DEVELOPING A NEW INOCULATION METHOD, AND EVALUATING THE POTENTIAL BIOLOGICAL CONTROL OF *RHIZOCTONIA SOLANI* BY *PENICILLIUM PINOPHILUM* ON SUGAR BEET

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ABSTRACT

Rhizoctonia solani causes damping-off, and root and crown rot of sugar beet (*Beta vulgaris* L.) and overwinters as sclerotia and mycelia. Research was conducted to determine how best to produce large quantities of sclerotia and mycelia *in vitro*, and compare their pathogenicity with traditionally used colonized barley grains to sugar beet *in vitro* and *in vivo*. The greatest number of sclerotia was produced on amended clarified V8 medium and sclerotia caused more disease compared to barley inoculum in the greenhouse. The bio-control potential of *Penicillium pinophilum* on *R. solani* AG2-2 on sugar beet was evaluated *in vitro* and *in vivo*. Results showed that the presence of *P.pinophilum* with *R.solani* reduced damping-off by 75% and thus have the potential to be developed as a bio-control agent for this pathogen.

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TABLE OF CONTENTS

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

CHAPTER ONE. LITERATURE REVIEW

Sugar Beet Domestication and US Commercialization

Sugar beet is an economically important crop of the large order Caryophyllales, supplying approximately 25% of sugar worldwide (Draycott, 2006). The sugar beet genome is diploid with 2n = 18 chromosomes and the estimated genome size is 731 Mbp (megabases/millions of base pairs) (Dohm et al., 2014). The sugar beet wild ancestors are the sea beet (*Beta vulgaris* subsp. *maritima*) which reside in the family, Amaranthaceae and sub-family, Betoideae (Cooke and Scott 1993; Romeiras et al., 2016) (Figure A.1. and Figure A. 2.). About 1500 years ago, sugar beet was introduced to China from Arabia. As it had high economic value in many countries, improvement of the crop has been extensively explored. It is a biennial crop with a sugar-rich tap root in the first year and a flowering seed stalk in the second. Currently, the crop is cultivated mainly in temperate regions between 30° and 60° N from Cairo to Helsinki (Harveson 2017; Zhang et al., 2016).

In the USA, sugar beet production was done in 1838 in Northampton, MA, and the first successful sugar factory was set up in 1879, in Alvarado, CA, (Francis, 2006). Sugar beet provides about 55% of the total sugar produced domestically, while sugar cane contributes 45% (Harveson 2002; USDA-ERS, 2020). Sugar beet is currently grown in 11 states of the USA which includes Minnesota, Idaho, North Dakota, Michigan, Nebraska, Montana, California, Wyoming, Colorado, Oregon, and Washington. Sugar beet thrives well in temperate climatic conditions but can also be produced in warm climates.

The Red River Valley (RRV) of western Minnesota and eastern North Dakota is the largest area of producers of sugar beet in the United States. The first sugar beet factory was established in the RRV in 1926 in East Grand Forks (Shoptaugh, 1997). Currently, there are three sugar beet cooperatives in the RRV: American Crystal Sugar Company, Minn-Dak Farmers' Cooperative,

and Southern Minnesota Beet Sugar Cooperative are located in Minnesota and North Dakota. These sugar beet cooperatives contribute approximately 57% of the US sugar beet acreage. This has created a huge economic impact of over $5 billion in the Upper Midwest. In USA, the total sugar beet planted area and yield was 1,132,000 acres and 28,600,000 tons, respectively in 2019 (USDA-ERS, 2020).

Since the mid-1970s growers in the US started joining together as farm-owned cooperatives, purchased the processing companies and managed the marketing and sales of their production. In the USA, private companies own the commercial seed production and the variety improvement programs. The USDA help to select and improve germplasm before making lines available to the seed companies for further development and commercialization. The varieties today are relatively high yielding and are moderately resistant to most of the common soil borne and foliar pathogens. For example, most varieties must have a minimum level of resistance to root pathogens such as *Rhizoctonia*, *Aphanomyces*, *Pythium*, *Fusarium*, sugar beet cyst nematode and viruses such as Beet Necrotic Yellow Vein and Curly Top (Harveson 2002; Heitefuss 2010; Li and Smigocki 2018). Thus, the commercialization of sugar beet advanced with the establishment of sugar processing houses and increased in efficiency in the field and factory. Improved seed and varietal choice have made commercialization easier as it has helped farmers choose the best varieties for better yield and quality (Gaskill 1968; Koch 2007).

However, several diseases are major limiting factors to sugar beet yield potential. Cercospora leaf spot is one of the most important and widespread foliar diseases in sugar beet. It is caused by a hemibiotrophic filamentous fungal pathogen, *Cercospora beticola*, which causes necrotic lesions and progressive destruction of the plant's foliage (Zhang et al., 2016). Research has been ongoing to map the genes that confer resistance to *C. beticola* (Grimmer et al., 2007).

Several other foliar diseases included alternaria leaf spot caused by *Alternaria tenuissima*, bacterial leaf spot caused by *Pseudomonas syringae* pv. *aptata* and powdery mildew caused by *Erysiphe polygoni* are occasionally observed in fields (Khan et al., 2019; Nikolic et al., 2018; Stojsin et al., 2015) in the RRV.

Damping-off and root rots caused by *A. cochlioides* and *R. solani* are common diseases found in the RRV. Since 2009, *R. solani*, which causes damping-off and root rot, has been listed by growers in the RRV as one of their most important issues.

Taxonomy and Biology of *Rhizoctonia solani*

The Rhizoctonia genus was first reported by DeCandolle in 1815. This is known as a large, diverse and complex group of fungi. *R. solani* Kühn was first reported in 1858 by Julius Kühn who observed the fungal pathogen on potato tuber (Sneh et al., 1996). The basidiomycetes fungus belongs to the class Agaricomycetes, order Ceratobasidiales and the family Ceratobasidiaceae with a teleomorphic (*Thanatephorus cucumeris* [Frank]Donk) stage. *R. solani* is found in nature mainly in the asexual stage, and primarily prevalent form is vegetative mycelia and/or sclerotia (Sumner 1996; Engelkes and Windels 1996; Herr 1996). The hyphae usually branch at a right angle with the presence of constriction at the base of the branch (Ajayi-Oyetunde and Bradley 2018; Christou 1962; Cubeta and Vilgalys 1997). Fungal colony appears brown on potato dextrose agar (PDA) and amended clarified V8 media (CV8) (Figure A .3. and Figure A. 4.).

R. solani contains more than three nuclei per hyphal cell which contributes to significant heterozygosity within a single cell (Sherwood 1967). The heterokaryotic genome of *R. solani* covers approximately 51.7 Mbp and it is predicated to encode 12, 726 genes (Wibberg et al., 2016). *Rhizoctonia* hyphae of several other species contains two nuclei known and are binucleate (Agrios, 2005).

Distribution and Host Range

R. solani is a cosmopolitan, devastating soil-borne pathogen causing consistent economic losses in more than 200 plant species included a wide range of cereals, tubers, oilseed crops, and vegetables as well as ornamental plants and forest trees (Buttner et al., 2002; Cubeta and Vilgalys 1997). This facultative saprophyte has a variety of disease name based on crop plants; for instance, rice sheath blight, bare patch on cereals, black scurf on potatoes, sugar beet seedling damping-off, and crown and root rot, as well as damping-off, root and stem rot on soybean (Heitefuss 2010; Shu et al., 2019; Singh et al., 2018).

This pathogen undergoes hyphal fusion which is known as anastomosis. There are 13 anastomosis groups (AGs) (Cubeta and Vilgalys 1997; O'Brien 1996). Several studies in sugar beet plants have shown the presence of the following AGs: AG-1-1B, AG-1-1C, AG-2-1, AG-2-2, AG-4, AG-5, AG-11, AG-K, and AG-3TB (Windels C.E., 1989; Zhang et al., 2015; Zhao et al., 2019). Among these AGs, the most destructive form is AG-2-2 to sugar beet, which has two subgroups, AG-2-2 IIIB and AG-2-2 IV (Brantner and Windels 2009; Strausbaugh et al., 2013; Zellner and Nottensteiner 2018).

AG-2-2 IIIB was reported predominantly in the Red River Valley and in southern Minnesota, while AG 2-2 IV was rarely reported in past years (Brantner and Windels 2009). AG 4 is mostly reported to cause post-damping-off of sugar beet (Nagendran et al., 2009). AG-2-2IIIB is aggressive to both seedlings and older sugar beet plants (Windels C.E. 1989) (Figure A.5. and Figure A.6.). The annual yield loss varies from field to field, and state to state, ranging from 2% to 60% (Neher and Gallian, 2011).

Infection Process and Symptoms

R. solani can survive as sclerotia for a prolonged period in the soil. The conductive condition for infection depends on soil moisture (25-100%) and soil temperature (20-35 ºC) (Bolton et al., 2010). Infections do not progress below 15°C (Harveson 2006). The fungus can penetrate into the host through forming an infection cushion, or it can produce an appressorium that penetrates through the cell wall and take nutrients from the plant cell. The pathogen colonizes inside the dead tissue, and overwinter inside the host tissue as sclerotia (Lee et al., 1995; Windels C.E., 1989) (Figure A.7.).

This necrotrophic pathogen is also familiar as a seed and soil-borne fungus. The sign of infection appears as dark brown/cankerous lesions below the soil surface, and it advances to the hypocotyls. Symptoms may appear as yellowing or wilting of leaves. Sign and symptoms appeared on above and below ground portions of the plant. Consequently, it is yielding to the wilting and complete collapse of cotyledons and immature death of seedlings (Heitefuss 2010). *R. solani* causes damping-off, and root and crown rot to young seedlings and older plants, respectively (Harveson 2002, 2006). Root and crown rot in sugar beet incurs poor yield and infected plants become more susceptible to heat or drought stress (Heitefuss 2010).

Disease Management of *Rhizoctonia solani*

R. solani damping-off as well as root and crown rot disease epidemics depend on the aggressiveness of the AGs, crops and cultivars, and the environment. Disease severity increases when the weather is warm and under wet field conditions. Generally, integrated pest management strategies that involves cultural practice, chemical control, host resistance and biological control are followed to reduce the pathogen propagules.

Cultural Control

Cover crops study have shown a significant effect in controlling *Rhizoctonia* infected fields. Cover crop (*Brassica*) used as green manure significantly reduced soil borne pathogens, particularly; *Rhizoctonia, Phytohpthora, Pythium*, *Sclerotinia* and *Fusarium* (Kundu and Nandi, 1985). There is evidence that neem (*Azadirachta indica*) used as a green manure and *Gliricidia* leaves reduced inoculum of *R. solani* in paddy field by improving the microbial community structure (Wen et al., 2017). However, cover crops are not commonly used in commercial fields to manage *R. solani*.

Agronomic tools such as crop rotation may help in reducing the disease. Sugar beet fields should be rotated at least every third year with non-host cereal crops such as wheat (Buhre et al., 2009; Koch et al., 2018); nevertheless, some AGs have the polyphagous nature to surmount this practice, for instance; AG 2-2IIIB strain of *R. solani* has a wide host range, including corn (*Zea mays* L.) and soybean (*Glycine max* L.) Merr.) (Engelkes and Windels, 1996). Studies in Europe showed that AG 2-2 IIIB caused root and stalk root of corn (Ithurrart et al., 2004). Another study in south-eastern US demonstrated root and brace rot of corn was caused by *R. solani* AG 2-2 IIIB (Sumner et al., 1999; Sumner et al., 1982).

Similar study in the Upper Midwest showed that AG 2-2 IIIB caused disease lesions on corn in crop rotation studies which included wheat, soybean and corn (Windels and Brantner, 2008). This research suggested that cultivation of corn in crop rotation to sugar beet may escalate propagules of AG 2-2 IIIB. Likewise, AG8 has been reported aggressive to both cereal and legume rotations (Hane et al., 2014). Also, AG1 and AG2 are aggressive to corn, canola, and soybean rotations (Bell and Sumner 1982; Pascual and Hyakumachi 2000). Another study in New York has observed that crop rotation become very narrowly effective in controlling *Rhizoctonia* in table

beets (Ohkura et al., 2009). In practice, growers in the Red River Valley typically plant wheat, although it is not very profitable, as the crop preceding sugar beet to reduce the inoculum potential of *R. solani*. In several production areas of the US, such as southern Minnesota, producers grow the more profitable corn and soybean crops in rotation with sugar beet. Since both of these crops are host of *R. solani* AG2-2 IIIB, root rot has become more widespread and problematic where this rotation is common.

Improving soil structure and drainage is useful in improving water infiltration, drainage, and aeration of plants (Bolton et al., 2010; Buhre et al., 2009). The availability of moderately resistant crop varieties with some reduction in yield is one way to avoid the significant yield loss in fields with a known history of severe disease (Behn et al., 2012). Certified seed free from sclerotia can also reduce the chance of crop damage.

Chemical Control

Fungicides such as azoxystrobin and pyraclostrobin (QoIs) and sedaxane, penthiopyrad, and fluxapyroxad (SDHIs) are widely used to control *R. solani*. The SDHI fungicides are typically used as fungicidal seed treatments while the QoIs may be applied at planting and foliarly (targeting the soil) to help control the pathogen (Markell and Khan, 2012; Khan 2020; Liu and Khan 2016).

Sugar beet growers prefer quinone outside inhibitors (QoI); azoxystrobin and pyraclostrobin. This helps to block electron transfer between cytochrome b and cytochrome c1 by binding to cytochrome b and it paves the halting of the ATP production (Balba 2007). These products are used as an in-furrow application at planting and as a foliar spray during the growing season (Khan et al., 2017; Khan et al., 2010). QoI fungicides typically have a high risk for the buildup of a fungicide resistant pathogen subpopulation, particularly when used in fields with consecutive or repeated applications. Similarly, succinate dehydrogenase inhibitor (SDHI), for

example, Penthiopyrad stops ATP production by binding to SDHI enzyme located in mitochondrial membrane (Hagerhall 1997). Penthiopyrad and other SDHIs are used as a seed treatment (Liu and Khan 2016). Furthermore, demethylation inhibitor (DMI) such as Prothioconazole is a sterol biosynthesis that distrupts plasma membrane structure to incur abnormal fungal growth and death (Georgopapadakou 1998). Greenhouse study at 26.7º C showed that azoxystrobin and prothioconazole to be effective against *R. solani* AG2-2IIIB (Khan et al., 2008). Producers in Minnesota and North Dakota indicated in an annual survey that the most commonly used fungicides were azoxystrobin, pyraclostrobin and prothioconazole to control *R. solani* (Carlson et al., 2013).

