tissues

Independently of the variety, germinating tubes were observed entering directly through stomata (Fig. 3.4 a), as well as producing dome-shaped appressoria on the guard cell (Fig. 3b). Secondary and tertiary branches (Fig. 3.4 c), also were observed entering plant tissue through stomata. After penetration, the fungal hyphae grew in the mesophyll tissues (Fig. 3.4 d-e). *C. beticola* grew on the surface and developed a dense fungal mat and was observed growing intercellularly after penetration (Fig. 3.4 f).

On the susceptible variety, conidia of *C. beticola* germinated randomly on the surface of sugar beet leaves and strands of hyphae grew without entering the stomata until 36 hours post inoculation (hpi) (Fig. 3.5). At 48- hpi, hypahe were observed entering through stomata. Germinating tubes were observed producing dome-shaped appressoria on the guard cell at 60-hpi and producing secondary and tertiary branches after that (Fig. 3.5). After penetration, the fungal hyphae grew in the mesophyll tissues. The secondary and tertiary hyphae formed on the leaf surface also entered the leaf through stomata. Starting at 84-hpi, *C. beticola* grew on the surface and developing a dense fungal mat and was observed growing intercellularly after penetration in the susceptible variety (Fig. 3.5).

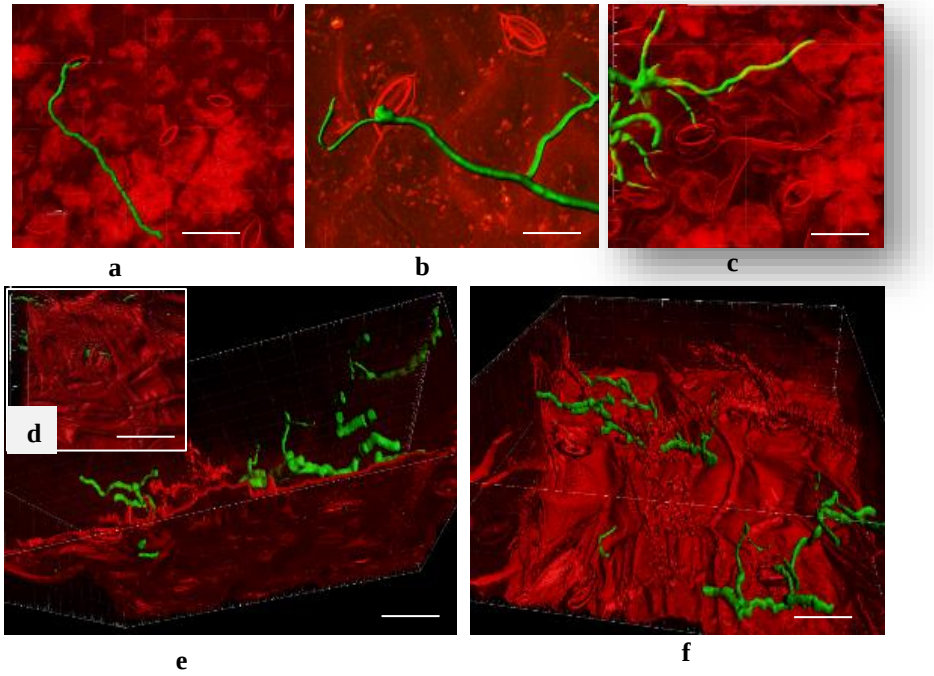

Figure 3.4. Penetration and subcellular growth of *C. beticola* in mesophyll tissue of sugar beet leaves (a-f).

On the resistant variety, *C. beticola* conidia germinated and grew on the leaf surface and directed towards the stomata but did not follow any patterns until 48-hpi (Fig. 3.6). However, germ tubes were observed penetrating the stomata at 60-hpi. Appressoria developed at 72-hpi and fungal hyphae progressed and developed through producing secondary and tertiary branches making fungal mats at 120-hpi. Direct fungal penetration by hyphae and by secondary or tertiary strands through stomata and into the mesophyll tissue of sugar beet leaves was observed at 120-hpi. As in the susceptible cultivar, some hyphal strands grew near and went across the stomata without penetrating into the mesophyll (Fig. 3.5). Starting at 84-hpi, *C. beticola* grew on the surface and developed a dense fungal mat (Fig. 3.5) and was observed growing intercellularly after penetration, in the susceptible cultivars.

On the resistant cultivar, *C. beticola* conidia germinated and grew on the leaf surface (Fig. 3.6) and directed towards the stomata but did not follow any patterns until 48-hpi. However, germ tubes were observed penetrating the stomata at 60-hpi. Appressoria developed at 72-hpi and fungal hyphae progressed and developed through producing secondary and

tertiary branches making fungal mats at 120-hpi. Direct fungal penetration by hyphae and by secondary or tertiary strands through stomata and into the mesophyll tissue of sugar beet leaves was observed at 120-hpi. As in the susceptible cultivar, some hyphal strands grew near and went across the stomata without penetrating into the mesophyll (Fig. 3.5).

3.4.3. Evaluation of Cell Death and Disease Severity

The susceptible and resistant variety responded variably to DAB staining and quantification of cell death of inoculated leaf samples showed distinct differences at each time point. The first symptoms of disease, tissue discoloration and death were visualized 48-hpi and 60-hpi in the susceptible and resistant variety, respectively (Fig. 3.7). Infected tissue in both varieties gradually became darker but the percentage of cell death was higher in the susceptible variety compared to the resistant variety (Fig. 3.7). After 120 hpi, the susceptible variety had 24.5% cell death, amount that was 3.3 times greater (*P=0.001*) than that of the resistant variety; similarly, the AUMPC for the susceptible variety was 609 units, which was 2.7 times greater (*P=0.01*) than that of the resistant variety (Table 3.2; Fig. 3.8 and Fig. 3.9).

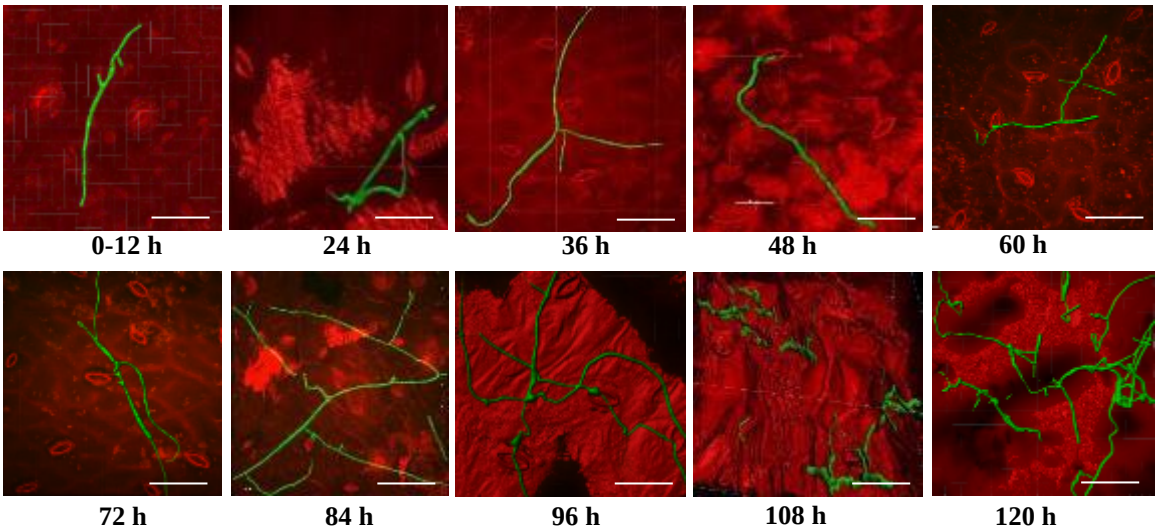

Figure 3.5. The growth and development of *C. beticola* before and after penetration on to susceptible variety (20-µm scale bar).

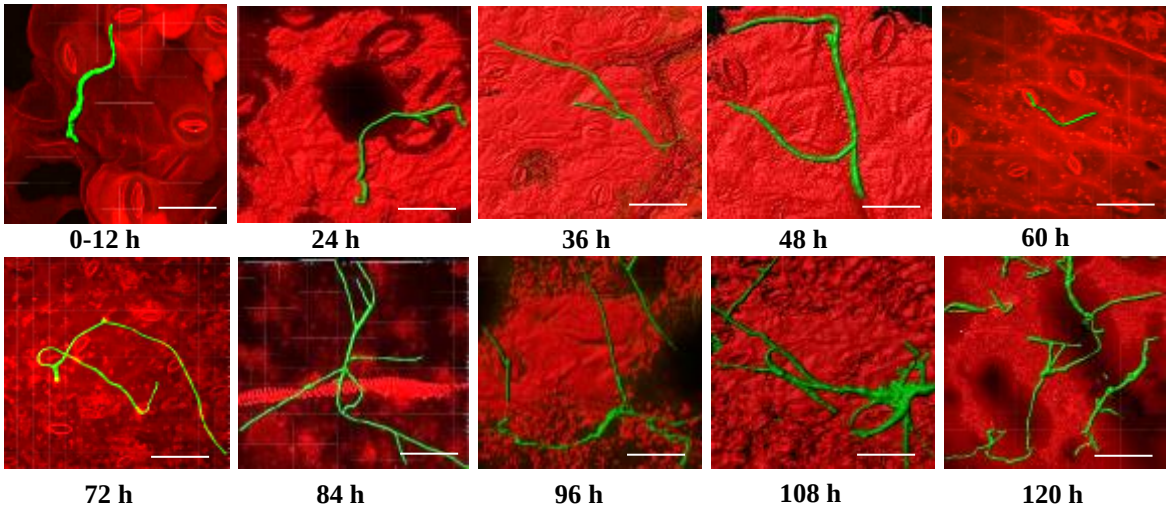

Figure 3.6. The progress of *C. beticola* on resistant variety from 0 hour to 120 hours was used for imaging under confocal microscopy (20-µm scale bar).

3.4.4. Evaluation of Fungal Biomass

A significant difference was recorded in the susceptible and tolerant cultivars in response to fungal accumulation and progress over time. The volume of fungal tissue produced 120 dpi within leaf tissues of the susceptible variety was 1.7 times higher ($P=0.01$) than the volume produced in the resistant variety (Table 3.2; Fig. 3.8 c-d). *C. beticola* grew 1.6 times faster ($P< 0.01$) in leaves of the susceptible variety than in the resistant variety (Table 3.2).

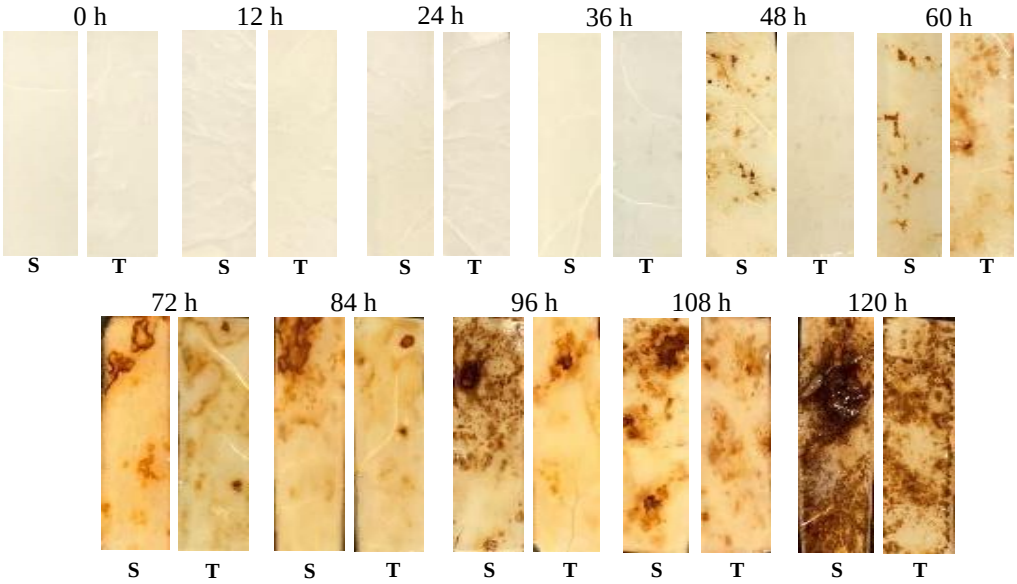

Figure 3.7. DAB staining of *C. beticola* inoculated leaf at different time points starting from 0 hour (non-inoculated check) to 120-hour post inoculation in susceptible and resistant variety at 12 hours interval. Here, h=hour, S=susceptible, R=Resistant varieties, respectively.

Table 3.2. Reaction of a susceptible and a resistant sugar beet variety to infection by *C. beticola* expressed as percentage of cell death surrounding infection points and fungal biomass measured 120 hours post inoculation.

Cultivar	Cell mortality		Fungal volume		
	Cell death (%)	AUMPC	Volume (μm^3)	Rate of volume increase (μm^3 h^{-1})	99% Confidence interval for rate
Susceptible	24.5 a	609 a	502 a	6.82	5.90 – 7.75
Tolerant	7.5 b	228 b	301 b	4.30	3.71 – 4.88

[1]AUMPC=area under the cell mortality progress curve. Values in each column sharing similar letters are statistically similar to each other at $P = 0.01$.

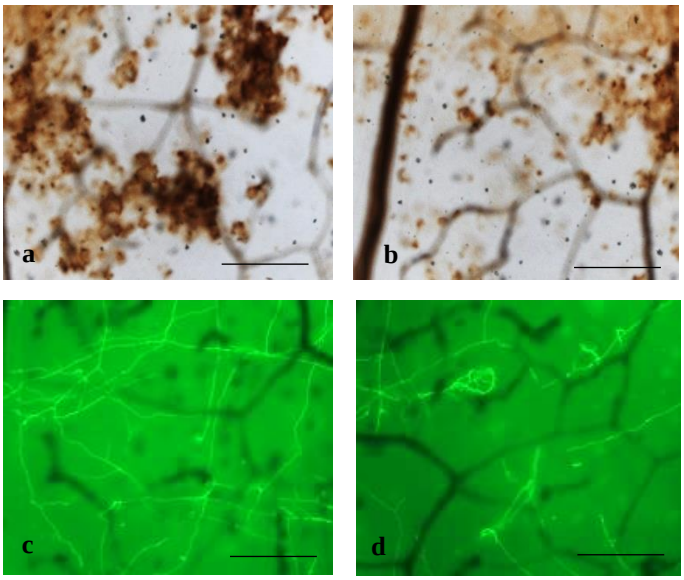

Figure 3.8. Evaluation of cell death and fungal biomass accumulation in sugar beet leaf. DAB staining of leaf samples in susceptible (a) and resistant (b) variety showing dark brown to black cell death at 120-hpi. Quantification of fungal bio-volume in susceptible (c) and resistant (d) varieties was calculated for each time point. Scale bar = 100 μm for Fig. a, b and 20 μm for Fig. c and d.

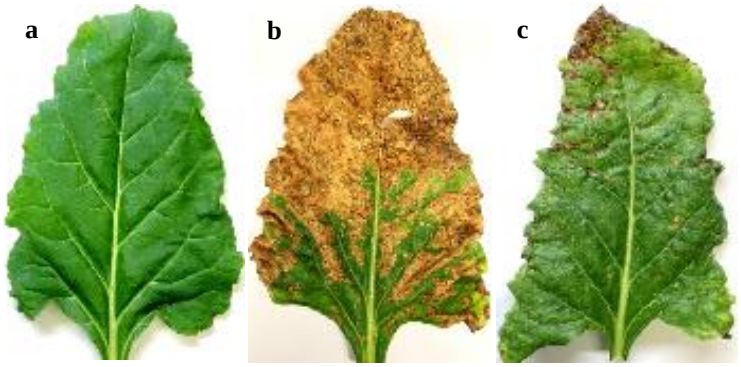

Figure 3.9. The non-inoculated check had no symptoms (a), disease severity of severity on the susceptible variety (b), and in the resistant variety were evaluated using 1-10 CLS rating scale.

3.5. Discussion

In order to understand and promote the resistance breeding progress, precise and reliable disease severity rating is very important (Montes et al., 2007; Bock et al., 2010). To assess the disease severity of CLS in sugar beet, the average percent of infected leaf area used used (Rossi et al., 1999; Wolf and Verreet 2002). In addition, disease severity was measured in replicated trials and characterized using temporal models and AUDPC. The genetic basis of disease resistance is very often complex (Lewellen and Whitney, 1976; Whitney and Lewellen,

1976; Koch et al., 2000). Partial resistance is quantitatively inherited and capable of limiting the rate of disease development (Rossi et al. 1999; Smith and Gaskill 1970; Schmittgen 2014). The quantitative host resistant reduces the chance of penetration, colonization and sporulation which play a significant role in suppressing the pathogen spread and the development of epidemics (Rivero do Vale et al., 2001; Bleiholder et al., 1972; Parlevliet, 1979). In this study, *C. beticola* enter leaf tissues of the susceptible variety 12 hours earlier; however, the amount of successful penetrations could not be quantified. Nevertheless, the growth of the pathogen in infected tissues of the susceptible variety was almost 1.7 times greater than that in the resistant variety. Further, when disease development in greenhouse was compared, CLS severity increased at a rate that was 1.3 times greater in the susceptible variety.

The cuticular components play a significant role in sensing fungal recognition (Gniwotta et al., 2005; Nielsen et al., 2000) and it has been reported that resistant sugar beet plants have thicker wax (Tsuba et al., 2002). A wet interface is needed for conidia to germinate (Gniwotta et al., 2005) but penetration of the stomata by the germ tubes is not influenced by the humidity (Vestal, 1933). The progress of conidia on stomata requires temperature and relative humidity of at least 15^0C and >60%, respectively (Solel and Minz 1972; Weiland and Koch 2004; Rees et al., 2007; Jacobsen and Franc 2009). In this study, inoculated plants were kept at 25 ^{o}C and during the first five days from inoculation the relative humidity was >90%. Occasional or enhanced penetration of hyphae through stomata is influenced by hydrotropism depending on constant saturated humidity or interrupted wetting (Rathaih, 1979). While light and darkness periods influence stomatal opening. Light has been reported that biotrophic fungi, like *Puccinia* spp. does not require light induced stomatal opening as these fungi can penetrate stomata in dark. However, the hemi-biotrophic *Cercospora zeae-maydis* is unable to develop appressorium on stomata in complete darkness (Kim et al., 2011). The WGA fluorescent staining method and confocal microscopy have been widely used and become very popular to

visualize, characterize the early spatial and temporal development of fungal structures in other host-plant systems, and keeping samples integrity while preparing slides for microscopy (Aylife et al., 2011; Diagne et al., 2013; Dugyala et al., 2015; Minker et al., 2018; Dickson and Kolesik 1999; Solanki et al., 2019). We have successfully used the protocols for investigating *C. beticola* infection mechanism into sugar beet the sugar beet plants and documented the fungal structures and progress over time. In our study, sugar beet leaves were incubated for five days under 14/10 h photoperiod daily. Decreased spore production is associated with quantitative disease resistance which interfere the disease epidemics in sugar beet. Besides, reduced lesion size is compromised due to higher tolerance to pathotoxin in resistant variety. Lucker et al. (2016) documented that lesion size and number of conidia were lower in resistant cultivar hence resulted in reduced disease severity.

Duration of development of CLS necrotic lesions varied with host genotype. We documented the biotrophic phase lasting for 5 to 6 days followed by formation of leaf spot in the susceptible and resistant varieties. *C. beticola* germ tubes grew and started entering through stomata at 48-hpi in the susceptible host while at 60-hpi, germ tubes were observed entering the stomata in the resistant variety. Fungal entry to mesophyll tissue occurred by direct conidial penetration through stomata and/or by the formation of appressoria onto the stomata in both cultivars. Fungal tropism towards the stomata did not follow any particular patterns and hyphal strands approached or went over the stomata without any penetration. Our findings on conidia germination, hyphal growth on the leaf surface, penetration, and the final development of leaf necrosis are similar to previous reports (Schmidt et al., 2008; Feindt et al., 1981, Weltmeier et al., 2011; Rossi et al., 2000; Lartey et al., 2007; Schmittgen, 2014). Barley-*Cochliobolus sativus* and peanut-*Cercospora arachidicola* interaction also resulted in varying response based on lesion size (Fetch and Steffenson 1999; Ricker et al., 1985).

Our findings on temporal rate of CLS disease development are similar to previous reports (Oerke et al., 2019; Leuker et al., 2015). The fungal entry and subsequent growth and its requirement of light are varied and highly depend on the physiology of pathogens and host-pathogen interactions. The susceptible and resistant cultivars showed distinct variation in fungal biomass accumulation at different time point post penetration. Microscopic observation and quantification of fungal biomass growing intercellularly was higher in the susceptible cultivar than the resistant cultivar. The resistant cultivar exhibited an approximately 60% reduction in fungal biomass compared with that observed in the susceptible cultivar, which concurred with the previous research (Weltmeier et al., 2011; Whitney and Lewellen, 1976). Leaf orientation, age, stomatal opening and number can regulate the intensity of successful penetration and disease severity (Feindt et al., 1981). We investigated the stomatal distribution and *C. beticola* inoculation to lower and upper surface of the sugar beet leaves. It revealed that there were no significant differences on stomatal numbers and disease severity in susceptible and resistant variety (data unpublished). It is assumed that subcellular growth and development of *C. beticola* in the resistant variety was repressed due to host basal resistance. Arens et al., (2016) reported that glucosylvitexin accumulation in resistant variety acts as a protectant. Niks and Kuiper (1983) reported that partially resistant barley showed reduced infection success and impaired growth and development of rust colonies. A number of genes regulates fungal recognition, cell signaling, and defense-related protein in quantitative resistance against *C. beticola* (Gottschalk et al., 1998; Nielsen et al., 1993).

In this research, we were able to determine the actual time of penetration of the fungus and post-penetration of mesophyll tissue. Accordingly, the resistant plants showed less cell death, and the disease severity was evaluated lesser than the susceptible host, although the progress of cell death and lesser disease severity was gradually increased in both varieties over time until final evaluation. We documented the proliferation success of *C. beticola* on sugar

beet leaves at early stage of penetration and explored the varietal response to fungal infection, which was associated with disease severity, fungal biomass accumulation, and percent cell death at different time points post inoculation. Temporal rate of disease development and infection mechanism were documented in for susceptible and resistant varieties in sugar beet plants. We investigated and documented a comparative the progression infection of *C. beticola* and disease development in susceptible and resistant varieties. The potential of this novel approach for fungal infection during early stage of penetration could be useful for understanding disease epidemiology, phenotyping and quantification of fungal growth in the other host-pathogen systems. Additionally, sugar beet-*C. beticola* interactions will add a new dimension to future research on pathogenicity genes involved at the early stage of host-pathogen interaction.

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4. COMPARISON OF CROWN AND ROOT INOCULATION METHOD FOR EVALUATING THE REACTION OF SUGAR BEET CULTIVARS TO *RHIZOCTONIA SOLANI* AG 2-2 IIIB

4.1. Abstract

Sugar beet (*Beta vulgaris* L.) is the second major economically important sugar-yielding crops in the world and the US ranked third in world sugar beet production. Sugar beet crown rot and root rot diseases caused by *Rhizoctonia solani* (Khün) are a serious threat for sugar beet production and processing. Prior to the adoption of Roundup Ready® sugar beet (RRSB) cultivars, crown rot was a serious problem caused by mechanical tillage operations required for weed control. Following the introduction and large-scale cultivation of RRSB, however, crown rot was reduced but root rot became severe. This necessitated reassessment of screening methods for development of *Rhizoctonia* resistant cultivars. In this study we evaluated two inoculation methods, *viz*. crown inoculation and root inoculation methods for development of Rhizoctonia root rot and assessed their efficacy to differentiate the reactions of sugar beet cultivars. The results of this study demonstrated that the root inoculation method is optimal for consistent disease rating of the germplasms in the greenhouse. It was concluded that the use of root inoculation method is convenient and accurate for screening of RRSB cultivars in a resistance breeding program.