The success of fungicide application depends on suitable timing that can offer long-term disease protection. Soil temperature and moisture are the two critical parameters that impact *R. solani* infection. Research shows that the mean daily soil temperature at the 10 cm soil depth needs to be at least 18 C for *R. solani* infection in sugar beet (Bolton et al., 2010).

Host Resistance

Genetic resistance is an effective way of managing *R. solani* mediated diseases, as it involves low cost, effective, sustainable and an eco-friendly approach. Nevertheless, it takes 8-10 years to develop a resistant cultivar (Sherf and MacNab, 1986; McGrath et al., 2015). Sugar beet resistance breeding to Rhizoctonia started in 1950 at Fort Collins, Colorado by the United Sates Department of Agriculture-Agriculture Research Service (USDA-ARS). Sugar beet cultivars have moderate resistance to *Rhizoctonia* that involves multiple genes regulating the phenomenon of quantitative resistance (Panella and Ruppel., 1996) (Gaskill, 1968) (Hecker and Ruppel, 1975). Partial resistant varieties are grown to minimize the level of disease incidence, but growers prefer

susceptible cultivars because of high yield potential (Behn et al., 2012; Brantner and Windels, 2009).

In the US, private companies are mainly involved for developing resistant varieties, and in many cases, they do not disclose the genetic background of their resistant cultivars. Furthermore, the resistance of cultivars to *R. solani* is evaluated by scoring disease reactions at the crowns and roots of older seedlings (Ruppel et al., 1979), thus resistance is not evaluated during seed germination. Moreover, earlier studies evaluated cultivars resistance to *R. solani* using colonized whole barley or wheat grains which, unlike sclerotia, are artificial inocula of the pathogen that require time, space and technical know-how to produce. Moreover, colonized grains are prone to contamination with other pathogens and may be consumed by birds and wildlife when applied in the field. One of the objectives of this study was (1) to develop a medium for production of *R. solani* sclerotia, and compare the pathogenic potential of sclerotia, mycelia, and colonized barley grains to selected commercial sugar beet cultivars under greenhouse condition.

Diversity in Ecosystem and Potential Role of Biological Agents

In the ecosystem, various types of interactions are occurring among all kinds of organisms, for instance; single-celled to multi-cellular organisms, pathogens are causing diseases while parasites are living on or in another living organisms to get their food (Sint and Traugott 2016). On the other hand, symbiosis illustrates that the two organisms living together regardless of the outcome. However, there are a number of two species interactions in the nature that has been divided into two broad types, for example; negative interactions and positive interactions. There are a number of negative interactions in the ecosystem for example; parasitism, competition, amensalism, predation, and neutralism (Preston et al., 2016).

In parasitism, one species gets the benefits at the expense of the other, for example, bacteria, fungi, nematodes, and viruses. In competition both species competes with each other directly or indirectly for light, water, and food, for example, weeds in a crop (Mohamad et al., 2015; Pereira 2003; Shimizu et al., 2018). In amensalism, one species is inhibited while other is unaffected, for example, many algae in nature. Another example is that bacteria-killing phenomenon of *Penicillium* (Naik et al., 2019). In predation, there are a number of predatory insects in nature, for example, praying mantis that kills other insects. Neutralism is a type of interaction where neither population affects the other, for example, cacti and tarantulas living in the desert.

Apart from these negative interactions, there are a number of positive interactions available in nature, for example, mutualism, commensalism, and protocooperation (Mahatma and Mahatma 2015).

The mutualism illustrates the favorable interactions to both species and it is obligatory, for example, lichens; a fungal partner (mycobiont) and an algae (Cyanobacteria/Photobiont). Another example of cellulolytic bacteria harbored in the rumen of the cattle (Du et al., 2019). The commensalism phenomenon illustrates the interaction of species-1 (for example; orchids) which is directly benefited by the others, while species-2 is unaffected. For example, in the rain forest, orchids grown on the trees without causing any problem (Grange et al., 2016; Rasmussen and Rasmussen 2018). Protocooperation is another form of mutualism where interaction is favorable to both species but it is not obligatory. In this type of interaction occurs in soil bacteria or fungi and in plants growing in the soil (Fowler and Garcia 1989).

As a component of integrated disease management, biocontrol strategies are environmentally safe, there is no chance of developing resistant biotypes, and it is convenient to use in greenhouse and field research.

Soil bacteria such as Rhizobacteria has shown the suppression effect on inoculum density of *R. solani* (Homma, 1996). Similarly, a commercial preparation of *Bacillus subtilis*, Kodiak has been used to reduce *R. solani* AG 2-2 IIIB infection in sugar beet. *Bacillus* strain MSU-127 and low rate of Azoxystrobin used in-furrow application improved sugar beet by about 16% and a foliar fungicide application at the 4-leaf stage also increased root yield by 17% (Jacobsen et al., 1997; Kiewnick et al., 2001).

Antagonistic mechanisms of *B. subtilis* have been elucidated at the molecular level. For instance, *B. subtilis* produce bacteriocins which is a low molecular weight peptide molecule that involves different mode of action. This included protoplasm vesicularization, pore formation, and cell disintegration. Subtilin is the most studied bacteriocin that found to inhibit bacterial growth (Caulier et al., 2019).

A wide diversity of secondary metabolites mediating antibiosis have been identified over the last two decades. Genome of most of the *B. subtilis* groups have revealed that 4-5% of genome devoted to antimicrobial peptides (AMPs) included ribosomal peptides (RPs) (bacteriocins and enzymes), the polyketides (PKs), the non-ribosomal peptides (NRPs) and volatile metabolites. Other type of enzymes are known to show antagonistic activities, quorum sensing, cell lysis or induction (Stoica et al., 2019).

Recently, field application of *B. subtilis* was found to be effective for the control late blight of potato caused by *Phytophthora infestans* (Kumbar et al., 2019). Other research showed that *Bacillus velezensis* LHSB1 strain controlled peanut stem rot caused by *Sclerotium rolfsii* (Chen et

al., 2020). Other greenhouse study on *Bacillus amyloliquefaciens* SB14 strain showed to reduce damping-off disease by 58% caused by *R. solani* AG-4 and 52% caused by *R. solani* AG 2-2 on sugar beet (Karimi et al., 2016).

Fungus-like *Laetisaria arvalis* Burds. (Division: Basidiomycota, Order: Corticiales, Family: Corticiaceae) was reported as a potential biocontrol agent for soil-borne pathogens included *Pythium* species and *R. solani* (Burdsall, et al., 1980). In another research, *Trichoderma harzianum* in a conidial suspension has been used to inhibit the growth of *R. solani* and reduced disease index by 65% (Barakat et al., 2007). Research on the yeasts *Candida valida*, *Trichosporon asahii* and *Rhodotorula glutinis* protected sugar beet root rot from *R. solani* AG 2-2, and promoted plant growth in vitro (El-Tarabily 2004). Over the last four decades, several studies on biological control have been initiated, nevertheless, there has not been much success achieved in the field compared to the greenhouse. It is likely that very complex heterogenous biotic and abiotic factors influence the potential role of biocontrol agents.

Taxonomy and Biology of *Penicillium pinophilum* (*Talaromyces pinophilus*)

The *Penicillium* is a large, diverse and ubiquitous genus that contains approximately 354 species. These are blue or green mold fungi that mostly exists as asexual (anamorph) stage. Some members of the genus are known to produce penicillin that is used as antibiotic to stop the growth of specific bacteria (Visagie et al., 2014).

The *Penicillium pinophilum* Hedgc, belongs to the genus *penicillium*. This species was first reported in 1910 (Thom, C. 1910). The synonymous name proposed as *Talaromyces pinophilus* (Samson, 2011). This species belongs to Fungi, division Ascomycota, class Eurotiomycetes, order Eurotiales and family Aspergillaceae. The genome of *P. pinophilum* covers approximately 36.51 Mbp and it is predicated to encode 13, 472 protein-coding genes. Among the genes, 64 secondary

metabolism gene clusters were annotated. In addition, 39 cellulose degrading and 24 starch degrading enzymes were identified (Li et al., 2017). This endophytic fungus is known to produce bioactive secondary metabolites including oxyskyrin, skyrin, dicatenarin, and 1,6,8-trihydroxy-3-hydroxy methylanthraquinone. These metabolites are involved to induce reactive oxygen species (ROS)-mediated apoptosis via mitochondrial pathway in cells (Koul et al., 2016). This study demonstrated that *P. pinophilum* (*T. pinophilus*) produce useful biomass-degrading enzymes and secondary metabolites.

Other researchers found that *P. pinophilum* inoculation in soil increased nutrient uptake (N, P, and K) in pomegranate (*Punica granatum* L.) that resulted improved plant growth, significantly higher leaf area index and photosynthetic rate (Maity et al., 2014). Research in India has shown that *P. pinophilum* can be used in biofertilizer formulation to supplement potassic fertilizer to pomegranate plant (Maity et al., 2019).

Another study has demonstrated the antagonistic potential of *P. pinophilum* and *P. bilaiae* in a dual-culture of phytopathogenic fungi including *Alterniaria alternata*, *Fusarium equiseti*, *Fusarium graminearum*, and *Fusarium verticilloides* (Taieb et al., 2019). This study demonstrated that the co-cultivation of plant beneficial fungi and phytopathogenic fungi may provide an effective strategy to simulate the production of bioactive metabolites, and thus possible help to identify novel compounds for crop protection.

Several studies on biocontrol of soilborne pathogens have been initiated, nevertheless, there has not been much success achieved in controlling *R. solani* in sugar beet. Lately, the biocontrol potential of *Penicillium pinophilum* (*Talaromyces pinophilus*) was reported to control *Pythium* and *Rhizoctonia*-induced damping-off in cucumber (Kazerooni et al., 2019). There are only a few fungicide chemistries which provide effective control of *R. solani*. Some countries do

not allow the use of fungicides for control of *R. solani* in sugar beet. It will be useful to develop other novel ways to manage this important pathogen of sugar beet. In this research, we evaluated the antagonistic effect of *P. pinophilum* to *R. solani* AG 2-2 IIIB on sugar beet in the laboratory and under greenhouse conditions.

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CHAPTER TWO. EVALUATION OF *RHIZOCTONIA SOLANI* SCLEROTIA MEDIATED PATHOGENICITY TO SUGAR BEET CULTIVARS IN GREENHOUSE STUDIES[1]

Abstract

Rhizoctonia solani causes damping-off, as well as crown and root rot of sugar beet (*Beta vulgaris* L). This pathogen overwinters as sclerotia or melanized mycelia. Traditionally, the resistance of cultivars to *R. solani* is evaluated by scoring disease reactions of the crowns and roots of older seedlings, instead of evaluating at seed germination. Most studies that have evaluated cultivar resistance to *R. solani* have used colonized whole barley grains as artificial inocula. Colonized grains are prone to contamination with other pathogens and are often lost to rodents/birds when applied in the field. Considering those limitations, a study was undertaken (1) to develop *in vitro* methods to generate natural sclerotia of *R. solani* on a large scale, (2) to compare pathogenic potentials of *R. solani* sclerotia, mycelia, and colonized barley grains for optimization of damping-off assays, and (3) to evaluate resistance of selected commercial cultivars to *R. solani*. Of six different culture media evaluated for their effects on sclerotia development, amended clarified V8 (ACV8) was the most suitable culture medium to grow and produce sclerotia on a large scale and 10% PDA was the least suitable. Three sizes of sclerotia tested were found to be equally effective in causing plant losses. Sclerotia inocula were comparable with mycelial discs and colonized barley grains in causing pre-emergence damping off under aseptic *in vitro*

[1] The material in this chapter was co-authored by M. E. Haque, D. Lakshman (USDA, ARS, Baltimore, Beltsville, MD 207052-350), Aiming Qi (School of Life and Medical Sciences, University of Hertfordshire, Hatfield, AL10 9AB, UK), M. Z. R. Bhuiyan1, Y. Liu, and P. Hakk, and M. F. R. Khan (University of Minnesota, St. Paul, MN USA). M. E. Haque had primary responsibility for collecting samples in the field and for interviewing users of the test system. M. E. Haque was the primary developer of the conclusions that are advanced here. M. E. Haque also drafted and revised all versions of this chapter. M. F. R. Khan served as proofreader and checked the math in the statistical analysis conducted by M. E. Haque.

conditions. Sclerotia also were equally or more effective than mycelia plug or barley grain inocula in reducing seedling emergence, damping off, and increased root rot ratings under greenhouse *in vivo* conditions. To conclude, sclerotia can be prepared on a feasible scale and used as natural inocula to screen response to *R. solani* in sugar beet.

Keywords: *Beta vulgaris*, Inoculum, Damping-off.