4.2. Introduction

Sugar beet (*Beta vulgaris* L.) is one of the two most economically important sugar-yielding crops in the world. The United States (US) ranks 3rd in the world sugar beet production. The sugar beet industry of North Dakota (ND) and Minnesota (MN) contribute 57% of the total US sugar beet production and it is valued at approximately $5 billion (Khan, 2018; ISO 2020; USDA-ERS, 2021). In the US, about 8.4 million metric tones of sugar are produced from sugar beet, which represents 55% of the total domestic sugar production (UDSA-ERS, 2021). Sugar

beet crown and root rot is a serious fungal disease caused by *Rhizoctonia solani* (Khün) in the United States and Europe (Franc et al., 2001; Buddemeyer and Märländer, 2004; Buhre et al., 2009). This disease is a serious threat for sugar beet growers and processors in all U.S. sugar beet growing states including California, Colorado, Idaho, Michigan, Minnesota, Montana, Nebraska, North Dakota, Washington, and Wyoming (Stump et al., 2004; Kirk et al., 2008; Strausbaugh et al., 2011). This pathogen, which is known to have a wide host range of plant species (Anderson, 1982; Salazar et al., 2000), exists primarily as mycelia in organic debris, survives for a long time in the soil through resting spores called sclerotia, and forms sexual spores rarely (Abawi et al., 1986; Harveson et al., 2009).

R. solani presents remarkable genetic variations and comprises 13-different anastomosis groups (AGs) and one bridging group, which are genetically distinctive populations. Each AG can be subdivided further into intra specific groups (Ogoshi, 1987, 1996; Cubeta and Vilgalys, 1997; Sharon et al., 2006). Isolates from AG 1, AG 2-2, and AG 4 affect early season plant population; however, isolates from AG 2-2 IIIB and AG 2-2 IV are genetically divergent and have a wider host range than originally reported (Windels and Brantner, 2007; Nehar and Gallian, 2011; Bolton et al., 2010; Strausbaugh et al., 2011; Wibberg et al., 2016). The isolates from these two groups are mostly responsible for crown and root rot of sugar beet in ND and MN. Crown rot infection is evident on sugar beet crown and occurs at or immediately below the soil line. It produces brown to blackish, sunken, and circular lesions that can coalesce to cover large areas on the root surface (Whitney and Duffus, 1986; Asher and Hanson, 2006). The disease sometimes occurs in patchy patterns in the field (Anees et al., 2010); however, continuous cultivation of susceptible sugar beet varieties and presence of significant inoculum potentiality often result in the loss of entire fields to the disease, which is favored by warm and moist environmental conditions (Khan and Bolton, 2016).

Annual yield loss due to Rhizoctonia root and crown rot is estimated at 30 to 60%. Sometimes the entire crop could be affected in the field and subsequently becomes a major concern for storage in the piles (Ruppel et al., 1979; Windels et al., 2009; USDA-ERS, 2017; Khan, 2018). Growers are recommended not to harvest sugar beet fields having more than 50% incidence since the affected beets cannot be stored safely in the storage piles (Khan et al., 2010). Close rotation and expansion of edible beans and soybeans into sugar beet growing areas can enhance the establishment of *R. solani* population in these fields and thus compound the risk of crown and root rot over time (Brantner and Windels, 2007). Prior to the adoption of Roundup Ready® sugar beet (RRSB) cultivars, crown rot was considered a serious problem when conventional sugar beet cultivation was mainstream in which several mechanical tillage operations were required for weed control. These mechanical weed control operations resulted in inoculum from infested soil being deposited on the crown of plants. Hence, weed management by avoiding covering plants with soil debris during cultivation was highly encouraged (Schneider et al., 1982; Harveson et al., 2002). Downpour rainfall can also spread infected soils into the crown causing infection (Khan, 2012).

RRSB cultivars are genetically engineered to be tolerant to the herbicide glyphosate. Since the deregulation in 2005, RRSB cultivars have been widely commercialized in the US and by 2010, about 98 to 100% of the area planted to sugar beet in the country was drilled with RRSB cultivars. The introduction of RRSB cultivars markedly reduced the frequency of weed control and its associated involuntary mechanical crown inoculation (Khan, 2014; Morishita, 2016; APHIS, 2020). Thus, the incidence of crown rot decreased. However, the incidence of sugar beet root rot has increased in commercial Rhizoctonia-resistant RRSB varieties. This necessitated reevaluation of possible environmental factors favoring root rots as well as of Rhizoctonia resistance screening methods commonly used by commercial varieties developers. Moreover, it is very hard to assign disease ratings, e.g., resistant, or moderately resistant, to

sugar beet germplasm evaluated under field conditions because of the unpredictable and non-uniform environments that result in patchy patterns of the disease and inconsistent reactions from one year or location to the next. The identification of a inoculation protocol that allows consistent response of germplasm to Rhizoctonia crown and root rot is needed.

The identification of an appropriate inoculation method is paramount. Since 1960s, crown inoculation of sugar beet was the inoculation method of choice to assess the varietal resistance to crown and root rot (Pierson and Gaskill, 1961; Gaskill, 1968, Behn et al., 2012; Buttner et al., 2004; Wigg and Goldman, 2020). This technique was replaced at the end of the 1990s by root inoculation methods (Engelkes and Windels, 1996; Scholten et al., 2001; Vagher et al., 2014; Inokuti et al., 2019; Wigg and Goldman, 2020); however, a comparison of both methods was never made. Independently of the plant part where the inoculum is placed, however, the final level of infection has been affected by location of the trials, level of resistance of the host, and environmental conditions prevalent during the study (Behn et al., 2012; Liu et al., 2019; Brantner and Chanda, 2020). Further, it has been reported that the type of inoculum used, e.g., mycelium suspension, mycelium-colonized pearl millet or mycelium-colonized barley grains, could evoke different levels of response to either crown or root inoculation (Buttner et al., 2004; Behn et al., 2012; Rajabi et al., 2012; Wigg and Goldman, 2020). The availability of these methods provides the opportunity to compare them and thus develop an inoculation protocol that consistently results in high disease pressure. As the initial step towards this protocol, we intend to compare the efficacy of crown and root inoculation methods on expression of disease of germplasm with under greenhouse conditions.

4.3. Materials and Methods

4.3.1. Sugar Beet Varieties

Seven commercial sugar beet cultivars, M 504, H-4302, C-101, C-467, B-86, B-85, and B-80 with varying levels of resistance to *R. solani* crown and root rot of sugar beet were used

(Table 4.1). Fungicide coatings on seed samples from these cultivars were removed by soaking and washing them in distilled water followed by surface disinfestation with 70% ethanol. Then seeds were rinsed thrice with autoclaved water and air dried. Three seeds from each cultivar were sown in each pot and ten days later the pots were thinned down to a single vigorous plant.

4.3.2. Experiment Location and Conditions

Research was conducted at the Agricultural Experiment Station (AES) Greenhouse of North Dakota State University, Fargo, ND, USA in 2020. In the greenhouse, plants were grown in plastic pots measuring 10x7x12 cm (T.O. Plastics Inc., Clearwater, MN, U.S.A.), filled with peat mix (Sunshine mix 1, Sun Gro Horticulture Ltd.; Alberta, Canada). At planting time, 20 g of slow-release fertilizer 15-9-12 (N-P-K) (Osmocote, Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) was added to each pot. The greenhouse room was set to maintain 12 h photoperiod at 25 ± 2°C (Argus Control Systems Ltd.; British Columbia, Canada). Plants were regularly watered and monitored until they were subjected to inoculation.

4.3.3. Preparation of Inoculum

The *R. solani* AG 2-2 IIIB isolate MKBH-1 was grown for 10-days on potato dextrose agar (PDA) medium (Sigma Aldrich, MO, USA) amended with streptomycin sulphate at 200 mg/L (Fig. 4.1 a). This isolate was previously obtained from sugar beet plants collected from ND in 2019 and hyphal tip purified. Sterilized barley grains were inoculated with mycelial plugs of *R. solani* pure culture and incubated as described by Noor and Khan (2014) (Fig. 4.1 b).

4.3.4. Crown and Root Inoculation and Evaluation of Disease Severity

Delivery of inoculum to infect plants was performed by both crown and root inoculation methods. Plants were inoculated at 4-leaf growth stage. For root inoculation, a hole was made 2.5 cm (1 inch) below the soil line and 2.5 cm to the side of the taproot, a single colonized barley grain was dropped, and the hole was covered with the peat mix. For crown inoculation

method, colonized barley grain was placed on the sugar beet crown and covered with a fine layer of greenhouse soil to avoid inoculum displacement during the experiment. For each inoculation method, four plants of each cultivar were inoculated with colonized barley grain in each replication. Also, four plants were inoculated with sterile, non-colonized barley grains to make sure the barley grains alone would not result in symptoms like the ones caused by the pathogen. Individual plants were evaluated for disease severity 28-days post inoculation (dpi). For the evaluation, roots were carefully pulled by hand and washed under tap water. A categorical severity scale from 0 to 7 was used (Torres et al., 2016). In this scale, 0 = no visible disease symptoms, 1 = 1-5% root surface with visible lesions, 2 = 6-10 % root surface with visible lesions, 3 = 11- 25% root infection, 4 = 26 - 50% root infection, 5 = 51 - 75% root infection, 6 = > 75% root infection, and 7 = entire root completely deteriorated or dead plant (Fig. 4.2).

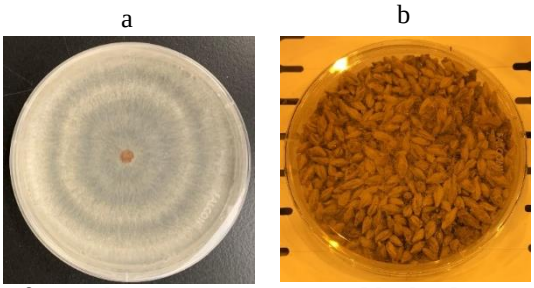

Figure 4.1. a) Pure culture of *R. solani* AG 2-2IIIB, and b) barley inoculum prepared following Noor and Khan (2014).

Figure 4.2. Typical root rot symptoms scoring was evaluated using 0-7 scale.

4.3.5. Data Analyses

The greenhouse experiment layout was a completely randomized design (CRD) with factorial arrangement. A treatment consisted of either crown or root inoculation of one of the seven cultivars and each treatment had four replications. The same experiment was conducted one more time. The Shapiro-Wilk test showed that the disease scores from the two experiments were not normally distributed ($p < 0.01$). This, combined with the fact that severity data was categorical, prompted us to use non-parametric statistics to analyze the disease score data. To determine whether the variances of both trials were homogeneous and could be combined for analysis, the non-parametric Levene's test was conducted (Nordstokke et al., 2011). This nonparametric method uses disease severity score ranks calculated by the rank procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) instead of means or medians to compare variances. Data collected from plants inoculated with non-colonized barley grains was not included in the analyses. An analysis of variance of the ranks was conducted using the generalized linear mixed model procedure (Proc Glimmix) of SAS. Treatment's least square mean (lsmeans) ranks were compared using the Tukey test with P=0.05. In addition, the relative treatment effects of root rot disease severity for each treatment and their 95% confidence intervals were estimated and compared using Brunner's LD_CI SAS macro as described by Shah and Madden (2004). Briefly, if R_{ik} is the rank among all observations (N), the mean rank for the ith treatment combination was determined as:

$$R_{i.} = \frac{1}{n_i}\sum_{k}^{n_i} R_{ik}$$

The dot subscript indicates the mean rank over all the four replications (n_i) for the ith treatment combination with the rank position (k). The relative treatment effect of the ith treatment combination was estimated from the mean rank as:

$$RE_i = \frac{1}{N}\left\{R_{i.} - \frac{1}{2}\right\}$$

4.6. Results

All plants in the seven cultivars inoculated with colonized barley grains through both crowns and roots showed typical crown and root rot symptoms from 0 to 7 scales (Torres et al., 2016) while plants inoculated with non-colonized barley seeds did not develop symptoms (Fig. 4.2 and 4.3). Therefore, data from these treatments were excluded from data analyses. The non-parametric Levene's test indicated the variances of the trials were statistically similar to each other ($P = 0.2436$) and therefore the trials were combined for analyses. The combined analysis of variance revealed the effect of inoculation methods was significant ($P < 0.0001$) but that of cultivars and their interaction with inoculation methods were not ($P = 0.3190$ and 0.4023, respectively). Overall, treatments inoculated using the crown method had an lsmean rank of 15 compared to 42 of treatments inoculated using the root method. The 28 dpi were long enough to allow development of severe root rot symptoms that facilitated varietal characterization and evaluation of the differences between root and crown inoculation methods. In general, cultivars infected using the crown inoculation method developed significantly ($P < 0.05$) less disease severity than those inoculated using the root inoculation method (Table 3.1, Fig. 4.4). However, there were no significant differences ($P = 0.05$) in severity among cultivars inoculated using the same method (Fig. 4.4). In general, the median disease severity of crown-inoculated plants was 3, which is equivalent to no more than 25% of root surface tissues affected, except for cultivar B-80 which had a median severity of 3.5 which has a range of 26 to 50% of root damage. On the other hand, the median disease severity of root-inoculated plants was 6, which equates to more than 75% of root surface being affected (Table 4.1).

Figure 4.3. Phenotypic variations of disease symptoms of seven sugar beet cultivars inoculated by crown and root inoculation method where non-inoculated check having healthy roots without any visual symptoms 28-dpi under greenhouse conditions.

Neither of the inoculation methods evaluated in this study were completely in agreement with what seed companies consider their cultivars' reaction to the disease are. The crown inoculation method suggested that H-4302, B-86, and C-101 were resistant (median reaction ranging between 2 and 2.5) while the seed companies considered the latter two to be susceptible (Table 4.1). On the other hand, however, the root inoculation method considered all materials as susceptible.

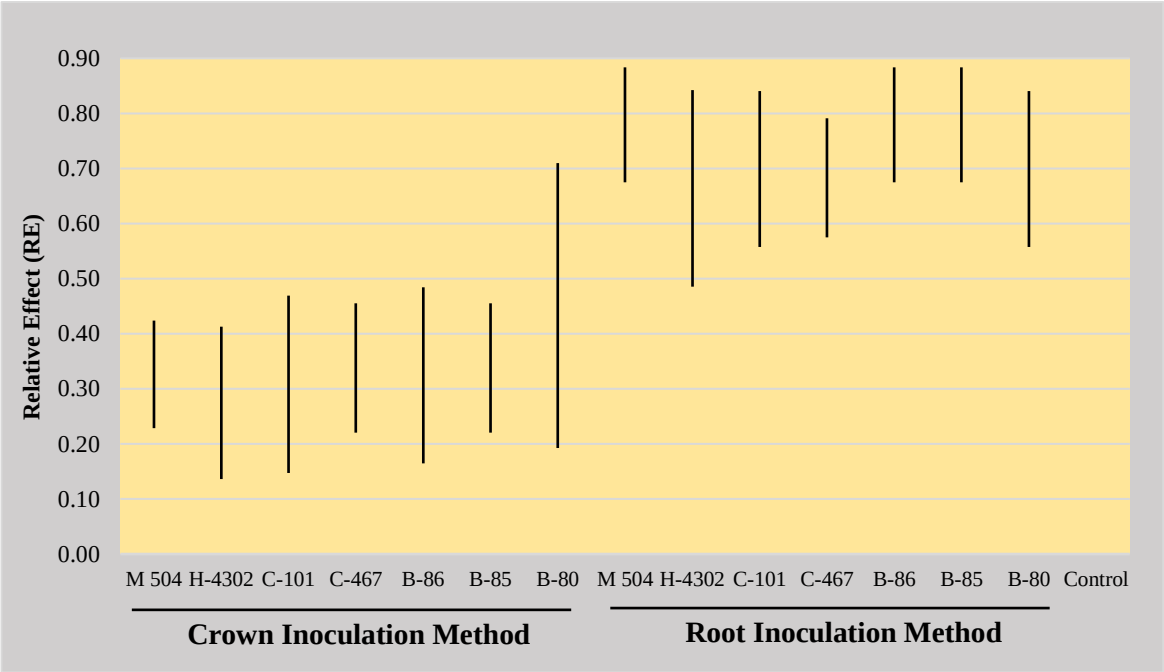

Figure 4.4. Effect of crown and root inoculation methods on the disease severity of seven sugar beet cultivars were measured by the relative effect (RE) (Solid circle). The bars represent 95% confidence interval of the estimated relative effect.

Table 4.1. Effect of crown and root inoculation methods on response of seven sugar beet cultivars to *Rhizoctonia solani* AG 2-2IIIB in greenhouse condition.

	Disease severity (1-7)[1]				Cultivar appraisal by seed companies	
	Median		Least square mean rank[2]			
Cultivars	Crown	Root	Crown	Root	Rating	Reaction[3]
M-504	3.0	7.0	14 b	43 a	4.5	S
H-4302	2.0	6.5	9 b	40 a	3.6	R
C-101	2.5	6.5	13 b	39 a	4.8	S
C-467	3.0	6.0	17 b	41 a	4.4	MR
B-86	2.5	7.0	15 b	45 a	4.7	S
B-85	3.0	7.0	18 b	44 a	4.5	S
B-80	3.5	6.5	22 b	40 a	4.3	MR
Main effect[4]	3.0	6.0	15 b	42 a	-	-

[1] Plants inoculated at 4-leaf growth stage with barley grains colonized with *R. solani* AG2-2 IIIB mycelia. Disease severity assessed 28 days post inoculation following Torres et al., (2016) where 0= no visible disease symptoms, 1=1-5% root surface with visible lesions, 2=6-10 % root surface with visible lesions, 3=11-25% root infection, 4=26-50% root infection, 5=51-75% root infection, 6= greater than 75% root infection, and 7= root completely deteriorated or dead plant.

[2] Least square mean ranks with different letters in both columns are statistically different according to Tukey means separation test ($P = 0.05$).

[3] S=susceptible, MR=moderately resistant and R=resistant.

[4] Main effect= median severity across two trials and four replications per trial; least square mean ranks with different letters are statistically different from each other according to Tukey mean separation test with $P = 0.05$; "-" = no analysis was conducted.

4.5. Discussion

We assessed and compared crown and root inoculation method to evaluate the resistance rating of sugar beet cultivars against *R. solani* AG 2-2 IIIB in greenhouse conditions. The use of effective inoculum, and appropriate experimental conditions and methods of inoculation are considered necessary for proper and dependable evaluation of the reaction of germplasm to a disease (Dhingra and Sinclair, 1995). Greenhouse trials are expected to produce severe and uniform disease infection when using effective inoculum (Buttner et al., 2004) while field trials may produce variable pressure due to the patchy patterns of *R. solani* crown and root rot incidence and unpredictable environmental conditions making the former a better alternative (Ruppel et al., 1979; Van Brüggen et al., 1996). Under greenhouse conditions, the root inoculation method could be considered a better alternative than crown inoculation because it does not require extra care to keep the inoculum from drying (Panella and Hanson, 2001; Buttner et al., 2004; Behn, 2012). Although both crown and root inoculation methods take almost same amount of time, drying and displacement of inoculum from the crown region could be a disadvantage both in greenhouse and field conditions. In fact, the root inoculation method was verified as more strigent by causing significantly higher root rot severity scores than the crown inoculation method within the same given time period. Previouly, it was reported that inoculum placement around or in contact with the root at 2-2.5 cm depth provided favorable conditions for disease development (Gaskill, 1968; Engelkes and Windels, 1994; Inokuti et al., 2019). Moreover, in a greenhouse trial the researcher does not need to wait for plants to mature to make evaluation; this saves time.

Taken together, artificially prepared *Rhizoctonia* inoculum on barley is highly effective for both crown and root inoculation, as it casued obvious symptoms in all inoculated plants. The discrepancy between the reaction of cultivars in the trial and the seed companies' assessment may be due in part to disease escape. In greenhouse trials, escaping infection is less likely to occur since each plant was exposed to similar amounts of inoculum and incubated under a disease-conducive environment. In a way, ratings obtained under controlled conditions may reflect more accurately the true reaction of the cultivars to the disease. In contrast, under field conditions the inoculum distribution and its concentration through the soil may be less uniform (Anees et al., 2010). In addition, seed companies base their assessments on data collected in multiple years and locations with a broad range of environments. Their assessment may reflect what farmers might see rather than the true reaction of the cultivar to the disease. Considering this, it is logical to assume that when searching for germplasm resistant to *R. solani*, researchers should use an inoculation method that allows for the expression of the true nature of the germplasm reaction to the disease. We conclude that the preferred protocol for screening germplasm for resistance to Rhizoctonia root and crown rot should be colonized barley seeds deposited to the side of roots rather than on crown, followed by evaluation of disease reaction 28 days after inoculation.

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5. IDENTIFICATION AND CHACTERIZATION OF *Sclerotinia sclerotiorum,* CAUSAL AGNET FOR ROOT ROT AND NECROSIS IN SUGAR BEET

5.1. Abstract

Sugar beet is major sugar yielding crop in the US states of North Dakota and Minnesota. Sugar beet root samples collected from Moorhead, MN had rot symptoms along with whitish mycelia growth and blackish sclerotia on their external surface of root. Pure cultures were obtained from infected roots through planting method on PDA media. *S. sclerotiorum* was identified based on morphological features and furthered confirmed molecularly by sequencing of the Internal Transcribed Spacer (ITS) region and matching homology with reported ITS of the fungus. Pathogenicity of *S. sclerotiorum* was confirmed through mycelial inoculation of seeds and roots in laboratory and greenhouse conditions. Inoculated seeds showed a range of symptoms that included pre- and post-emergence damping off, wilting, black discoloration of root, constricted collar region and stunted seedling growth. In laboratory conditions, roots were artificially wounded using a cork borer and inoculated by mycelial plug. This resulted in noticeable root decay and growth of whitish, cottony mycelia and sclerotia externally. Transverse sections of the diseased root showed brown to black discoloration and rotting of internal tissue. Root inoculation of 4-weeks old sugar beet plants was achieved by depositing colonized barley grains in a greenhouse, resulting in brown to black lesions and necrosis of root tissue when evaluated at 28 days post inoculation. *S. sclerotiorum* was isolated from inoculated roots showing infection and was found identical to pure isolates recovered from the field samples. These findings could be useful for sugar beet growers in Minnesota, allowing better management of this pathogen in field and storage conditions before its widespread future occurrence.

5.2. Introduction

Root rot diseases are considered the most important limiting factor for sugar beet production worldwide. Root rot disease of sugar beet caused by *R. solani*, *Aphanomyces* sp., *Fusarium* sp., *Pythium* sp., *Phytophthora* sp., *Rhizopus* sp. etc. results in significant yield losses every year (Berkeley, 1994; Jacobson, 2006; Khan, 2017). *Sclerotinia sclerotiorum* (Lib.) deBary, is an important fungal pathogen that affects many broad leaf plant species. *S. sclerotiorum* causes significant yield losses to a wide range of crops including sugar beet (Boland and Hall, 1994; Purdy, 1979). This pathogen is cosmopolitan and is prevalent in many US states including those in the North Central region (Bradley and Lamey, 2005). *S. sclerotiorum* is responsible for more than US$ 200 million loss annually in the United States (USDA, 2005). Moreover, North Dakota and Minnesota are two leading sugar beet growing states that produce 57% of the US sugar beet, which results in over $5 billion in total economic activity. This fungus form sclerotia, a hard bodied, black colored resting spore at adverse environmental condition, which can survive for many years in nature (Adams and Ayers, 1979; Bell and Wheeler, 1986).

Sclerotia and ascospores can serve as primary inoculum for epidemics caused by *S. sclerotiorum.* Under favorable conditions, apothecia developed from sclerotia (Adams and Ayers, 1979) and which then produces ascospores (sexual spores) which are considered as the primary source of inoculum in many pathosystems (Abawi and Gorgan, 1979; Willets and Wong, 1980). Ascospores require a film of water to germinate (Shahoveisi and del Río Mendoza, 2020; Bolton et al., 2006). Senescing flower parts serve as the primary source of nutrients for ascospores as they fall on leaves, petioles or stem (Inglis and Boland, 1990; Turkington and Morrall, 1993). Infection is favored by cool to moderate daily temperatures with a maximum < 85 ^{0}F or 29 ^{0}C, and a daily average of 9 or more hours of leaf wetness (Shahoveisi and del Río Mendoza, 2020; Workneh and Yang, 2000). *S. sclerotiorum* causes a wide range of symptoms including stem rot, leaf blight, head rot, stalk rot, root decay and

crown rots etc. On roots, symptoms appear as water-soaked lesions that expand and become depressed. As lesions develop, they can girdle the root surface. Whitish cottony mycelia grow on the lesions and develop blackish, hard coated, globose, circular and or variably shaped sclerotia on root externally, which are unique features for morphological identification (Kohn, 1979). At advanced stages of disease, infected tissues become necrotic and disintegrate. Sclerotia may be dislodged by wind or during harvesting and may be distributed vertically in the soil profile by land preparation and irrigation (Brown and Butler, 1936; Cook et al., 1975; Steadman et al., 1975). Root rot symptoms due to *S. sclerotiorum* are very similar to those caused by *Rhizoctonia solani* or *Sclerotium rolfsii* root rot. It has been reported that Sclerotinia leaf blight of sugar beet is becoming an emerging problem for many areas in North Dakota, Minnesota (Khan et al., 2020; Khan et al., 2021) and Montana (M. Khan, Personal communication) in the US. The germinating hyphae from overwintering sclerotia can initiate infection in neighboring plants at the soil line as well as underground portions of the plant. While some Sclerotinia diseases, such as basal stalk of sunflower (Huang and Hoes, 1980; Underwood et al., 2020) showed this type of infection, there is no evidence that *S. sclerotiorum* could infect the roots of sugar beet plants in North Dakota (ND) and Minnesota (MN). In this paper, we investigated the pathogenicity of isolates of *S. sclerotiorum* to seedling emergence and root rot of sugar beets.