Introduction

Sugar beet (*Beta vulgaris*, L.) contributes approximately 20% of worldwide sugar production while sugarcane contributes the rest (Dohm et al., 2014). In the US, sugar beet accounts for 55% of total sugar production (USDA, ERS, 2019). *Rhizoctonia solani* Kühn is among the soil-borne pathogens that affect sugar beet stands and sugar yields. This genetically complex soil-borne fungus causes pre- and post-emergence damping-off, and root and crown rot (Windels and Nabben 1989; Harveson et al., 2006). *R. solani* has 13 anastomosis groups (AGs; AG 1 to AG 13), some of which are host-specific while others have wide host ranges (Carling et al., 2002; Parmeter et al., 1969). Sugar beet is prone to the AG 2-2 strain (Parmeter et al., 1969). The main AG subgroup that causes significant yield losses is AG-2-2 IIIB (Brantner and Windels 2009; Windels et al., 1997). The primary inocula of the pathogen in nature are mycelia and sclerotia. This pathogen can survive in the soil for many years in the form of sclerotia (Sherwood 1967; Adams and Papavizas 1970; Papavizas 1970). The sclerotia germinate under humid conditions and are often stimulated to germinate by the root exudates of seedlings (Flentje et al., 1963). The host-pathogen interactions are generally initiated by mycelia that penetrate into the root cortex causing infections to the tissue (Armentrout and Downer 1987; Christou 1962). *Rhizoctonia* symptom phenotyping in the field often varies due to biotic and abiotic factors (Bolton et al., 2010; Behn et al., 2012). *R. solani* rarely produces basidiospores/sexual spores but they do not produce any asexual spores or conidia

(Cubeta and Vilgalys 1997). Traditionally, Rhizoctonia colonized-barley-grains are prepared in the laboratory and used for greenhouse as well as field study (Ruppel et al., 1979; Behn et al., 2012). The barley act as a carrier that contains the crusted form of mycelia, nevertheless, the artificial barley inoculum often does not simulate the pathogenic aggressiveness of *R. solani* in evaluation of sugar beet cultivars in the fields. This can also be difficult to relate disease severities obtained with colonized-barley-grains inocula in greenhouse studies to those results with colonized-barley-grains inocula in evaluation of sugar beet cultivars in the fields (Paulitz 2002; Paulitz and Schroeder 2005; Mahmoudi and Ghashghaie 2013; Webb and Calderon 2015). In this research we tried to develop an alternative inoculum which is natural such as mycelia and sclerotia *in vitro*. Since *R. solani* generally produces overwintering sclerotia, and it can be convenient to use the same number of fungal biomass for field or greenhouse evaluation of cultivar resistance or susceptibility to *Rhizoctonia*. As a novel approach to use of vegetative propagule such as mycelia or sclerotia of *R. solani* to simulate natural pathogenic infection and evaluate host resistance. This study evaluated the development of sclerotia on six different artificial media and then compared *Rhizoctonia* sclerotia, mycelia and colonized barley grains for infection severity on sugar beet cultivars in the greenhouse.

Materials and Methods

Fungal Isolates of *R. solani*

Five *R. solani* isolates were collected from affected sugar beets from a field in Hickson, North Dakota (ND), USA. Genomic DNAs (Norgen Biotek Corp, Fungi DNA Isolation Kit #26200) (Table B.1.) of the five isolates were used for polymerase chain reaction (PCR) with the internal transcribed spacer (ITS) (Sharon et al., 2008). Subsequently, PCR products were flushed by E.Z.N.A ®Cycle Pure Kit Omega Bio-tek (Norcross, GA) and four samples were sequenced by

GenScript (Piscataway, NJ). The sequences were identical, and BLASTn analysis showed 100% sequence homology to *R. solani* AG 2-2 IIIB (Genbank accession: MN128569). Those isolates were maintained on an amended clarified V8 (ACV8) culture medium.

Evaluation of Culture Media for Production of Sclerotia of *R. solani*

Microbial media play a significant role in the optimum mycelial growth and differentiation of different fungal species. Six media: amended clarified V8 (ACV8), 50% potato dextrose agar (50% PDA), 10% PDA, methylene-benomyl-vancomycin (MBV), cornmeal agar (CMA) and water agar (WA), were prepared following the "Manual of Microbiological Culture Media" (Difco and BBL Manual 2009) (Table B. 2.). The experimental design was a completely randomized design (CRD) with four replications. Mycelial discs (4.5 mm, Cork Borer) of *R. solani*, AG 2-2IIIB cut from the 7-day old mother colony were transferred onto each of the six media (Table B. 3). After inoculation and plates were sealed with parafilm, the plates were incubated at 23±2 ºC in incubator. This experiment was conducted twice. Radial growth was measured using a digital caliper (Pittsburg 6" Composite DC, Item 93293) at four time points after transfer: 2-day, 4-day, 6-day, and 8-day, respectively. The number of sclerotia was counted at four-time points after transfer: 7-day, 14-day, 21-day, and 28-day, respectively.

Evaluation of Sclerotia Size Effects on Damping-Off

Three groups of sclerotia were categorized with a measuring scale based on their size: large (≥4.00 mm), medium (<4.00 mm but ≥2.00 mm), and small (<2.00 mm but ≥0.5 mm). To evaluate the effect of sclerotia size on inoculum potential, an experiment was conducted in a humidity chamber at 25°±2º C and 85% relative humidity. Plastic pots (27 x 13 x 13 cm, T.O. Plastics, Inc.; Clearwater, MN, USA) were filled with vermiculite and perlite mixer (PRO-MIX FLX) amended with osmocote (N-P-K:15-9-12) fertilizer (Scotts Company; Marysville, OH). Ten sugar beet seeds

(Crystal 101RR) were sowed at 2 cm deep furrow at 1 cm apart (Noor and Khan 2015) for each treatment. One sclerotium was placed, next to each seed at the same depth and covered with mixer. Three-size groups of sclerotia were used exclusively in furrow to inoculate sugar beet cultivar Crystal 101RR, with a completely randomized design of four treatments (including non-inoculated check) with four replications. Four plastic pots were considered for each treatment/category. This experiment was conducted twice. The seedlings damping-off were counted at 14 days post-inoculation (dpi).

In Vitro Inoculation on PDA Using Three Forms of _R. solani_ Inocula

To compare the efficacies of the three forms of inocula- sclerotia, mycelia and colonized-barley grains- sugar beet seeds of Crystal 101RR were co-cultured with each form of inoculum on PDA with four replications. A non-inoculated check with four replications was used as a control. Sugar beet seeds were washed with 70% ethanol for 1 minute and rinsed twice with sterile water. Seeds were dried on sterile blotter paper under the laminar airflow cabinet. Three seeds were placed with sterile forceps at 1 cm apart on each culture plate followed by each form of inocula being placed close to each seed. This experiment was conducted in a growth chamber at 25°±2º C. This experiment was conducted twice. Germination data were recorded at 7 days post inoculation (dpi).

Greenhouse Evaluation of Cultivars' Susceptibility to _Rhizoctonia_ Inocula

To determine if inoculum type had an effect on cultivar response to Rhizoctonia root rot, seven commercial sugar beet cultivars and four forms of inocula were arranged in CRD with four replications in a greenhouse. Root rot ratings (figures in brackets) of these seven commercial cultivars were reported (sbreb.org/research/, Research Report 2018) as follows: Crystal 101RR (4.50), Crystal 467RR (3.94), Hilleshog 4302RR (3.71), Maribo MA 504 (4.25), BTS 8606 (4.24),

BTS8500 (4.36) and BTS80RR52 (3.96). Three forms of Rhizoctonia inocula, colonized-barley grains, sclerotia, and mycelia plug, along with autoclaved-barley grains as a control, were used to inoculate each cultivar. Plastic pots (27 x 13 x 13 cm, T.O. Plastics, Inc.; Clearwater, MN, USA) were filled with vermiculite and perlite mixer (PRO-MIX FLX) amended with osmocote (N-P-K:15-9-12) fertilizer (Scotts Company; Marysville, OH). Ten sugar beet seeds of each cultivars were sowed at 2 cm deep furrow at 1 cm apart (Noor and Khan 2015). One colonized-barley grain, one sclerotia, one mycelial plug and one autoclaved barley seed as mock inoculation was placed, respectively, next to each seed at the same depth and covered with mixer. The greenhouse temperature during the experiment period was 25 ± 2ºC, with 80% relative humidity, and a 12-hour photoperiod. Plants were watered as needed to maintain adequate soil moisture conducive for plant growth and disease development.

The seedling emergence, and damping-off were recorded at 14 days post-inoculation (dpi) and 42 dpi, respectively. At 56 dpi, plants were removed from pots, and roots were washed and rated for root rot disease severity using a modified 1-7 rating scale, where 1 = clean roots and no infection, 2 ≤ 5% of root surface with black/brown symptoms, 3 = 5-25% of root surface with black/brown symptoms; similarly, 4 = 26-50% , 5 = 51-75%, 6 = 75-100% of root surface with black/brown symptoms, and 7 = dead plants (withered) (Ruppel et al., 1979).

Statistical Analyses

Experiments were conducted twice as a complete randomized design (CRD) with four replicates. Levene's test of homogeneity of variances was performed across the two experiments before the data were combined. Data in all the experiments were analyzed using R-studio (Version 3.6.1, St. Louis, Missouri, USA). The data were subjected to analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (LSD) was used to separate treatment means using

the same R-package (3.6.1). Treatment means were distinguished by the calculated Fisher's LSD at p = 0.05 probability level. Non-parametric analysis (Kruskal-Wallis test and Pairwise. Wilcox. Test) in R was performed for categorical data (root rot ratings) across the type of inocula and for the cultivars.

Results

Culture Media Suitability for Radial Growth and Sclerotia Production

Six culture media were used to identify their effects on the radial growth of mycelia. There was a significant difference among the media, time points, interaction effects (Culture media × Days) on the radial growth of mycelia, and the number of sclerotia (Table B.4) at $p \leq 0.001$.

A mean comparison test was performed for radial growth, and the number of sclerotia developed across six media. The mean radial growth of mycelia was significantly varied over the four different time points (LSD=0.06). The highest and lowest mean radial growth were observed in ACV8 and WA media, respectively (Fig. C. 1. A). Mycelial growth was fast between the 2 to 4-day period in all media, and later advanced more slowly. The interaction effect (culture media × time points) was significant for the mycelial growth (Table B.4).

Six culture media were used to identify their respective effects on the number of sclerotia. The ANOVA Table B.4. showed significant variation among the culture media at $\alpha = 0.05$, with the least significant difference 4.29 at 95% confidence level. The mean number of sclerotia varied significantly over the four different time points (Fig. C.1.B). The lowest and maximum number of sclerotia were recorded at 7 days and 28 days, respectively (Table B.4). The highest mean number of sclerotia was observed in ACV8 (152.62), followed by CMA (80.77). No sclerotia were observed in WA (Fig. C.1.B). The lowest mean number of sclerotia was observed in 10% PDA

(12.5). The interaction effect (culture media × time points/days) showed a significant variation on the mean number of sclerotia, at $\alpha = 0.05$ according to LSD 7.91 at 95% confidence level.

Inoculum Potentials of Different Sizes of Sclerotia

Mean comparison tests showed that all sizes of sclerotia significantly reduced plant stands at 14 dpi compared with the control treatment ($p<0.05$), and they all caused the same 60% of plant losses ($p>0.05$) (LSD = 1.15 at $p=0.05$) (Fig. C. 3). The inoculum potentials of sclerotia sizes had equal capacities to infect sugar beet seedlings.

Observing Damping-Off *In Vitro*

The three different forms of *R. solani* inocula tested all resulted in visual damping-off at 7 dpi, while 100% seedling emergence was observed in the non-inoculated check (Fig. C. 4.). All three inoculum types yielded a similar severity of damping-off symptoms under *in vitro* conditions. Notably, damping-off defined as seeds were not germinated, and germinated by wilted.

Effect of Forms of *Rhizoctonia* Inoculum on Sugar Beet Cultivar Response in The Greenhouse

The effects of inoculum forms on cultivars were significantly different on percentage of emergence at 14 dpi ($p<0.05$) and percentage damping off at 42 dpi ($p<0.05$) (Table B.5.). Among the three inoculum forms, sclerotia inoculum resulted in the lowest mean emergence (42.86%) when averaged across all cultivars, with the non-inoculated check showing the highest mean emergence (96.07%) (Table B.5.). Colonized barley grains and mycelial forms resulted overall emergence 64.29% and 54.29%, respectively for all cultivars. The highest mean emergence was observed in Maribo MA 504 (72.50%), followed by BTS 8500 (69.38%) (Table B.6.). There was overall similar significance level between in the mean emergence of BTS 80RR52 and BTS 8600 (66.25%). The lowest mean emergence was found in Crystal 101RR (54.38), which was followed

by Crystal 467RR (58.13%) (Table B.6.). Among the three forms of *Rhizoctonia* inocula, the highest damping-off was observed with sclerotia (74.29%), followed by mycelial inoculum (68.50%), when averaged across all cultivars (Table B.6.). The lowest overall mean damping-off was found in colonized barley inocula (54.29%). The highest mean damping-off was found in Crystal 101RR (55.01%), followed by BTS8500 (54.39%). The lowest mean damping-off was observed in Maribo MA 504 (44.37%) and BTS 80RR52 (44.38%). The percent stand for each inoculum source and cultivar was directly and inversely correlated with percent damping-off (i.e, if damping-off was 60%, percent stand was 40%) (Table B.6.).

The effects of inoculum forms on root rot ratings at 56 dpi were significant ($p<0.05$)(Kruskal-Wallis Chi-squared = 76.598, df = 3, p-value < 2.2e-16). Our analyzed p value (p-value < 2.2e-16) was smaller that suggest there were significant difference among the inocula forms. It further suggests to run pairwise.wilcox.test in R (Table B.9). This helps to differentiate the individual treatment or inocula effects on the cultivar. Mycelia and sclerotia were statistically non-significant. It showed p-value of 0.34 which was higher than 0.05. Sclerotia and colonized barley grains were statistically significant, since the pairwise p-value (0.0009) was smaller than 0.05 (Table B.9).

There was highest mean root rot with sclerotia, when averaged across all cultivars, while the colonized barley grains had lowest mean root rot ratings (Table B. 8.). When averaged across the three inoculum methods, root rot ratings varied among cultivars (Table B. 8). BTS 8606, BTS 8500, and Crystal 101RR had higher root rot ratings than the other four cultivars tested in the greenhouse (Table B.7.).