5.3. Materials and Methods

5.3.1. Isolation and Identification of *S. sclerotiorum*

During a visit to a sugar beet commercial field in Moorhead MN (46.9190 N, 96.70610 W) in September 2020, the presence of numerous wilting plants was noted. A survey of the field revealed the percentage of affected plants was approximately 5-10%. Affected plants had blighted lower leaves and necrotic lesions near soil line on their taproots (Fig. 5.1 a). Sugar beet root samples with necrotic lesions accompanied by whitish mycelial mats and blackish

sclerotia on the infected root surface were collected from a field and was taken to the NDSU Sugar beet Pathology Laboratory for isolation (Fig. 5.1 b). About 30-40% of infected root samples presented necrotic lesions and presence of sclerotia. The samples were washed with running tap water, immersed in an aqueous solution of 10% sodium hypochlorite for 1 min, rinsed thrice with sterile water, and dried in laminar airflow hood to remove excess moisture from the samples. Root pieces of approximately 5 mm were plated on potato dextrose agar (PDA) media amended with 200 mg/L of streptomycin sulphate and kept in dark for 3 days at 22 ^{0}C (Fig. 5.2).

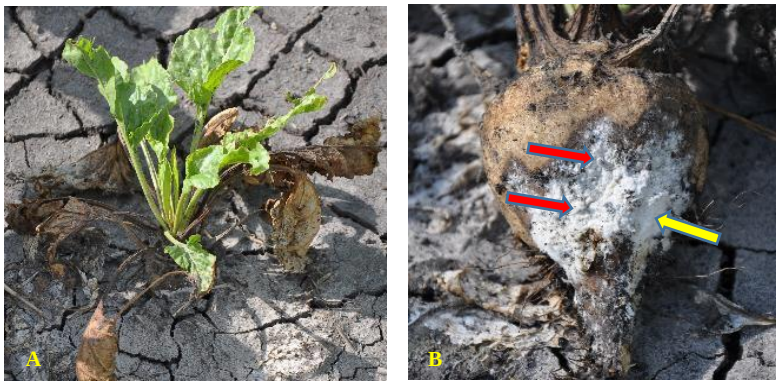

Figure 5.1. Sugar beet plant sample collected from an experimental field at Moorhead, MN which had blighted leaves and root rot symptoms near soil line (A); diseased beet roots presenting brown to black, discolored and necrotic lesions sometimes covered with whitish cottony mycelia (yellow arrow) and blackish sclerotia (red arrows) (B).

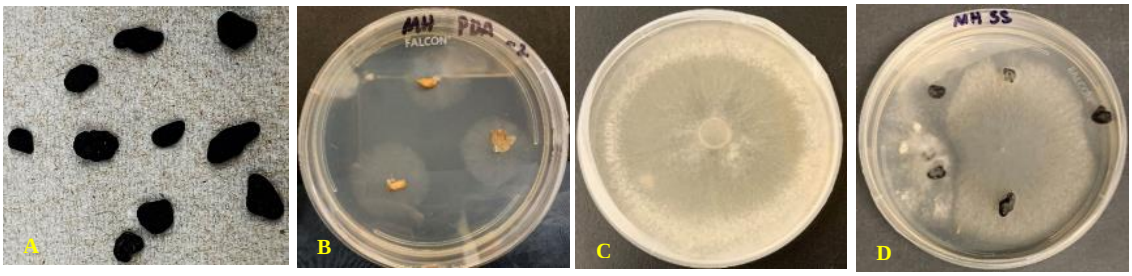

Figure 5.2. Isolation and identification of S. sclerotiourm from the diseased roots. A) Sclerotia recovered from sugar beet roots infected by *S. sclerotiorum*, B) Creamy white mycelia growing from infected sugar beet root tissues onto PDA, C) 5-days old culture of *S. sclerotiorum* showing whitish cottony mycelial growth, and D) 14-days old *S. sclerotiorum* culture showing blackish sclerotia developed on PDA plate.

5.3.2. Seed Inoculation with *S. sclerotiorum* Mycelia

To fulfill Koch's postulates, three commercial sugar beet cultivars, C-572, H-9739 and S-655, were inoculated at planting time using mycelial plugs from the *S. sclerotiorum* isolate, MHSS-1. Since the commercial seeds were coated with fungicides, the seeds were washed with distilled water, surface disinfested in 70% ethanol, and rinsed thrice with autoclaved water. Groups of ten seeds per cultivars were sown in plastic trays (25 x 12 x 10 cm) filled with FLX soilless mix (PRO-MIX, Quakertown, PA) amended with Osmocote (N-P-K: 15-9-12) fertilizer (Scotts Company; Marysville, OH). The trays were arranged in a completely randomized design and the study was conducted twice with 3-replications and 10 seeds per replicate each time (Figure 5.3). A 5-mm diameter agar plug containing actively growing hyphal tips from a 2-3 days old fungal colony were used for seed inoculation. Individual seeds were inoculated by placing one plug to its side in direct contact and covered with the soilless mix. Inoculated pots were incubated in a mist chamber at 25 ^{0}C for five days and then transferred to a greenhouse room. Fourteen days post inoculation, seedling emergence and disease incidence were assessed.

5.3.3. Root Inoculation and Evaluation of Disease Symptoms

The test of pathogenicity by root inoculation was done by two ways: inoculating fungal mycelial agar plug through wound in room condition, and root inoculation with colonized barley grains with fungal mycelia in greenhouse, respectively. Pathogenicity of the causal agent, *S. sclerotiorum* was attained by placing a fully colonized agar plug over a same size wound using a sterile cork borer. Four sugar beet roots (cv. M 504) were inoculated, equal number of roots were inoculated with agar plug without fungal mycelia, serving as a check, and the experiment was repeated. Roots were incubated at room temperature (25 ^{0}C) for 21 days and disease symptoms were evaluated. In the second test, four-week-old sugar beet plant (cv. B-8606) roots were inoculated by artificially prepared colonized barely grains (Fig. 5.5 c).

Individual barley grains were placed at close contact with the root 1-inch deep from the soil line and covered by soil. The barley inoculum preparation method was adapted from Noor and Khan (2014) with slight modifications. Three plants of each variety were inoculated by one colonized barley grain. Equal numbers of plants were considered as non-inoculated checks and the experiment was repeated. The Greenhouse was set to maintain a 14/10 h photoperiod and a 24 h temperature of 25 ± 2°C during the experiment. Plants were regularly watered. Disease symptoms were evaluated at 28-days post inoculation (dpi). Roots were pulled by hand and washed under tap water followed by root rot evaluation.

5.3.4. Molecular Identification

Genomic DNA was extracted from three isolates, MHSS-1, MHSS-2 and MHSS-3 using Qiagen's DNeasy plant kit (Qiagen, Hilden, Germany). The internal transcribed spacer-5.8S rDNA region was amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTAT TGATATGC-3′) primers (White et al., 1990). The samples were sent for Sanger sequencing to Molecular Cloning Laboratories (MCLAB, San Francisco, CA).

5.4. Results

5.4.1. Identification of Pathogens Based on Morphological Features

The fungus developed hyaline, septate, multinucleate, branched, creamy white mycelia grew on PDA media plate (Fig. 5.2). No conidia and conidiophores were produced. Sclerotia was developed at the edge of growing media, which are hard, blackish in color. Sclerotia were measured approximately 4.8 to 5.0 mm in width and 5.5 to 6.3 mm in length. Based on macroscopic and microscopic observation of sclerotia and colony morphology, *S. sclerotiorum* was identified (Kohn, 1979).

5.4.2. Molecular Identification

The nucleotide sequences obtained from the isolates, MHSS-1, MHSS-2, and MHSS-3 showed 100% identity with *S. sclerotiorum* GenBank accessions MT393753, MG516658,

MW696199 and MW375456. The sequence obtained from one of the isolates retrieved from the MN field was deposited in the NCBI GenBank as accession MW786662. To our best knowledge, this the first report of identification of *S. sclertiorum* caused root rot in sugar beet in Moorhead, Minnesota, USA.

5.4.3. Seed Inoculation and Evaluation of Disease Incidence

Seedlings from inoculated seeds showed variable symptoms (Fig. 5.3). Pre-emergence damping off reduced plant emergence by 30 to 40%. Seedlings that emerged showed post-emergence damping off symptoms, with water-soaked lesions at the soil line accompanied by wilting, and wrinkling of cotyledons. These seedlings had thin and black discolored hypocotyl (Figs. 18c and d). All cultivars evaluated were highly susceptible to the disease. The pathogen reduced seedling emergence and infected those that emerged while seedlings developed from non-inoculated seeds were healthy, vigorous and symptomatic (Fig. 5.3 a). Average incidence of symptomatic seedlings was 65, 79, and 75% for the cultivars H-9739, C-572 and S-655, respectively. Similarly, seedling emergence was 98% for S-655, and 80% for other two cultivars. Seedling emergence was reduced about 14% (Fig. 5.4). The fungus was re-isolated from the infected seedling samples on PDA and subsequently the growth of whitish, cottony mycelia and sclerotia were found identical to the inoculated isolates recovered from the field samples.

5.4.4. Root Inoculation and Evaluation of Disease Symptoms

After 21 dpi, inoculated root showed evident root decay (Fig. 5.5) and the growth of cottony mycelia and sclerotia was formed externally (Fig. 5.5 a). Transverse section of inoculated sugar beet roots represented brown to black discoloration and rotting of internal tissues (Fig. 5.5 b). Mycelial growth and sclerotia was also found internally. Roots were inoculated with sterile agar plugs did not show symptoms. All inoculated roots developed

numerous lesions on the root surface and necrosis of root tissues (Fig. 5.5 d) while the non-inoculated roots had clear roots without any symptoms (Fig. 5.5 e).

Figure 5.3. Pre-emergence and post-emergence damping off (B) of sugar beet caused by *S. sclerotiorum* on cultivar H-4302. Asymptomatic seedlings showed healthy hypocotyls (C) while symptomatic seedling showed necrotic lesions on hypocotyls (D). Non-inoculated seeds were healthy and well-developed cotyledons (A). Symptoms observed 14-days post inoculation.

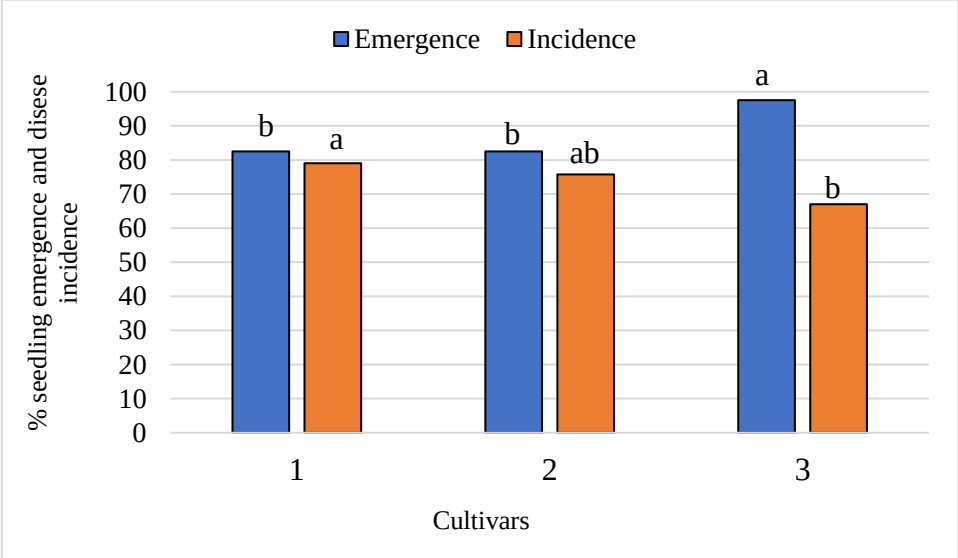

Figure 5.4. Percentage of seedling emergence and damping off incidence of three commercial sugar beet cultivars inoculated with *S. sclerotiorum* mycelial agar plugs at planting. Here, 1= C-572, 2=H-9739 and 3=S-655 sugar beet cultivars, respectively.