Discussion

Six culture media were evaluated to determine the most effective *in vitro* method of large-scale formations of sclerotia by *R. solani*. Hardar et al. (1981) demonstrated that sclerotia formation of *Sclerotium rolfsii* is induced in agar media within 3 days following scratching with scalpel to aerial mycelia. We demonstrated that ACV8 is a promising medium to produce *Rhizoctonia* sclerotia on a large scale, without the necessity of scratching the mycelia. The size of the sclerotia in our study varied significantly among the different culture media but the merits of inoculation potentials of different size of sclerotia remained similar (Garrett, 1956). This experiment showed an *in vitro* technique to prepare large scale sclerotia for pathogenic investigations.

Overwintering sclerotia, as well as melanized and moniloid mycelia are the primary source of infection during the seed germination stage in the field (Boland et al., 2004; Lee and Rush 1983). However, most previous pathogenetic studies were on adult beet plants using artificial inocula, mostly consisting of blended *Rhizoctonia* mycelia or *Rhizoctonia*-colonized cereal grains. Thus, those screening methods discounted the seed germination/seedling stage, which is the most vulnerable to stand losses due to damping off by *R. solani*. Liu et al. (2019) also demonstrated that most commercial resistant sugar beet cultivars are highly susceptible to *Rhizoctonia* damping off at the seed germination stage.

In vitro inoculation study with three different types of *R. solani* inocula showed that the pathogenesis of sclerotia and the mycelial plug was better than colonized-barley grains in causing damping-off. This result demonstrated a novel approach of in vitro inoculation with three different forms of *R. solani* inocula in PDA media for studying host-pathogen interaction. This study demonstrated the use of sclerotia or mycelial plugs as a substitute for colonized barley/wheat/oat

grains for evaluating the disease ratings of the commercial cultivars, as well as to simulate natural infection in the greenhouse.

In vivo inoculation studies in the greenhouse showed that all three forms of *Rhizoctonia* inocula were virulent and capable of damaging sugar beet plants. Inoculation with sclerotia showed more severe damping-off and root rot in the tested cultivars compared with the colonized-barley grains. This finding is in line with observations that sclerotia cause seed rot or pre-emergence damping-off by other research groups (Gaskill 1968; Naito and Makino 1995). This study was the first attempt to evaluate varietal resistance against *Rhizoctonia* damping-off on commercial cultivars using sclerotia and mycelial plugs. The results of this study suggest that sclerotia and mycelial plugs can be used as natural inocula to substitute for colonized barley grains in evaluating varietal resistance prior to release as a commercial cultivar. Recently, Liu et al. (2019) observed that sugar beet cultivars were highly susceptible to *R. solani* prior to attaining the six- to eight-leaf stage (4-5 weeks) after planting, regardless of the assigned level of resistance. The important response indicators of sugar beet cultivars include damping-off, root rot severity index, and stand count. Maribo MA 504 and BTS 80RR52 showed significantly lower damping-off compared to all other cultivars. Both cultivars showed the highest stand count and lowest root rot. This finding suggested that both Maribo MA504 and BTS 80RR52 can be used as resistant cultivars. Likewise, Crystal 101RR and BTS8500 were most susceptible among cultivars to damping-off. However, cultivation of Crystal 101RR and BTS 8500 in areas with existence of *R. solani* can be possible if the seed is protected by using fungicides. For example, use of a recommended dose of azoxystrobin in-furrow or another labeled fungicidal treatment is advised during the early growth stage, regardless of varietal resistance (Khan 2018).

Sclerotia mediated damping-off in greenhouse studies showed significant variation in response among the cultivars. This emphasized the need to screen cultivars in the early stage of growth with sclerotia/mycelial plug inoculation to get a more reliable resistance rating than seen with adult plant screenings. Although growers in North America and Europe commonly use effective fungicides such as sedaxane or seeds coated with fungicides to control post-emergence damping-off (Liu and Khan 2016), the seed companies need to consider age-dependent inoculation of plants for better evaluation of cultivars against susceptibility to *R. solani*.

In conclusion, an understanding of *Rhizoctonia* aggressiveness with natural inocula (sclerotia or mycelia) and screening of cultivars at the seed germination stage are essential for successful *R. solani* management. Future studies should evaluate interactions of cultivars at the seedling stage with other anastomosis groups of *R. solani* in order to minimize both stand and yield losses in sugar beets.

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CHAPTER THREE. *PENICILLIUM PINOPHILUM* CAN MITIGATE *RHIZOCTONIA SOLANI* DAMPING-OFF IN SUGAR BEET[2]

Abstract

Rhizoctonia solani is an economically important pathogen of sugar beet (*Beta vulgaris* L.) causing seedling damping-off, and root and crown rot. Cultural practices, partially resistant cultivars, and fungicides are among the methods used to manage *R. solani*. There has not been much success in using biological control for this pathogen in sugar beet. The objective of this research was to determine the efficacy of the bio-control potential of *Penicillium pinophilum* against *R. solani* AG 2-2 under laboratory and greenhouse conditions. *In vitro* co-culture of both fungi showed that *R. solani* growth was inhibited by *P. pinophilum*. A greenhouse inoculation study was done using sclerotia of *R. solani* and a conidia suspension of *P. pinophilum* to evaluate the response of a Rhizoctonia susceptible cultivar (Crystal 101RR). Treatments included *R. solani* sclerotia, *P. pinophilum* conidia suspension (1×10^6 conidia/ml), a combination of sclerotia with conidia suspension (1×10^6 conidia/ml), and a mock inoculation with water (control). One 2-cm deep furrow was made in the middle of peat filled trays into which 10 seeds were planted. Each treatment was applied adjacent to each seed and covered with peat. There were four replicates per treatment arranged in a completely randomized design. The sole sclerotia treatment caused 75% damping-off while the combination of sclerotia with *Penicillium* conidia suspension reduced damping-off by 75%. No damping-off incidences were observed with the *Penicillium* conidia

[2] The material in this chapter was co-authored by M. E. Haque, D. Lakshman (USDA, ARS, Baltimore, Beltsville, MD 207052-350), Aiming Qi (School of Life and Medical Sciences, University of Hertfordshire, Hatfield, AL10 9AB, UK), and M. F. R. Khan (University of Minnesota, St. Paul, MN USA). M. E. Haque had primary responsibility for collecting samples in the field and for interviewing users of the test system. M. E. Haque was the primary developer of the conclusions that are advanced here. M. E. Haque also drafted and revised all versions of this chapter. M. F. R. Khan served as proofreader and checked the math in the statistical analysis conducted by M. E. Haque.

suspension or the mock-inoculated control. It was concluded that *P. pinophilum* has the potential to reduce damping-off caused by *R. solani* and its mitigation mechanisms need further studies.

Keywords: Biological control, *Beta vulgaris*, Antagonistic, *R. solani* inocula.

Introduction

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris* [Frank] Donk) is a necrotrophic pathogen that causes damping-off, and Rhizoctonia crown and root rot diseases in sugar beet (*Beta vulgaris,* L.) (O'Brien 1996). This pathogen is of monocyclic infection and it overwinters in the soil and on crop debris as sclerotia (Sherwood 1967; Adams and Papavizas 1970; Papavizas 1970). Sclerotia germinate to form infective hyphae that penetrate into the root cortex and cause infections to the tissue (Armentrout and Downer 1987; Armentrout et al., 1987; Flentje et al., 1963). This soil-borne fungus varies in morphogenetic diversity including hyphal fusion or anastomosis, virulence, cultural appearance, and physiology of the biotypes (Carling et al., 2002; O'Brien 1996). There are 13 anastomosis groups (AGs) of *R. solani* (Carling et al., 2002; Parmeter et al., 1969), while the main AGs detrimental to sugar beet in Minnesota and North Dakota are AG 2-2 IIIB and AG 2-2 IV (Brantner and Windels 2009; Windels et al., 1997; Windels and Nabben 1989). Other AGs and sub-groups, including AG 4, AG 1, and AG 5 have also been reported in other US states but at low frequency (Windels et al., 1997). *R. solani* has been reported to reduce sugar beet yield by an average of 2% when combined with use of crop protection, while in the yield loss can be 30% to 50% (Neher and Gallian 2011).

Severities of damage caused by *R. solani* depend on characteristics of the AG, host, and environment. Integrated pest management (IPM) strategies are considered essential for minimizing disease severity. Cultural strategies such as crop rotation at least every third year with non-host cereal crops such as barely, wheat, oats, and corn are the best treatment to reduce primary inoculum

of *R. solani* (Behn et al., 2012; Boine et al., 2014; Buhre et al., 2009; Buttner et al., 2002; Dircks et al., 2014). Nevertheless, some AGs have a polyphagous nature to surmount this strategy, such as AG 2-2IIIB which has a wide range of hosts including corn and soybean (Engelkes and Windels 1996; Ithurrart et al., 2004). In the US, private breeding companies are developing varieties resistant to *R. solani,* and in many cases, they do not want to disclose the background of the gene. It takes time to develop quantitative resistant cultivars against *R. solani* even though resistant cultivars show poorer potential yields than susceptible commercial cultivars (Panella and Ruppel 1996; Ruppel et al., 1995). Chemical strategies such as seed treatment (Penthiopyrad), and in-furrow application of fungicides (azoxystrobin, pyraclostrobin, prothioconozole) at planting provide effective control in greenhouse and field research (Khan et al., 2017; Khan et al., 2010; Liu and Khan 2016; Secor et al., 2010). Among the quinone outside inhibitor (QoI) fungicides, Azoxystrobin, is the most widely used fungicide in major sugar beet growing states such as Minnesota, North Dakota, Montana, and Michigan (Harveson et al. 2002; Kirk et al. 2008). Although chemical controls provide some degree of control, fungi often develop resistant biotypes under selection pressure when used repeatedly in commercial fields. Recently, QoI resistance has been reported in AG2-2IIIB in turfgrass, and AG 3 in potato (Blazier and Conway 2004; Djebali et al., 2014; Olaya et al., 2012).

Alternatively, biocontrol strategies are environmentally safe, generally pose little risk of developing resistant biotypes, nevertheless, there has not been much success achieved in controlling *R. solani* in sugar beet with biocontrol agents in the field. Soil bacteria such as Rhizobacteria has shown the suppression effect on inoculum density of *R. solani* (Homma, 1996). Commercial preparation of *Bacillus subtilis,* Kodiak has been used to reduce *R. solani* AG 2-2 IIIB infection in sugar beet (*Beta vulgaris*). *Bacillus* strain MSU-127 and low rate of Azoxystrobin

used in-furrow application improved sugar beet by about 16% and a foliar fungicide application at the 4-leaf stage also increased root yield by 17% (Jacobsen et al., 1997; Kiewnick et al., 2001). Other greenhouse study on *Bacillus amyloliquefaciens* SB14 strain showed to reduce damping-off disease by 58% caused by *R. solani* AG-4 and 52% caused by *R. solani* AG 2-2 on sugar beet (Karimi et al., 2016). Thus, research study demonstrated that biocontrol agents are often convenient to use in greenhouse rather than to use in the field.

Recently, field application of *Bacillus subtilis* found to be effective to control late blight of potato caused by *Phytophthora infestans* (Kumbar et al., 2019). Other research showed that *Bacillus velezensis* LHSB1 strain controlled peanut stem rot caused by *Sclerotium rolfsii* (Chen et al., 2020).

Furthermore, the biocontrol potential of *Penicillium pinophilum* (*Talaromyces pinophilus)* has been reported in reducing soil-borne pathogens *Pythium* and *Rhizoctonia*-induced damping-off in cucumber (Kazerooni et al., 2019). The objective of this research was: to evaluate the biological potential of *P. pinophilum* in controlling *R. solani*-induced damping-off in sugar beet.

Materials and Methods

Fungal Isolates of *R. solani* and *P. pinophilum*

Clones of *R. solani* AG 2-2 IIIB (Genbank accession: MN128569), isolate #MN569 was maintained on amended clarified V8 (ACV8) (25º C ± 2º C). Sclerotia and mycelia were developed from subcultures on ACV8, and were used for in vitro and in vivo study.

Five isolates of *P. pinophilum* were obtained from sugar beet tap roots collected in 2018 from a field in Moorhead, MN (46.8738° N, 96.7678° W). The fungal colonies were observed with blue-green velvety and white margins on the root periphery. Conidia were hyaline, globose, and conidiophores were densely penicillated. The morphological characteristics of the fungus closely

matched those of *Talaromyces* species (Yilmaz et al., 2014). A single spore isolation method was used to prepare five independent isolates and genomic DNAs were extracted from those isolates. For the PCR assay, the internal transcribed spacer (ITS) ITS4/ITS5 primers were used to amplify the ITS genomic region. PCR products were cleaned via E.Z.N.A ®Cycle Pure Kit, OMEGA and sent to Sanger sequencing by GenScript (GenScript, Piscataway, NJ). A Blastn analysis of the ITS sequences of the five isolates showed 100% alignment to *Talaromyces pinophilus* (*Penicillium pinophilum*), accession no. AB455516.1 (596 bp genomic sequence) with E-value of 0. The amplified genomic sequence (539 bp) was submitted to NCBI (GenBank accession no. MK757839.1). Conidia suspension of *P. pinophilum* were prepared from the clone of MK757839, and were used for *in vitro* and *in vivo* study.

In Vitro Co-culture of Two Forms of *R. solani* Inocula and Conidia of *P. pinophilum* on 50% Potato Dextrose Agar (50% PDA)

To understand the potential of *P. pinophilum* as a growth suppressor to *R. solani*, two forms of *Rhizoctonia* inocula – sclerotia and mycelial plug (6 mm^2) were individually co-cultured with conidial suspensions of *P. pinophilum* (1×10^6 conidia/ml) on 50% PDA with four replicates. Each replicate contained 4-sclerotia or 4-mycelial plugs, one in each quarter of the culture plate using sterilized forceps. *P. pinophilum* conidia suspensions of 200 µl were transferred immediately adjacent to each sclerotium/mycelium plug using a dropper. Four replicates of non-conidia suspension (only autoclaved water) were used as mock-inoculations that contained only sclerotia or only mycelial plugs, and were arranged in the plates as described above. All the plates were sealed with parafilm and kept in an incubator at 25±2ºC. This experiment was conducted twice. Microscopy was performed using VWR N. A. 0.30 at three time points: 4, 5, and 6 days post treatment initiation.

In Vitro Inoculation of Seeds on 50% PDA Using *R. solani* and *P. pinophilum*

The efficacy of *P. pinophilum* as a biocontrol agent of *R. solani* on sugar beet seeds placed on 50% PDA plates was evaluated. Four treatments were included: (1) one mycelial plug of *R. solani*/seed; (2) conidiophore plug of *P. pinophilum*/seed; (3) mycelial plug of *R. solani*/seed+ conidiophore plug of *P. pinophilum*; and (4) non-inoculated seeds. Sugar beet seeds were washed with 70% ethanol for 1 minute and rinsed twice with sterile water. Seeds were then dried on sterile blotter paper under the laminar airflow cabinet. Three seeds were placed with sterile forceps at 1 cm apart on each culture plate followed by each form of inocula being placed close to each seed. Four replicates per treatment were evaluated. All the plates were wrapped with parafilm and kept in a growth chamber at 25±2ºC. This experiment was conducted twice. Germination observations were recorded at 7 days post inoculation (dpi).

Greenhouse Evaluation of Antagonistic Potential of *P. pinophilum* to *Rhizoctonia* Inocula

A greenhouse study was done to further evaluate the potential of *P. pinophilum* in reducing *Rhizoctonia* damage. Four treatments were applied to a *Rhizoctonia* susceptible cultivar - Crystal 101RR (root rot rating of 4.5 and considered not to have either quantitative nor qualitative resistance to Rhizoctonia Root Rot) as follows: (1) one *R. solani* sclerotium; (2) *P. pinophilum* conidia suspension (1×10^6 conidia/ml); (3) combination of sclerotium with conidia suspension (1×10^6 conidia/ml), and (4) mock-inoculation (autoclaved water) per seed. Plastic pots (27 x 13 x 13 cm, T.O. Plastics, Inc.; Clearwater, MN, USA) were filled with vermiculite and perlite mixer (PRO-MIX FLX) amended with osmocote (N-P-K:15-9-12) fertilizer (Scotts Company; Marysville, OH) and the pots were arranged in a completely randomized design. Ten sugar beet seeds were sowed in each plastic pot in 2 cm deep furrow at 1 cm apart (Noor and Khan 2015). Four replicates per treatment were evaluated. Each treatment was applied next to each seed at the

same depth and covered with mixer. The greenhouse temperature during the experiment period was 27 ± 2ºC, with 80% relative humidity, and a 12-hour photoperiod. Plants were watered as needed to maintain adequate soil moisture conducive for plant growth and disease development.

Seedling emergence and damping-off were recorded at 14 days post-inoculation (dpi) and 28 dpi, respectively. Percent stand counts and root rot ratings data were collected at 42 dpi. At 42 dpi, surviving plants were removed from pots, and roots were washed and rated for root rot severity using a modified 0-7 rating scale, where 0 = clean roots and no infection, 1 = ≤ 10% of root surface with black/brown symptoms, 2 = ≥10-20% of root surface with black/brown symptoms, 3 = ≥20-30% of root surface with black/brown symptoms; similarly, 4 = ≥30-40% , 5 = ≥40-50%, 6 = ≥50-60% of root surface with black/brown symptoms, and 7 = ≥ 60% dead plant (withered) (Ruppel et al., 1979).

Statistical Analyses

Experiments were conducted twice as a complete randomized design (CRD) with four replicates. Categorical/discrete root rot severity data were transformed to a percent of disease severity index (%DSI) using the following modified formula: $\%DSI = \left[\frac{\{(a\times0)+(b\times1)+(c\times2)+(d\times3)+(e\times4)+(f\times5)+(g\times6)+(h\times7)\}}{\{(a+b+c+d+e+f+g+h)\times i\}}\right] \times 100$, where *a*, *b*, *c*, *d*, *e*, *f*, *g*, and *h* represent the number of plants with disease scores of 0, 1, 2, 3, 4, 5, 6, and 7, respectively, and *i* represents the highest root rot severity rating (Li et al., 2014). Levene's test of homogeneity of variances was done to determine whether two trials could be combined for analysis. Data were analyzed using R-studio (Version 3.6.1, St. Louis, Missouri, USA). Fisher's Protected Least Significant Difference (LSD) was used to separate treatment means using the same R-package (3.6.1). Treatment means were distinguished by the calculated Fisher's LSD at p = 0.05 probability level.

Results

In Vitro Growth Inhibition of *R. solani* Inocula by *P. pinophilum* on 50% PDA

Co-culture of the two fungi showed a consistent growth suppression of *R. solani* inocula (sclerotia and mycelia) by propagules of *P. pinophilum* (Fig. E.1.). Microscopic examination showed that *P. pinophilum* inhibited the hyphal proliferation of *R. solani* over time, as shown at 4, 5, and 6 days post treatment initiation (Fig. E. 2.). In the plates without *P. pinophilum*, *R. solani* sclerotia and mycelia proliferated vigorously on 50% PDA. Both the independent culture of sclerotia and mycelia initiated sclerotia production at 14 days post treatment initiation (Fig. E.1. A and Fig. E.1. C), while no sclerotia were observed with the *P. pinophilum* conidia suspension treatment (Fig. E.1. B and Fig. E.1. D).

In Vitro Inoculation of Seed with *P. pinophilum* Reduced *R. solani* Damping-off

Co-cultivation of sugar beet seed and mycelia of *R. solani* demonstrated 100% damping-off at 7 dpi in 50% PDA, while 90% seedling emergence was observed in the combined treatment of mycelia of *R. solani* with conidia suspension of *P. pinophilum*. No damping-off incidences were observed in the non-inoculated controls or the sole conidia treatments (Fig. E. 3.). The results show that conidia suspension suppressed mycelial proliferation, inhibited infections and mitigated damping-off under ambient conditions.

Greenhouse Evaluation of *R. solani* Mediated Damping-off via Conidia of *P. pinophilum*

Effects of treatments were significant ($p<0.05$) **(**Table 3.1.). At 28 dpi, the highest mean damping-off was 75% in the sclerotia treatment, while the mean damping-off was 25% in the combined treatment of sclerotia and propagules of *P. pinophilum* (Table 1). No damping-off incidences were observed in the mock-inoculated control and the treatment with only conidia of *P. pinophilum* (Table 3.1.). Overall, the treatments were significant for stand counts and root rot

rating ($p<0.001$). Table 3.1. showed the mean stand counts and root rot ratings (%DSI = % disease severity index) at 42 dpi. The highest mean stand count was observed in mock-inoculated control (95%), followed by the treatment with the sole conidia suspension (94%). The lowest mean stand count was 25% in the treatment with sclerotia. The combined treatment of sclerotia and conidia showed 75% stand count. Among the four treatments, the most severe mean root rot was observed in the treatment with *R. solani* sclerotia, while there was no root rot with the combined sclerotia and conidia of *P. pinophilum* treatment. Likewise, the mock-inoculated control and exclusive conidia suspension of *P. pinophilum* treatment did not show any root rot. Furthermore, the combined sclerotia and conidia of *P. pinophilum* treatment did not demonstrate negative effects on the root length, shoot length, fresh weight and dry weight (data were not shown here). All these parameters did not differ significantly when compared to those observed with the mock-inoculated control.

Discussion

This study demonstrated microscopically that mycelia growth of *R. solani* was inhibited by spore propagules of *P. pinophilum*. This study also demonstrated macroscopically that the production of *R. solani* sclerotia was inhibited in the combined co-culture of the two organisms. Conversely, monoculture of *R. solani* sclerotia and mycelia initiated new sclerotia production at 14 days post treatment initiation. This study provided evidence of inhibitory activity by *P. pinophilum* against *R. solani*, but the mechanism of this activity was not examined. Other researchers showed the antagonistic role of *Trichoderma harzianum* derived platelet-activating factor-acetylhydrolase (PAF-AH) in suppressing *R. solani* (Ibrahim 2017; Yu et al., 2014). Biocontrol agents generally involve in the production of antimicrobial compounds that inhibit the growth of other microorganisms.

Furthermore, *in vitro* co-cultivation of sugar beet seeds (i.e from a susceptible cultivar), *R. solani* inocula (mycelia), and propagules of *P. pinophilum* showed that this combined treatment at 7 dpi reduced damping-off by 80% compared to levels with co-cultivation of sugar beet seeds and mycelia of *R. solani* alone. Previously, the biological potential of *Talaromyces flavus* and *Trichoderma spp* have been demonstrated to control sugar beet damping-off disease in the greenhouse (Kakvan et al., 2013). The biological role of *P. pinophilum* has been reported recently to inhibit *Rhizoctonia* and *Pythium*-induced damping-off in cucumber (Kazerooni et al., 2019). Recent studies on *Streptomyces atrovirens* and *Trichoderma lixii* have shown the bio-control activity against *Rhizoctonia solani* in tomato (Solanki et al., 2019). Likewise, our study postulates the biological potential of *P. pinophilum* to control *R. solani* mediated damping-off in sugar beet. Other research has shown the potential of *T. flavus* and *Trichoderma spp* to control potato stem rot caused by *S. sclerotiorum* (Ojaghian 2011).

R. solani survives in soil as sclerotia or as melanized mycelia, forms which are the primary source of infection during the seed germination stage in the field (Boland et al., 2004; Lee and Rush 1983). We, therefore, preferred to use sclerotia in the greenhouse evaluations. We observed that sole sclerotia inoculation was aggressive and capable of causing the highest damping-off in 28 days. Others have also observed that sclerotia cause severe damping-off in sugar beet (Gaskill 1968; Naito and Makino 1995). In this study, the combined treatment of sclerotia and conidia of *P. pinophilum* shown 25% damping-off. Among the four treatments, the highest root rot was exclusively observed in the treatment of sclerotia. As expected the mock-inoculated check, and conidia suspension did not show any root rot. The combined treatment (Sclerotia-*R. solani* + Conidia of *P. pinophilum*) on Rhizoctonia susceptible cultivar did not show any root rot, either. These results provide evidence that the novel *P. pinophilum* significantly inhibited the root rotting

potential of *R. solani* and inhibited the growth of *R. solani* sclerotia. Therefore, the biocontrol agent *P. pinophilum* has the potential to be part of a *R. solani* management package in sugar beet in *R. solani* infested areas.

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APPENDIX A. LITERATURE REVIEW: SUPPLEMENTAL FIGURES

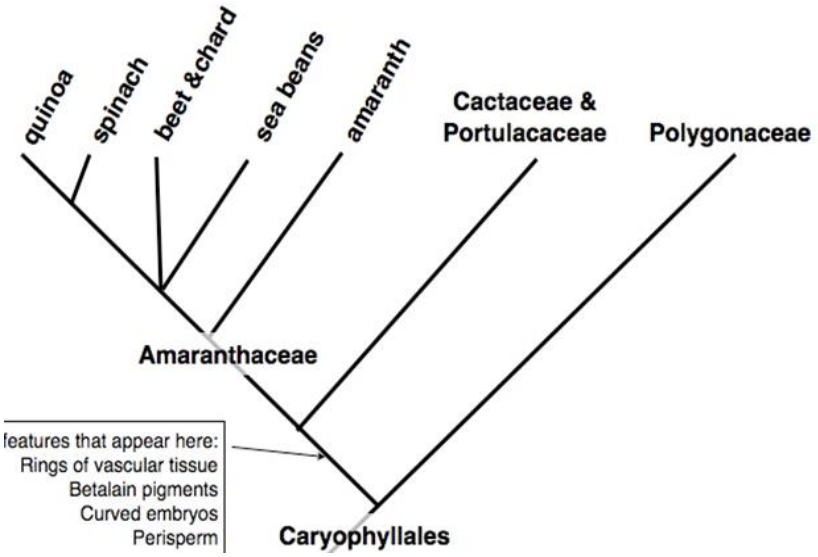

Figure A.1. Position of Amaranthaceae family under the order of Caryophyllales.

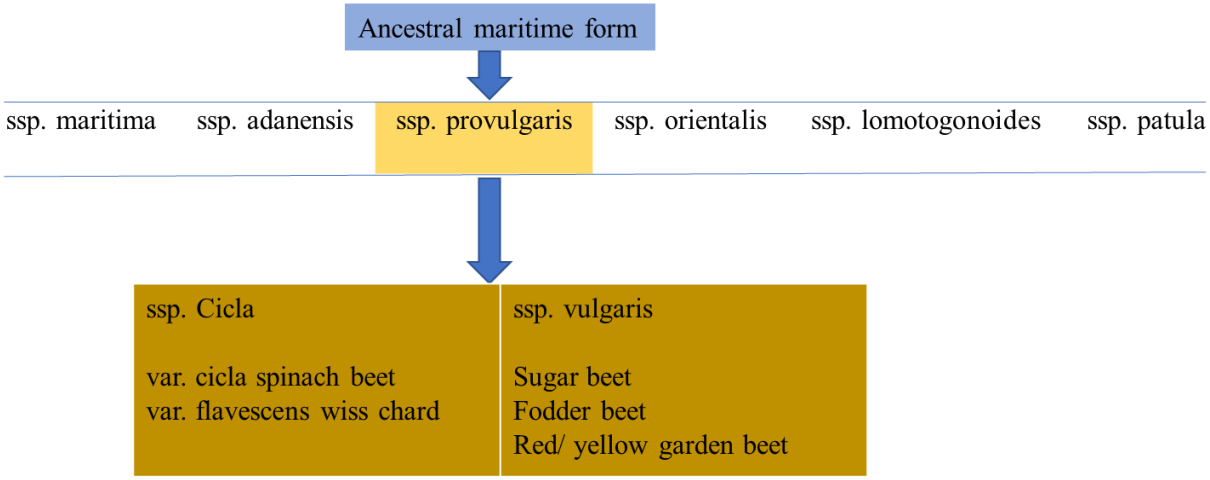

Figure A.2. A flow chart showing evolutionary affinities of *Beta vulgaris*, modified sketch of Ford-Lloyd and William (1975).