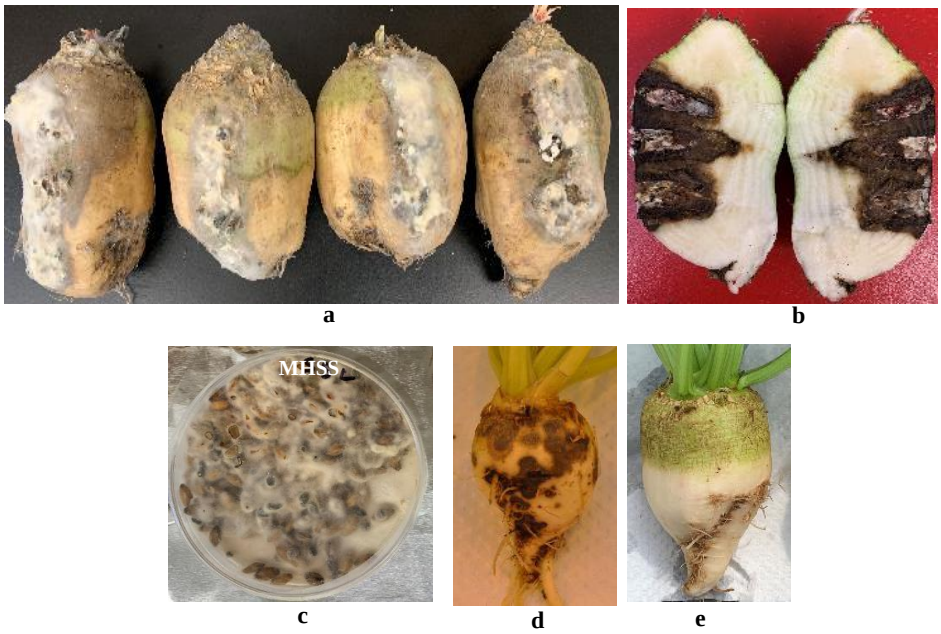

Figure 5.5. Inoculated sugar beet showing characteristic root decay at 21 dpi. (a) whitish cottony growth of mycelia and sclerotia developed on point of inoculation; (b) transverse section of inoculated beet represented brown to black discoloration, rotting of internal tissues and black sclerotia; (c) Root inoculation by artificially prepared barley grains by fungal mycelium at greenhouse; (d) scattered lesions developed on root surface and root rot symptoms when evaluated at 21 dpi; and (e) the non-inoculated root showing no diseased symptoms.

5.5. Discussion

This chapters described the symptomology of Sclerotinia root rot of sugar beet and its pathogenicity to sugar beet seeds and roots in room and greenhouse conditions. This pathogen is capable of causing pre- and post-emergence damping off if its mycelia get in contact with underground plant tissues. Besides, diseased beets in the storage condition may affect significant economic loss. Currently, growers are highly recommended to ingtegrates multiple approaches for Sclerotina management in order to curb serious economic losses for many crops annually. Therefore, more research endeavors are warranted for better understanding of this sugar beet disease and its effective management.

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6. IDENTIFICATION OF *Rhizopus arrhizus* FISHER CAUSING ROOT ROT IN SUGAR BEET IN NORTH DAKOTA AND MINNESOTA, USA

6.1. Abstract

North Dakota (ND) and Minnesota (MN) are two leading sugar beet producing states in US jointly contributing 57% of domestic production, which results in more than $5 billion economic activities. Sugar beet plant samples exhibiting wiltinghat had typical wilting and root rot symptoms were collected from Hickson, ND and Foxhome, MN in 2020. The fungus *Rhizopus arrhizus* (Synonym: *Rhizpus oryzae* Schipper) was isolated from infected tissues cultured on potato dextrose agar media (PDA). The identification of the fungus was done based on morphological features and microscopic visualization, but was further confirmed by DNA sequencing. *R. oryzae* pathogenicity was confirmed through seed and root inoculations in lab and greenhouse. *In-vitro* inoculation of sugar beet seeds with mycelial agar plugs in PDA plates showed reduced seedling growth and emergence. In the greenhouse, seeds inoculated with young growing mycelial agar plug showed seedling rot, wilting and abnormal growth of cotyledons when evaluated at 10 days post inoculation (dpi). Four-weeks old sugar beet plants were inoculated with fungal spore suspension by the dipping method. At 21-dpi, inoculated roots showed characteristics root rot and wilting symptoms. Re-isolation of the fungus was found to be similar to pure culture of the isolates retrieved from the field samples. These findings added a new insight on and better understanding of the fungus in seedlings and root rot of sugar beet. A further plan of action is needed for its management in the effort to prevent crop loss.

6.2. Introduction

R. arrhizus Fischer (synonym: *R. oryzae* Schipper) is a thermophilic fungus, commonly found on rotted plant residues and is common in most agricultural soils throughout the world. *R. arrhizus* and other *Rhizopus* species are known to be storage pathogens causing soft rot of

sugar beet roots and of other plants, e.g. sweet potatoes (Benada et al., 1984; Szymczak-Nowak, 1992; Takada et al., 1998; Holmes and Stange, 2002). Rhizopus root rot caused by *R. arrhizus* and *R. stolonifer* has been reported to occur in Arizona, California, Wyoming and Colorado in the USA and Alberta and Ontario in Canada and in Italy, Iran, France and many countries that made up the former USSR (Jacobson, 2006). In pure culture, *R. arrhizus* appears as creamy white, called mycelium and later becomes gray colored as spore-bearing structures (reproductive structure) called sporangia form. The sporangia are spherically shaped and turned to a blackish color at maturity, appearing like a pinhead and giving the fungal mass a dark appearance. It produces grayish brown spores, which spread through air currents and represents poorly developed rhizoid. *R. arrhizus* is capable of infecting and destroying artificially injured sugar beets most rapidly between 30° and 40 °C, in contrast to a relatively low temperature requirement for the optimum growth of R stolonifera in culture at 24 ^{0}C and with highest infection capability at 14 ^{0}C to 16 ^{0}C (Hilderbrand and Koch, 1943). The *R. arrhizus* and *R. stolonifer* Vuillemin (synonym: *R. nigricans* Ehrenberg) are considered weak pathogens that can damage sugar beet when the beet is stressed by excessive soil moisture, high temperature, poor surface drainage in the field, mechanical damage to the crown or insect damage (Pitt and Hocking, 1985; Vincelli and Burne, 1989). Infected plants show characteristic symptoms of wilting of the foliage. The foliage rapidly wilts and becomes dry and brittle, collapsing on the crown and appearing similar in appearance to plants affected by Rhizoctonia crown and root rot. Diseased root tissues showed symptoms of deterioration. Gray to brown lesions are seen on the taproot and diseased tissue turned dark and spongy. Infected roots emit acidic odor. A whitish mycelial growth is sometimes evident on the root surface.

Sugar beet root rot was reported previously in Arizona, California, Wyoming and Colorado in the US and in Canada (Alberta and Ontario), Italy, Poland, France Iran, and Russia (Hilderbrand and Koch, 1943; Vincelli and Burne, 1989; Jacobson, 2006; Moliszewska and

Wisniewski, 2006). Soft rot on soursop, longya Lily and potato, and head rot in sunflower caused by *R. arrhizus* have been reported in Mexico, China and other parts of the world (Hahm et al., 2014; Park et al., 2014; Cui et al., 2019; Palemon-Alberto et al., 2020). Rhizopus root rot has been reported in the states of Arizona, California, Colorado, Wyoming and Michigan (Schneider and Whitney, 1986; Hanson, 2020) but not in North Dakota and Minnesota in the USA. The objective of this study was to identifiy and chacterize the *R. arrhizus* causing sugar beet root rot.

6.3. Materials and Methods

6.3.1. Collection of Samples, Isolation and Identification of *R. arrhizus*

Symptomatic sugar beet roots were collected during a field visit to Hickson, ND (46.6694 N, 96.8104 W) and Foxhome, MN (46.2774 N, 96.3100 W) in October 2020. Field survey showed approximately 5-10% plants were affected and had typical symptoms including wilting of the foliage and rotted taproot (Moliszewska and Wisniewski, 2006) (Fig. 6.1 a-b). Collected root samples were washed and isolation of fungi from rotted rot were done by tissue planting methods (Windels and Lamey, 1998) in potato dextrose agar (PDA) media (Fig. 6.1 c-f).

6.3.2. Molecular Identification

The fungal genomic DNAs of 3-isolates each from Hickson, ND and Foxhome, MN locations was extracted using Qiagen's DNeasy plant kit (Qiagen, Hilden, Germany). The ITS rDNA of the isolates was amplified and sequenced using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 primers (5'-TCCTCCGCTTATTGATATGC-3'), as described by White et al. (1990). The purified PCR products were sent for Sanger sequencing to Molecular Cloning Laboratories (MCLAB, San Francisco, CA).

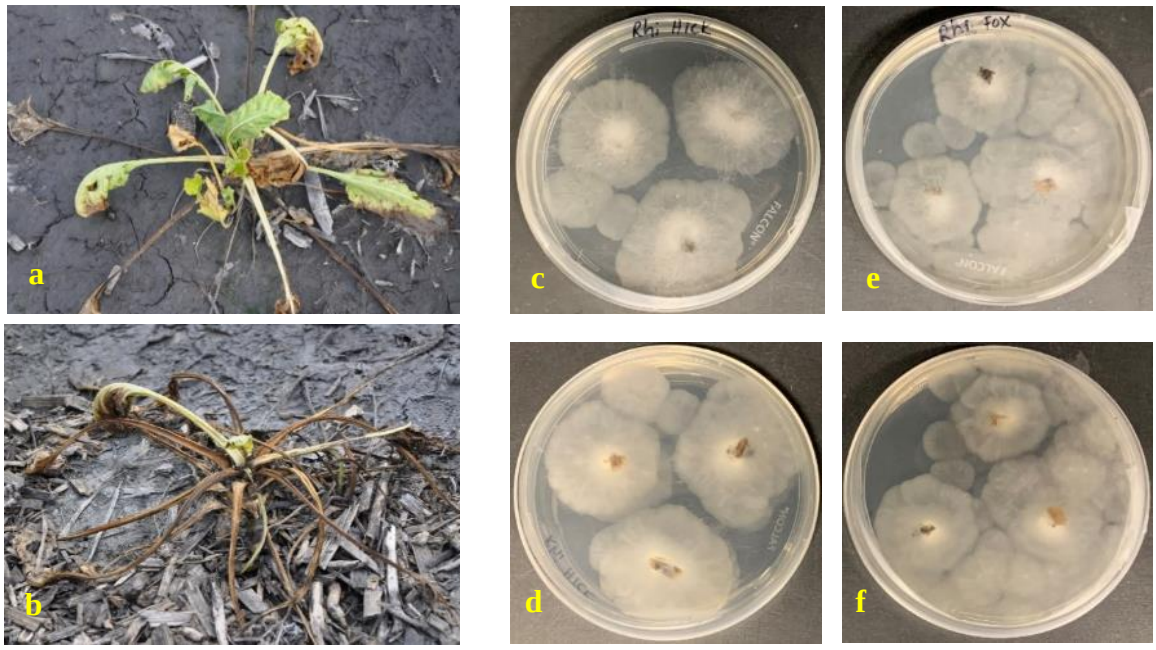

Figure 6.1. Diseased sugar beet samples (a, b) had typical Rhizopus root rot symptoms; Sugar beet plant collected from Foxhome, MN (a) and Hickson, ND (b) in 2020. *R. arrhizus* was isolated from surface sterilized disease tissue in PDA media (c-f). Front (c, e) and underside view (d, f) of Hickson and Foxhome isolates producing *R. arrhizus*, respectively.