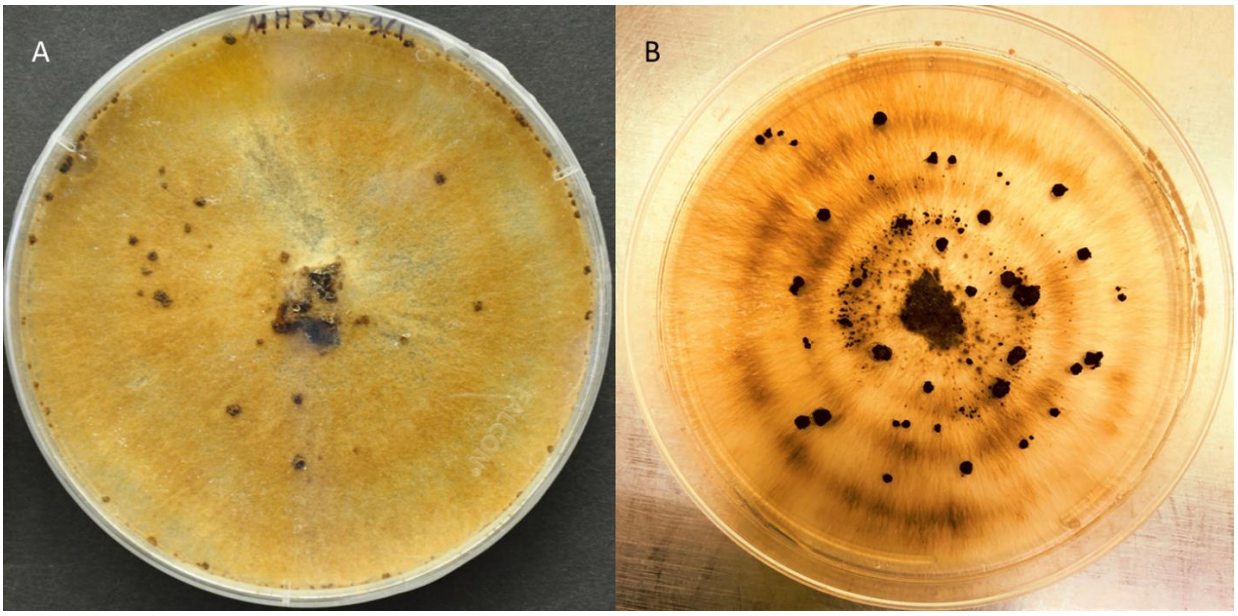

Figure A.3. *R. solani* in culture, A. potato dextrose agar (PDA) and B. amended clarified V8 (CV8) plate showing the formation of vegetative mycelium and sclerotia, respectively (Taken by Haque 2019).

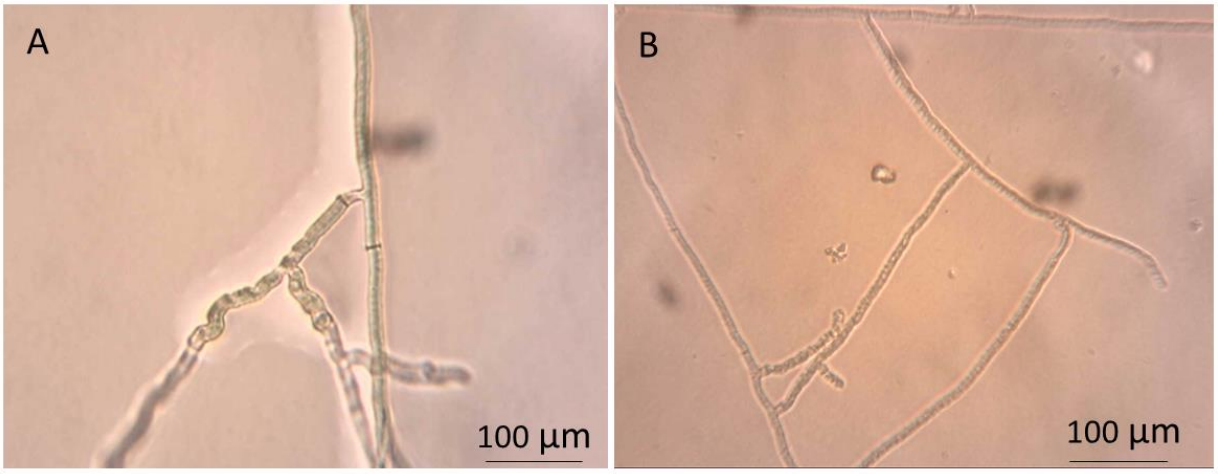

Figure A.4. Microscopic views of *R. solani,* A. hyphae, B. anastomosis reactions between two hyphal strands (Taken by Haque 2019).

Figure A.5. Dark brown/cankerous lesions below the soil surface and hypocotyl rot caused by *R. solani* in sugar beet A. Cotyledonary stage, B. Two leaf stage, C. Four leaf stage (Taken by Haque 2018).

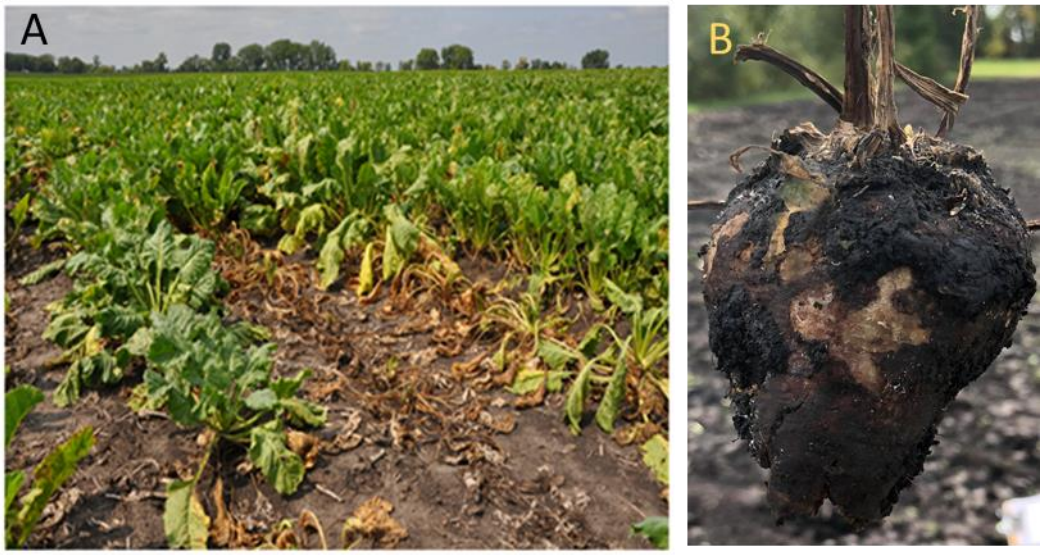

Figure A.6. A. *R. solani* infected sugar beet field, B. Rhozoctonia root rot of sugar beet (Taken by Haque 2018).

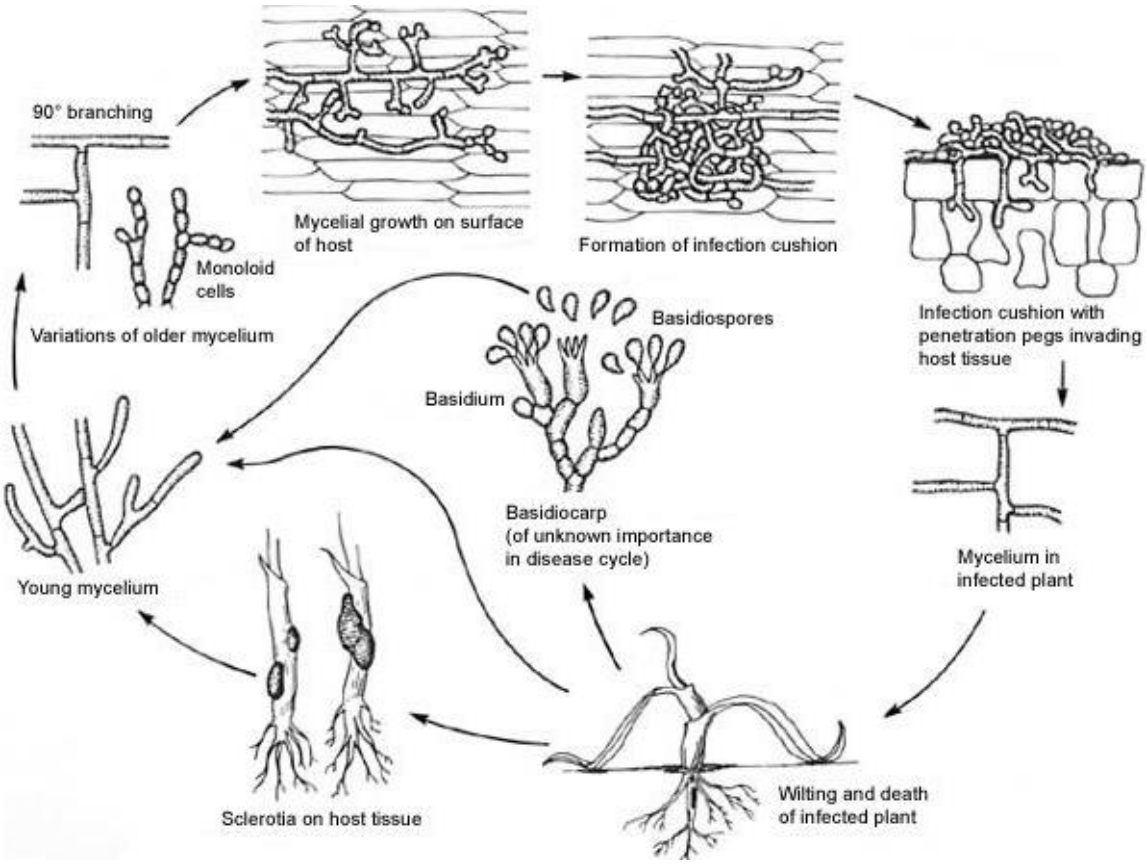

Figure A.7. A typical life cycle of *Rhizoctonia solani* (Collected from American Phyto pathological Society website accessed on 30 April 2018).

APPENDIX B. EVALUATION OF *RHIZOCTONIA SOLANI* SCLEROTIA MEDIATED PATHOGENICITY TO SUGAR BEET CULTIVARS IN GREENHOUSE STUDIES: SUPPLEMENTAL TABLES

Table B.1. Fungi DNA isolation protocol (Norgen Biotek Corp. Product # 26200, 26250)

Sl. No.	Major Steps		Description
1	Lysate preparation	1.	Placed ≤100 mg of fungi mass into a DNase-free 1.7 mL microcentrifuge tube, used beads (4/5) and added 500 µL of Lysis Buffer L and 1 µL of RNAse A to the sample immediately after homogenization and vortex for 20 seconds to mix.
		2.	Incubated at 65ºC for 10 minutes. Occasionally mixed the lysate 2- or 3-times during incubation by inverting the tube.
		3.	Added 100 µL of Binding Buffer I, mixed thoroughly and incubated for 5 minutes on ice.
		4.	Assembled a filter column with one of the provided collection tubes. Pipetted the lysate into Filter Column and spin for 2 minutes at 20,000 x g (~Transfer only the clear supernatant from the flow-through into a DNAase-free microcentrifuge tube using a pipette.14,000 RPM).
		5.	Added an equal volume of 70% ethanol to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortexed to mix. Proceed to Step 2.
2	Binding to Column	1.	Assembled a spin column to the provided collection tubes.
		2.	Applied up to 650 µL of the clarified lysate with ethanol onto the Spin Column and centrifuge for 1 minute at 10,000 × g (~10,000 RPM). Discard the flow through and reassembled the spin column with the collection tube.
3	Column Wash	1.	Applied 500 µL of Solution WN to the column and centrifuge for 1 minute at 10,000 × g (~10,000 RPM).
		2.	Discarded the flow through and reassembled the spin column with its collection tube.
		3.	Applied 500 µL of Wash Solution A to the column and centrifuged for 1 minute at 10,000 × g (~10,000 RPM).
		4.	Discarded the flow through and reassembled the spin column with its collection tube.
		5.	Spined the column for 2 minutes at 20,000 x g (~14,000 RPM) in order to thoroughly dry the resin. Discarded the collection tube.
4	DNA Elution	1.	Placed the column into a fresh 1.7 mL Elution tube provided with the kit.
		2.	Added 100 µL of Elution Buffer B to the column and incubated for 1 minute at room temperature.
		3.	Centrifuged for 1 minute at 10,000 x g (~10,000 RPM).
5	Storage of DNA	1.	The purified genomic DNA stored at 2-8°C for a few days. For longer term storage, -20°C is recommended.

Table B.2. Composition of six culture media

Media	Components--------- Quantity (g)/volume (ml)
ACV8 media	Agar: 15 g
(1L or 1000 ml)	Centrifuge V8 Juice: 100 ml
	Distilled Water: 900 ml
	Calcium Carbonate: 1 g/L
	Added Rifampicin @ 30 mg/L after autoclaved the media
50% PDA media	Agar: 15 g
	Potato Dextrose Broth: 12 g
	Distilled Water: 1000 ml
	Added 85% lactic acid @ 1ml/L after autoclaving media and pH adjusted to 3.5
10% PDA media	Agar : 15 g
	Potato Dextrose Broth: 2.4 g
	Distilled Water water : 1000 ml
	Added 85% lactic acid @ 1ml/L after autoclaving media and adjusted pH to 3.5
MBV media	Difco Bacto Agar : 10 g
	Difco Cornmeal Agar : 10 g
	Distilled water : 1000 ml
	Autoclaved and cooled to 45-50^{0}C and added Metalaxyl (30 mg), Benomyl (5 mg), Vancomycin (200 mg) and Amphotericin (0.5 mg)
CMA media	Corn Meal infusion : 2 g
	Agar :20 g
	Distilled Water water :1000 ml
Water Agar media	Agar :15 g
	Distilled water :1000 ml

Table B.3. Morphological characters of *R. solani*, AG 2-2IIIB in six different culture media

Characters	ACV8	50% PDA	10% PDA	MBV	CMA	WA
Colony color	brown to dark brown in the center	Light brown to dark brown in the center	Light Off white	Whitish	Whitish	Transparent
Texture	Uniform Distinct circular pattern of mycelia	Dense growth at central region but lightly loose at border	Dense at the border but very loosely distributed mycelia	Medium dense and distinct circular growth	Circular and densely arranged mycelia	Very loosely spread over the media surface
Number of sclerotia (max-min)	450-200	100-50	40-20	100-50	200-100	No
Position of sclerotia	Mostly in the center	Whole plate but less in center	Border not in the center	Center but very few at border	Whole plate	No
Size	Mostly large. 0.5 to 4 mm	Small to Medium	Medium 1-5 mm	Small to medium	Small to medium 0.5-5mm	No

Table B.4. Combined ANOVA for culture media, days and their interaction on radial growth and number of sclerotia (*R. solani*)

Parameters	**Sources of variation**	**Df**	**Mean Sq**	**F-value**	**Pr(>F)**
Radial growth	Culture media	5	6.69	88.43	< 2e-16 ***
	Days	3	151.806	2006.53	< 1.8e-12 ***
	Culture media × Days	15	0.406	74.7217	< 1e-10 ***
Number of sclerotia	Culture media	5	51089	1009.5	< 8.6e-16 ***
	Days	3	59378	1173.3	< 1.5e-12 ***
	Culture media × Days	15	12463	246.3	< 1.5e-16 ***

Significance. Codes: '***' 0.001, P<0.001

Table B.5. Combined ANOVA for *Rhizoctonia* inocula, cultivars and interaction effect on emergence, and damping-off.

Parameters	**Sources of variation**	**Df**	**Mean Sq**	**F-value**	**Pr(>F)**
Emergence at 14 dpi	*Rhizoctonia* inocula	3	14648.5	182.5179	2.235e-08 ***
	Cultivars	6	633.3	9.5000	8.938e-05 ***
	Cultivars × *Rhizoctonia* inocula	18	171.4	5.2258	1.105e-06 ***
Damping-off at 42 dpi	*Rhizoctonia* inocula	3	2870.24	65.1622	1.999e-06 ***
	Cultivar	6	580.65	11.5901	2.401e-05 ***
	Cultivars × *Rhizoctonia* inocula	18	222.32	6.5209	3.754e-08 ***

Significance. Codes: '***' 0.001, P<0.001

Table B.6. Percentage of seedling emergence at 14 dpi and percentage of damping-off at 42 dpi. Means followed by the same letters are not significantly different at p=0.05.

Indicator	Cultivar	Forms of inoculum				
		Colonized barley grains	**Mycelia**	**Sclerotia**	**Autoclaved barley grains**	**Cultivar mean**
Emergence (%) At 14 dpi	BTS80RR52	62.50	55.00	52.50	95.00	66.25b
	BTS8500	62.50	62.50	55.00	97.50	69.38ab
	BTS 8606	65.00	57.50	45.00	97.50	66.25b
	Crystal 101RR	55.00	45.00	22.50	95.00	54.38d
	Crystal 467RR	65.00	45.00	27.50	95.00	58.13cd
	Hilleshog 4302RR	65.00	55.00	37.50	97.50	63.75bc
	Maribo MA 504	75.00	60.00	60.00	95.00	72.50a
	Inoculum mean	64.29b	54.29c	42.86d	96.07a	
Damping-off (%) At 42 dpi	BTS80RR52	52.5	57.5	67.5	0.0	44.38d
	BTS8500	72.5	72.5	72.5	0.0	54.38a
	BTS 8606	57.5	67.5	80.0	0.0	51.25b
	Crystal 101RR	60.0	75.0	85.0	0.0	55.01a
	Crystal 467RR	50.0	65.0	75.0	0.0	47.50bc
	Hilleshog 4302RR	47.5	77.5	67.5	0.0	48.12bc
	Maribo MA 504	40.0	65.0	72.5	0.0	44.37d
	Inoculum mean	54.29c	68.58b	74.29a	0.0d	

Table B.7. Non-parametric analysis for root rot ratings at 56 dpi caused in cultivars

Cultivar	Mean Rank Severity	Variance	Lower Limit	Upper Limit	Min	Max
BTS80RR52	2.18	0.83	1.52	2.84	1	4
BTS8500	2.93	3.13	2.27	3.59	1	7
BTS 8606	3.06	2.99	2.41	3.72	1	7
Crystal 101RR	3.18	3.61	2.52	3.84	1	7
Crystal 467RR	2.43	0.94	1.77	3.09	1	4
Hilleshog 4302RR	1.87	0.37	1.21	2.53	1	3
Maribo MA 504	2.01	0.53	1.33	2.66	1	3

Root rot disease severity using a modified 1-7 rating scale, where 1 = clean roots and no infection, 2 ≤ 5% of root surface with black/brown symptoms, 3 = 5-25% of root surface with black/brown symptoms; similarly, 4 = 26-50%, 5 = 51-75%, 6 = 75-100% of root surface with black/brown symptoms, and 7 = dead plants (withered) (Ruppel et al., 1979).

Table B.8. Non-parametric analysis for root rot ratings at 56 dpi caused by forms of inocula

Inocula	Mean Rank Severity	Variance	Lower Limit	Upper Limit	Min	Max
Autoclaved barley grains	1	0.0	0.64	1.35	1	1
Colonized barley grains	2.3	0.22	1.97	2.7	2	3
Mycelia	3.07	0.50	2.81	3.4	2	5
Sclerotia	3.7	0.50	3.35	4.07	2	7

Root rot disease severity using a modified 1-7 rating scale, where 1 = clean roots and no infection, 2 ≤ 5% of root surface with black/brown symptoms, 3 = 5-25% of root surface with black/brown symptoms; similarly, 4 = 26-50%, 5 = 51-75%, 6 = 75-100% of root surface with black/brown symptoms, and 7 = dead plants (withered) (Ruppel et al., 1979).

Table B.9. Comparison between the inocula forms through pairwise.wilcox.test ($p<0.05$).

Pairwise inocula forms and its p-value	Colonized barley grains	Autoclaved barley grains	Mycelia
Autoclaved barley grains	1.20e-11	-	-
Mycelia	0.00017	1.50e-11	-
Sclerotia	0.0009	2.30e-11	0.34003^{NS}

NS, non-significant

APPENDIX C. EVALUATION OF *RHIZOCTONIA SOLANI* SCLEROTIA MEDIATED PATHOGENICITY TO SUGAR BEET CULTIVARS IN GREENHOUSE STUDIES: SUPPLEMENTAL FIGURES

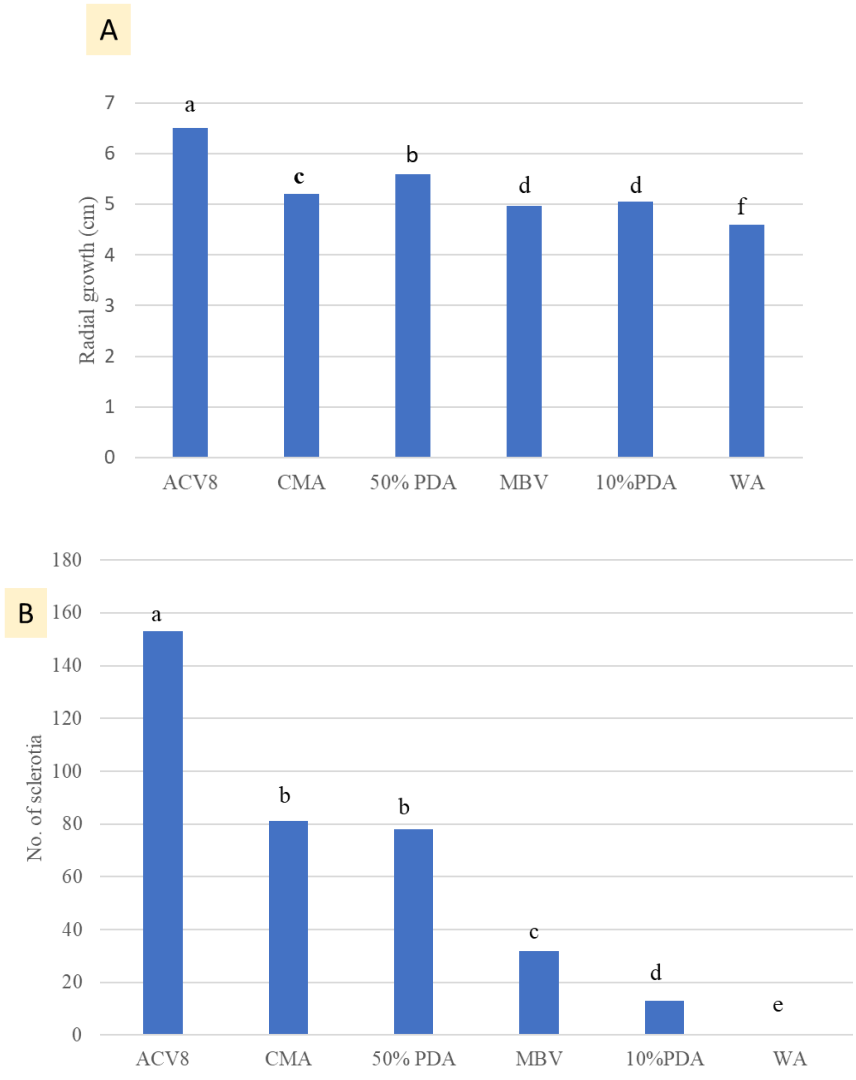

Figure C.1. LSD test for radial growth of mycelia, number of sclerotia, and four time points (days) on six different media ($p<0.05$). A, means radial growth of mycelia the x axis shows the culture media, while the y axis indicates average radial growth over four time points (2, 4, 6, and 8 days) at LSD = 0.06. B, means number of sclerotia on six culture media, the x axis shows the culture media, while the y axis indicates average number of sclerotia over four time points (7, 14, 21, and 28 days) at LSD = 4.929. Means followed by the same letters are not significantly different.

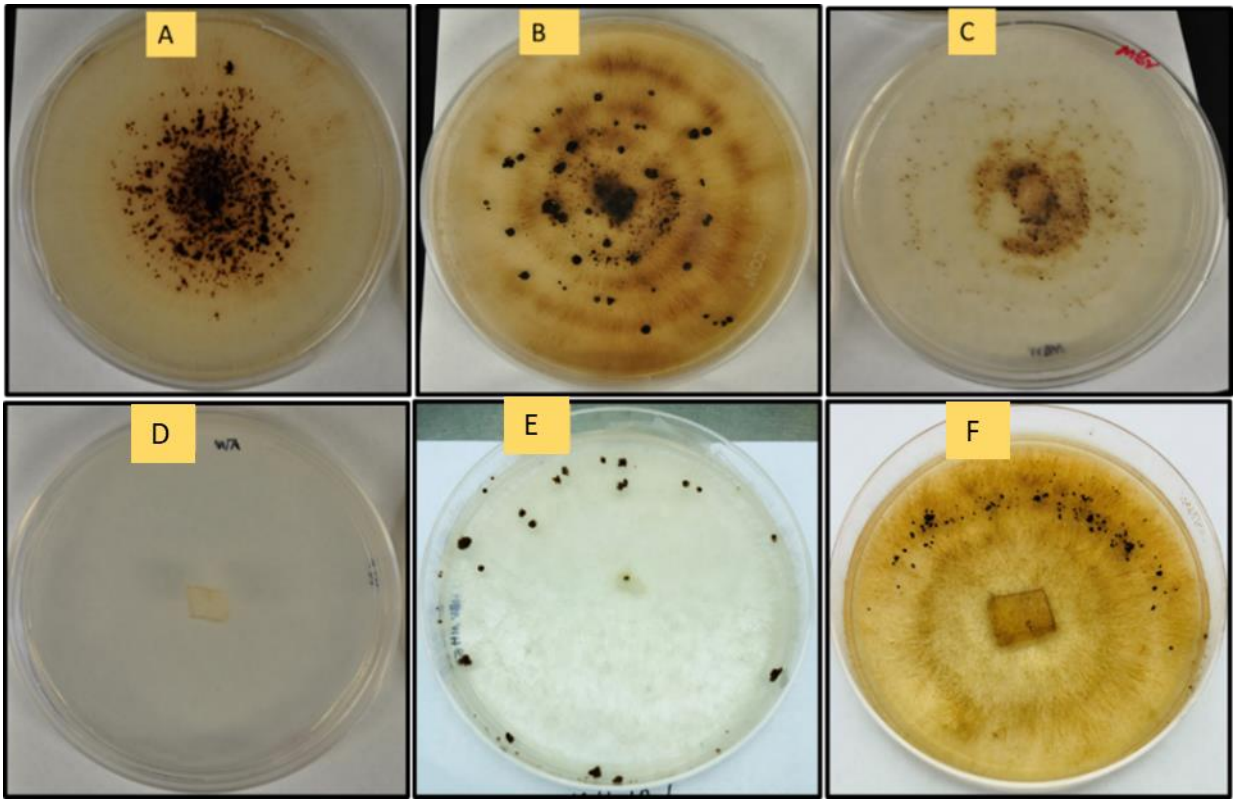

Figure C.2. Visual difference in development and formation of sclerotia on six culture media at 28 days after transfer. Cultural media are: A: amended clarified V8 (ACV8), B: CMA: cornmeal agar (CMA), C: MBV: methylene-benomyl-vancomycin (MBV), D: WA: water agar (WA), E: 10% PDA: 10% potato dextrose (10% PDA), and F: 50% PDA: 50% potato dextrose agar (50% PDA).