6.3.3. *In-vitro* Inoculation of Seeds using Mycelial Agar Plug

Sugar beet seeds of four commercial cultivars, H-4302, M-504, B-8606 and SV-655 were used for *in-vitro* inoculation. Fungicide coatings were washed with distilled water followed by surface disinfestation in 70% ethanol and rinsed thrice with sterile water. Three seeds of each cultivar were placed in a 15 cm PDA plates and triplicated. One 5 mm diameter agar plug of actively growing mycelia was kept adjacent to each seed. Seedling emergence and disease symptoms were evaluated at 10 dpi.

6.3.4. Seed Inoculation at Greenhouse Condition

To confirm the pathogenicity of the fungus, we studied seed and root inoculation in the greenhouse at North Dakota State University, USA. Additionally, we assayed on seedling emergence and mortality in *in-vitro* conditions. Two commercial cultivars of sugar beet, H-4302 and M-504 were inoculated by mycelial plug of *R. arrhizus* isolates recovered from Hickson, ND and Foxhome, MN at planting. Fungicides coatings of seeds were washed by distilled water followed by surface disinfestation with 70% ethanol. Ten seeds of each cultivar

were sown in a furrow of plastic trays (25 X 12 X 10 cm) filled with FLX soilless mix (PRO-MIX, Quakertown, PA) and with osmocote (N-P-K: 15-9-12) fertilizer (Scot Company; Marysville, OH). The pots were arranged in a completely randomized design and the study was repeated with 3-replications and 10 seeds per replicates each time (Figure 3). Fungal mycelial agar plugs of 5 mm in diameter from a 2-3 days old culture were used for seed inoculation. One mycelial agar plug was inoculated adjacent to individual seeds and covered by soilless mix. Inoculated pots were kept in humidity, chamber-maintained RH >80%, and temperature 28±2 ^{0}C for five days and transferred to the greenhouse room. At 14-dpi, seedling emergence and disease incidence were assessed.

6.3.5. Sugar Beet Root Inoculation

R. arrhizus were grown in PDA media at 25±2 ^{0}C for 7 days in dark conditions. Culture plates were then flooded with sterile water using a glass rod to release spores. Spore suspensions were adjusted to 1 X 10^4 using a hemacytometer. Four weeks old sugar beet plants of two sugar beet cultivars, H-4302 and M-504 were used for root inoculation by *R. arrhizus* spores. Pots were arranged in a completely randomized design, four pots (one plant per pot) and repeated. Each plant was dipped into the spore suspension (10 ml/plant) for 1 minute and planted again in the pots (Hanson 2010). Plants were kept in the humidity chamber for 5 days where RH was >80% and temperature maintained at 28±2 ^{0}C. After 21-dpi, roots were evaluated for disease symptoms.

6.4. Results

The fungus produces creamy white colonies in PDA media containing profuse growth of mycelia. Hyphae were hyaline and coenocytic (Fig. 6.2 a, d, and e) and later became brown to black due to abundant growth of blackish sporangia (Fig. 6.2 a and d). Long, slender, flexible sporangiophores rose from the junction of stolons and rhizoids. Rhizoids were less developed (Fig. 6.2 b). Sporangiophores produce a head-like outgrowth at the tip called columella, which

holds globose shaped sporangia (Fig. 6.2 c). Sporangiospores release at maturity from sporangia and disseminates through air currents (Fig. 6.2 f). The fungus, *R. arrhizus* was identified based on morphological features through macroscopic and microscopic observation (Schipper and Stalpers, 1984).

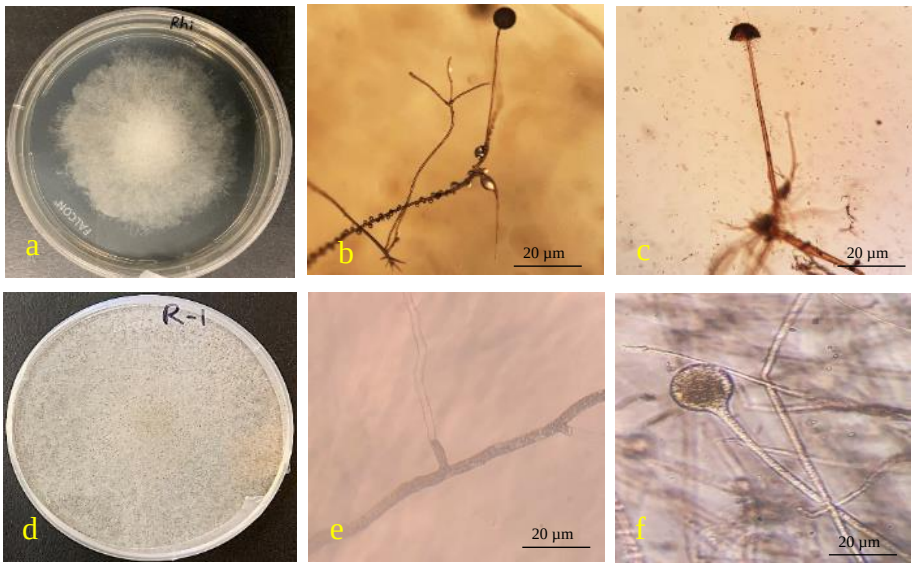

Figure 6.2. Pure culture of *R. arrhizus* (A, D) and other morphological structures (B, C, E, F). Blackish globose sporangia and reduced rhizoids growth (B); long, slender sporangiophore holds cap-like structure columella (C); Coenocytic mycelia (E); and sac like structure sporangia contains globose shaped sporangiospores (F) observed under microscope.

The nucleotide BLAST search of Hickson and Foxhome isolates showed 100% similarity with *R. arrhizus* GenBank accessions MN421950, MN088368, MH046841, MT540020, MT540019 etc. Only one isolate of each location, Hickson (RND-1) and Foxhome (FND-1) was deposited in the NCBI GenBank as accession number MW819939 and MW819941, respectively. Non-inoculated seeds gave rise to healthy asymptomatic seedlings (Fig. 6.3 a). Emerged seedlings from inoculated seeds represented variable symptoms including seedling rot, damping off, and reduced radicle development, respectively (Fig. 6.3 b-d). Seedling emergence was affected up to 5 to 10%. The fungus was re-isolated from the infected

seedlings in PDA media. The growth and development of creamy white mycelia and numerous blackish sporangia were identical to the isolates retrieved from the field samples (Fig. 6.4 a-d).

Figure 6.3. Seed inoculation with mycelia plug in greenhouse showing seedling rot and damping off (b-d), whereas non-inoculated seeds showing vigorous growth of seedlings (a).

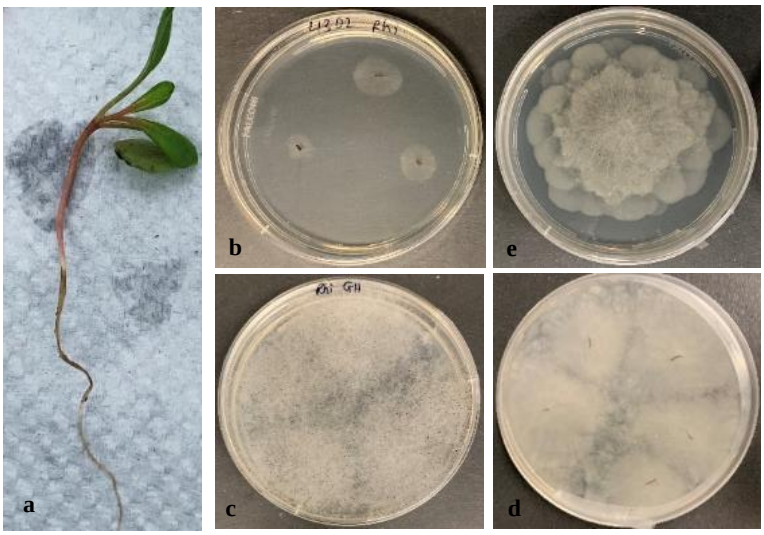

Figure 6.4. Re-isolation of *R. arrhizus* from infected seedlings (a). Seeds were inoculated by mycelial plugs and evaluated at 14 days post inoculation. Pure culture was developed on PDA plates from diseased seedling hypocotyl (b-d).

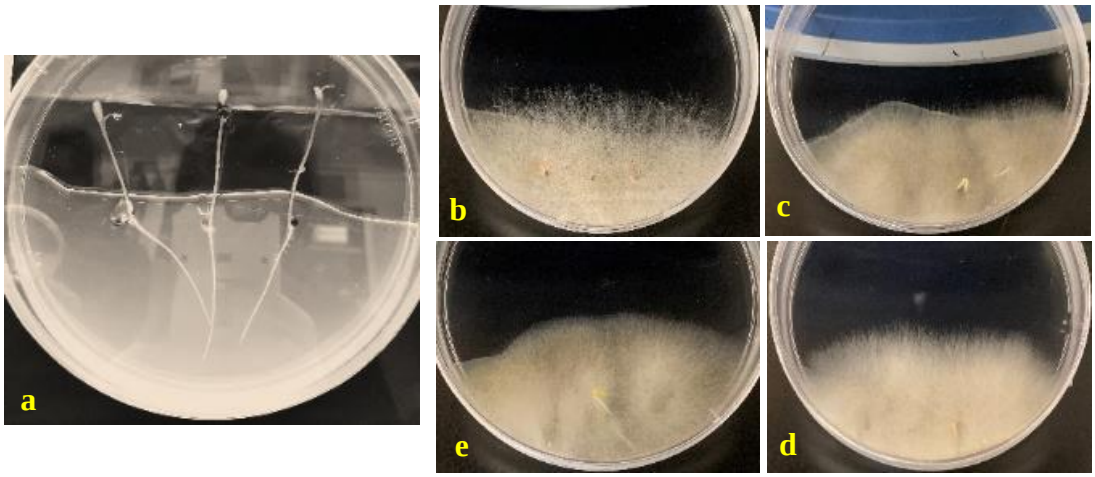

Figure 6.5. *In-vitro* inoculation of sugar beet seeds in PDA plates by mycelial plug of *R. arrhizus*. Inoculated seeds showed reduced germination, and seedling rot (b-e) (Clockwise). Later seedlings became dead and replaced by fungal structures. Non-inoculated seeds developed well-developed seedlings (a).

Seedling emergence and disease incidence were recorded at 10 dpi. Non-inoculated seeds had no disease symptoms and seedlings were healthy and vigorous (Fig. 6.5 a). Inoculated seeds showed reduced germination, limited growth of some seedlings and completely no germination of seeds. Later seedlings became rotted, dried and replaced by fungal hyphae and sporangiospores (Fig. 6.5 b-d). Inoculated plants of two cultivars showed brown to dark brown discolored roots and later became wilted, which represented reduced root development (Fig. 6.6 a), and growth of whitish mycelial mass was found on roots near the soil line (Fig. 6.6 c). Non-inoculated plants were healthy and had no disease symptoms (Fig. 6.6 b).

Figure 6.6. Inoculated roots showing rot symptoms. Dense fungal mycelial network was visible on and around root surfaces near the soil line (c). Taproots become rooted and internal tissues become disintegrated (a). Non-inoculated root had no disease symptoms (b).

6.5. Discussions

Economic production of sugar beet is restricted by many soil borne pathogens including *R. solani, Pythium* sp., *Aphanomyces* sp., *Rhizopus* sp., *Fusarium* sp. etc. (Berkeley, 1994; Jacobson, 2006; Khan, 2017). This is the first report on the occurrence of root rot disease of sugar beet caused by *R. arrhizus* in the states of North Dakota and Minnesota. This paper demonstrated the natural occurrence of the disease and characterized the pathogenicity of *R. arrhizus* on sugar beet seeds and roots in lab and greenhouse conditions. Field infection many continue in storage. Commercial cultivars of sugar beet seeds resulted in seedling rot and wilting when exposed to mycelia of fungus. Sugar beet roots showed root rot when artificially inoculated with spore suspensions of *R. arrhizus*. As this fungus is capable of causing seedling rot and hampered seedling emergence, growers need to address this issue accordingly for its management to ensure expected yield. The synergistic interactions of the Rhizopus root rot with the Rhizoctonia crown and root rot (Hanson, 2010) is favored by mechanical damage by cultivation instruments and or insects' damages coupled with predisposition by prolonged wet conditions and relatively high temperature (30^0 to 40^0C) (Pitt and Hocking, 1985; Gilman,

1957). Further investigations of the disease for proper management of sugar beet root rot is warrented to save yield losses in the field and storage conditions.

6.6. References

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APENDIX A. SUPPLEMENTARY TABLE

Table A1. Contract analysis of the treatments of field experiment at Foxhome, MN in 2019, 2020 and 2022.

	2019	2020	2021	2019 and 2021 (combined)
Contrasts	Pr> F	Pr> F	Pr> F	Pr> F
Penncozeb vs Penncozeb +Cerium Elite	0.3514	0.3689	0.2114	0.1031
Badge SC vs Badge SC + Cerium Elite	0.2058	0.765	0.7325	0.9781
Ins vs Ins+CE	0.2881	0.2615	0.9997	0.9857
Rotation vs Rotation+CE	0.8419	0.5633	0.1732	0.9155
Penn vs Penn+Com	0.7587	0.6729	0.5878	0.0266
Badge vs Badge+Com	0.0492	0.81	0.9042	0.8403
Ins vs Ins+Com	0.1845	0.0035	0.6378	0.2172
Rotation vs Rotation+Com	0.8864	0.1873	0.1827	0.5313
Penn vs Penn+Trans	0.3586	0.9911	0.7127	0.5741
Badge+ vs Badge+Trans	0.3798	0.7635	0.2972	0.9032
Ins vs Ins+Trans	0.3181	0.0907	0.3786	0.8449
Rotation vs Rotation+Trans	0.6861	0.7942	0.8261	0.2807
Penn vs Penn+CE, Penn+Com and Penn+Trans	0.8957	0.5923	0.8868	0.0715
Badge vs Badge+CE, Badge+Com and Badge+Trans	0.3292	0.9224	0.7359	0.8862
Ins vs Ins+CE, Ins+Com and Ins+Trans	0.1679	Pr> F	0.8663	0.5623
Rotation vs Rotation+CE, Rotation+Com and Rotation+Trans	0.8871	0.3689	0.2335	0.8179

Here, Penn=Penncozeb, Ins=Inspire XT, CE= Cerium Elite, Com= Complex, Trans=Transfix, respectively.

APENDIX B. SUPPLEMENTARY FIGURES

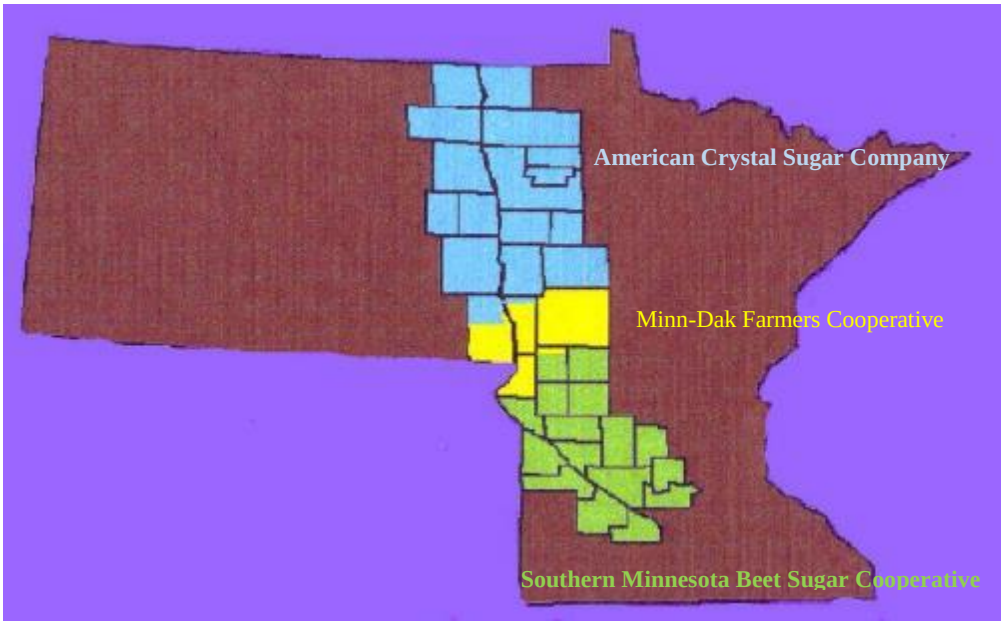


Figure B1. Sugar beet cooperative in North Dakota and Minnesota.

A

B

Figure B2. A) Inoculation *C. beticola* followed by application of fungicides-adjuvants spray and B) Spraying of fungicides-adjuvants followed by *C. beticola* inoculation

Figure B3. Generation-III Research Sprayer for fungicides adjuvants spray located at NDSU greenhouse.

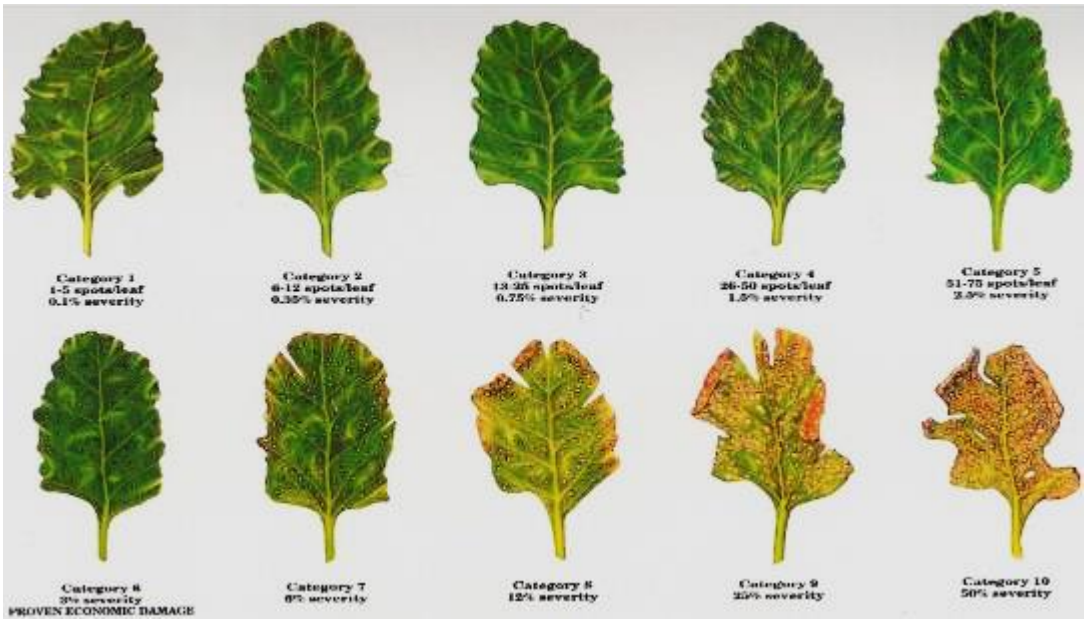
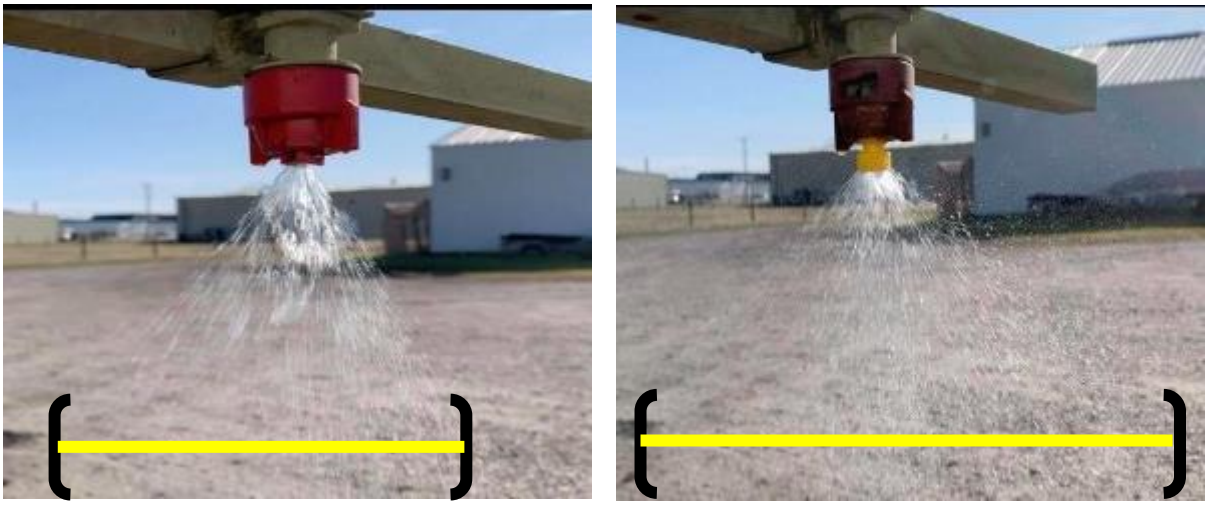


Figure B4. Sugar beet CLS disease severity 1-10 scale (Jones and Windels 1998)



a. Conventional Flat Fan Nozzle **b. Turbo Twin Jet**

Figure B5. a) Comparison of the conventional flat fan nozzle, and b) Turbo twin jet nozzle spray width and spray droplets.

a **b**

Figure B6. a) Droplets of fungicides, b) fungicides mixed with adjuvants

Figure B7. Spraying fungicides and adjuvant in the field using Trubo Twinjet nozzles

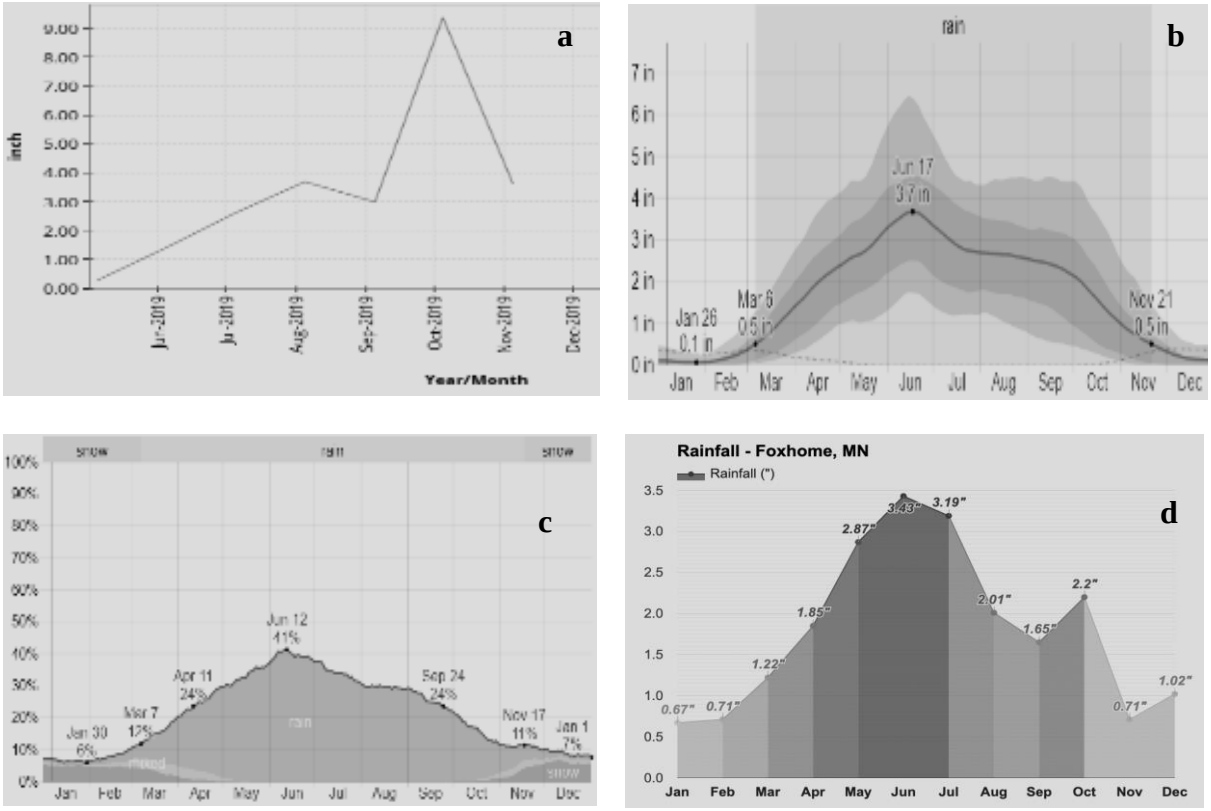


Figure B8. a) Monthly total rainfall in 2019, b) Average monthly rainfall in 2020, c) daily chance of rainfall in 2020, and d) monthly total rainfall in 2021 in Foxhome, MN. Source: North Dakota Agricultural Weather Network (NDAWN).