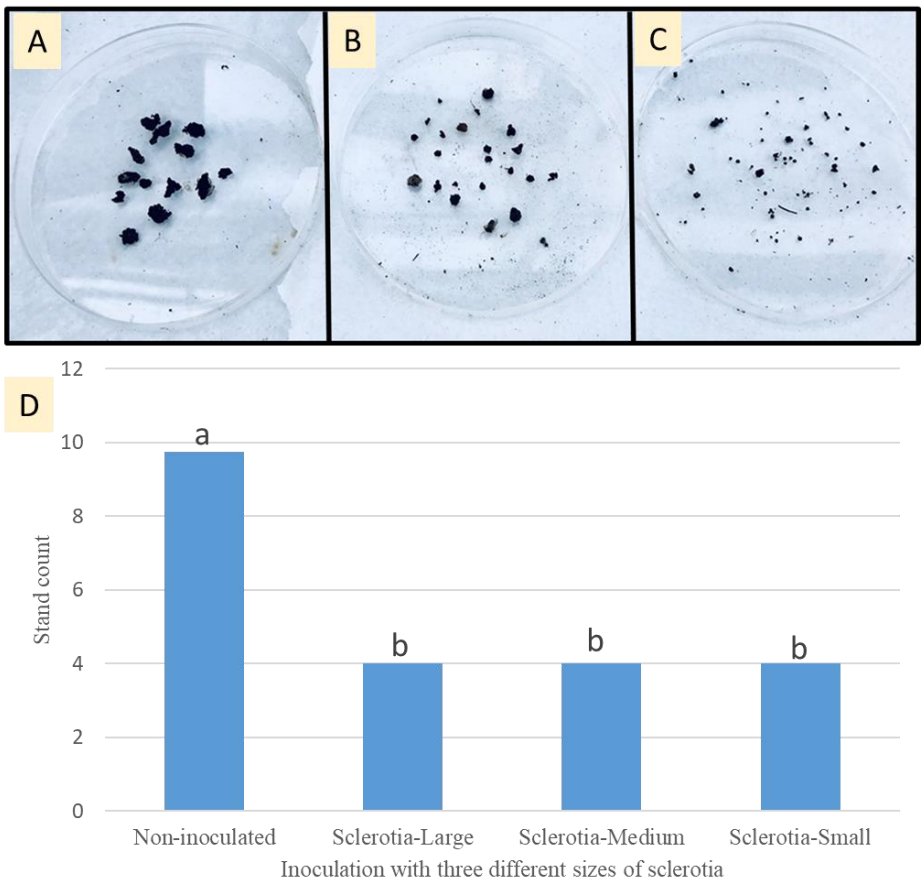

Figure C.3. Three different categories of sclerotia and its pathogenicity test. A, Large (≥4.0 mm). B, (<4.00 mm but ≥2.00 mm). C, small (<2.00 mm but ≥0.5 mm) and D, stand count at 2 weeks of post-inoculation. Mean followed by the same letters are not significantly different at $\alpha = 0.05$ at which LSD 1.15, and MSE =0.562.

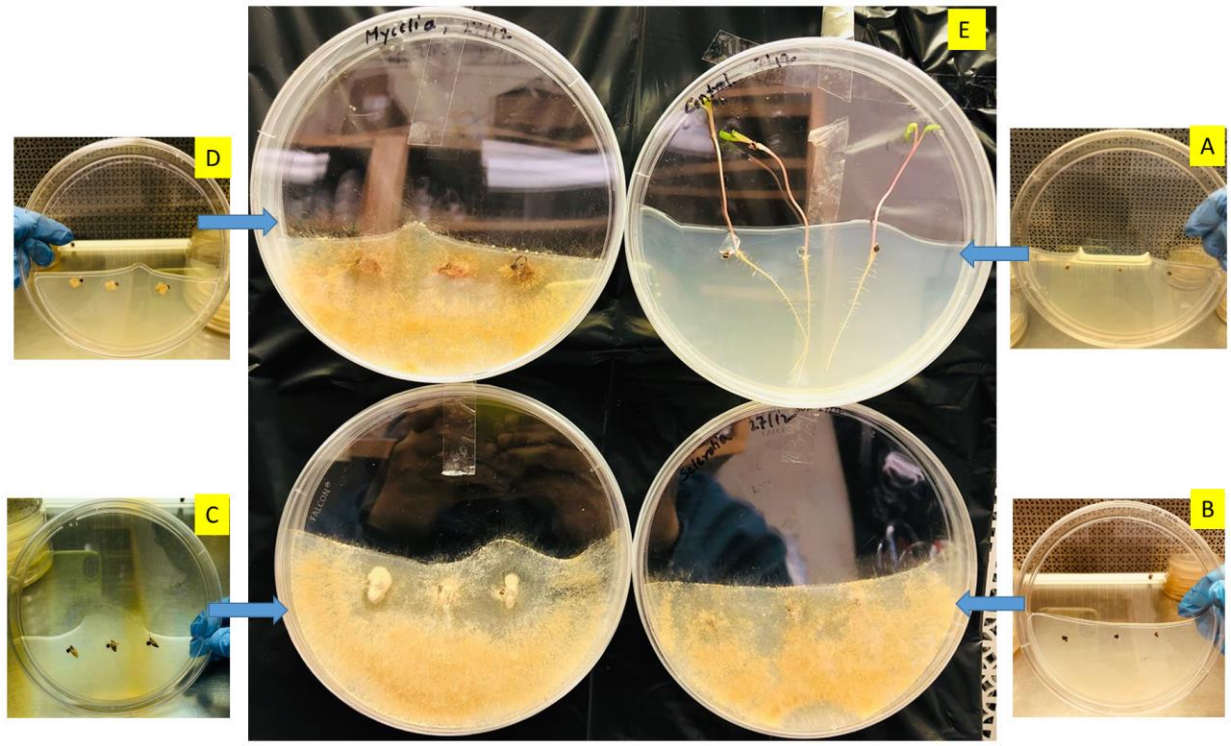

Figure C.4. *In vitro* inoculation on PDA media using three forms of *R. solani* inocula and a control: A: with only seed (control check), B: with seed and sclerotia, C: with seed and colonized barley grains, and D: with seed and mycelia plug, E: Center picture illustrates the corresponding pre-emergence damping-off at 7 dpi. Only non-inoculated (control) resulted in seedling development

APPENDIX D. *PENICILLIUM PINOPHILUM* CAN MITIGATE *RHIZOCTONIA SOLANI* DAMPING-OFF IN SUGAR BEET:

SUPPLEMENTAL TABLE

Table D.1. Percentage of damping-off at 28 dpi, and plant stand counts and root rot severity (%DSI) at 42 dpi in a Rhizoctonia susceptible cultivar - Crystal 101RR under greenhouse conditions. Means followed by the same letters are not significantly different at p = 0.05.

	at 28 dpi	**at 42 dpi**	
Treatment/Inocula	**Damping-off**	**Stand count (%)**	**% DSI***
Mock-inoculated Check	0.0c	95a	0.0b
Sclerotia of *R. solani*	75a	25c	80a
Sclerotia of *R. solani* + Conidia of *P. pinophilum*	25b	75b	0.0b
Conidia of *P. pinophilum*	0.0c	94a	0.0b

$* \%\text{DSI} = \left[\frac{\{(a\times0)+(b\times1)+(c\times2)+(d\times3)+(e\times4)+(f\times5)+(g\times6)+(h\times7)\}}{\{(a+b+c+d+e+f+g+h)\times i\}}\right] \times 100$, where *a*, *b*, *c*, *d*, *e*, *f*, *g*, and *h* represent the number of plants with disease scores of 0, 1, 2, 3, 4, 5, 6, and 7, respectively, and *i* represents the highest root rot severity rating (Li et al., 2014).

APPENDIX E. *PENICILLIUM PINOPHILUM* CAN MITIGATE *RHIZOCTONIA SOLANI* DAMPING-OFF IN SUGAR BEET: SUPPLEMENTAL FIGURES

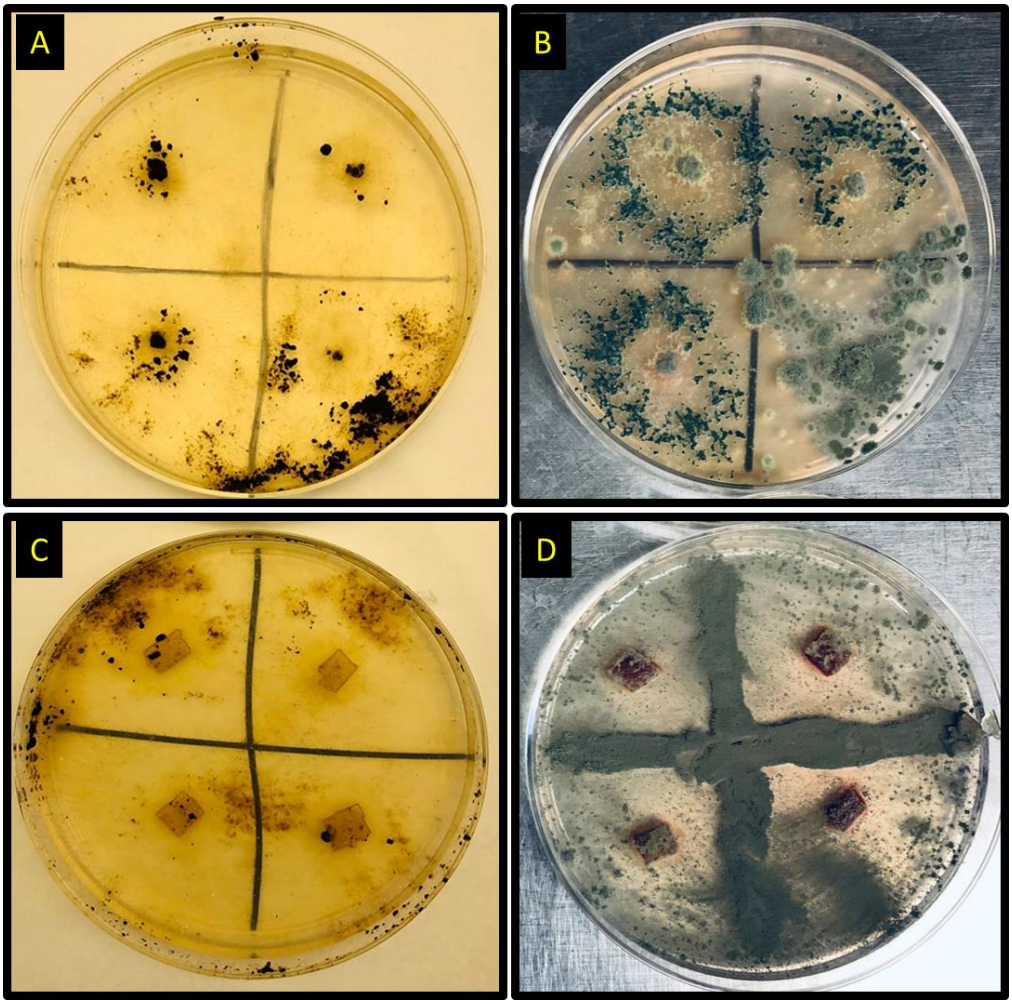

Figure E.1. Contrasting view of growth inhibition of *R. solani* inocula (sclerotia and mycelial plugs) by propagules of *P. pinophilum* on 50%PDA at 21 days after co-cultivation- A. *R. solani* sclerotia alone germinated and produced B. Co-cultivation of *R. solani* sclerotia and conidia of *P. pinophilum* resulted inhibition of germination of *R. solani* sclerotia, C**.** Mycelial plug of *R. solani* alone germinated and produced sclerotia D. Co-cultivation of mycelia plug of *R. solani* and propagules of *P. pinophilum*- resulted in growth inhibition of *R. solani.*

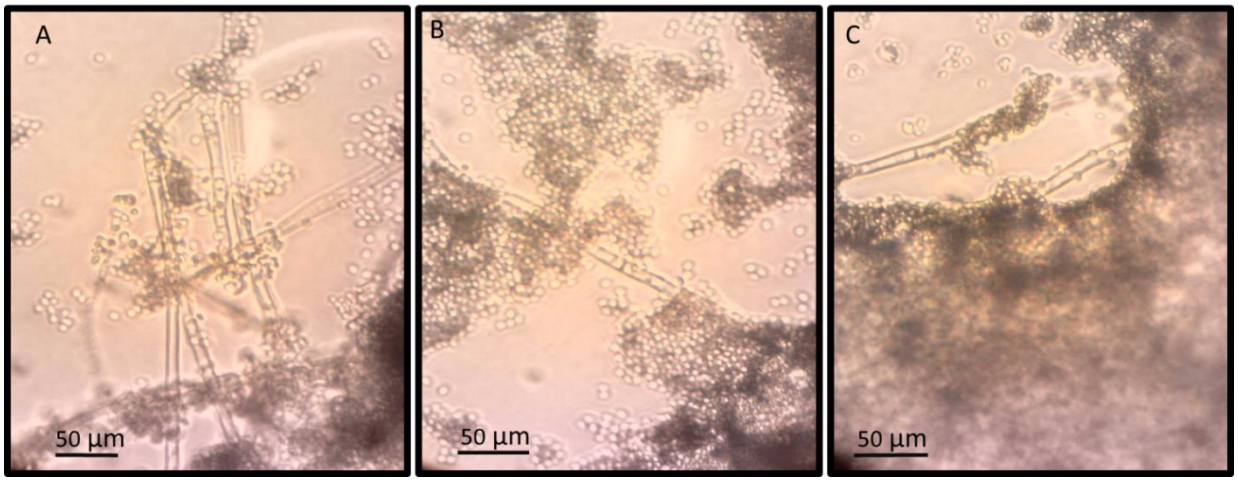

Figure E.2. Co-cultivation of *R. solani* and *P. pinophilum* showed growth inhibition of *R. solani* hyphae by conidial mass of *P. pinophilum* on 50%PDA at three time points-A. 4-days after co-culture, B. 5-days after co-culture, C. 6-days after co-culture. Magnifications was 10x.

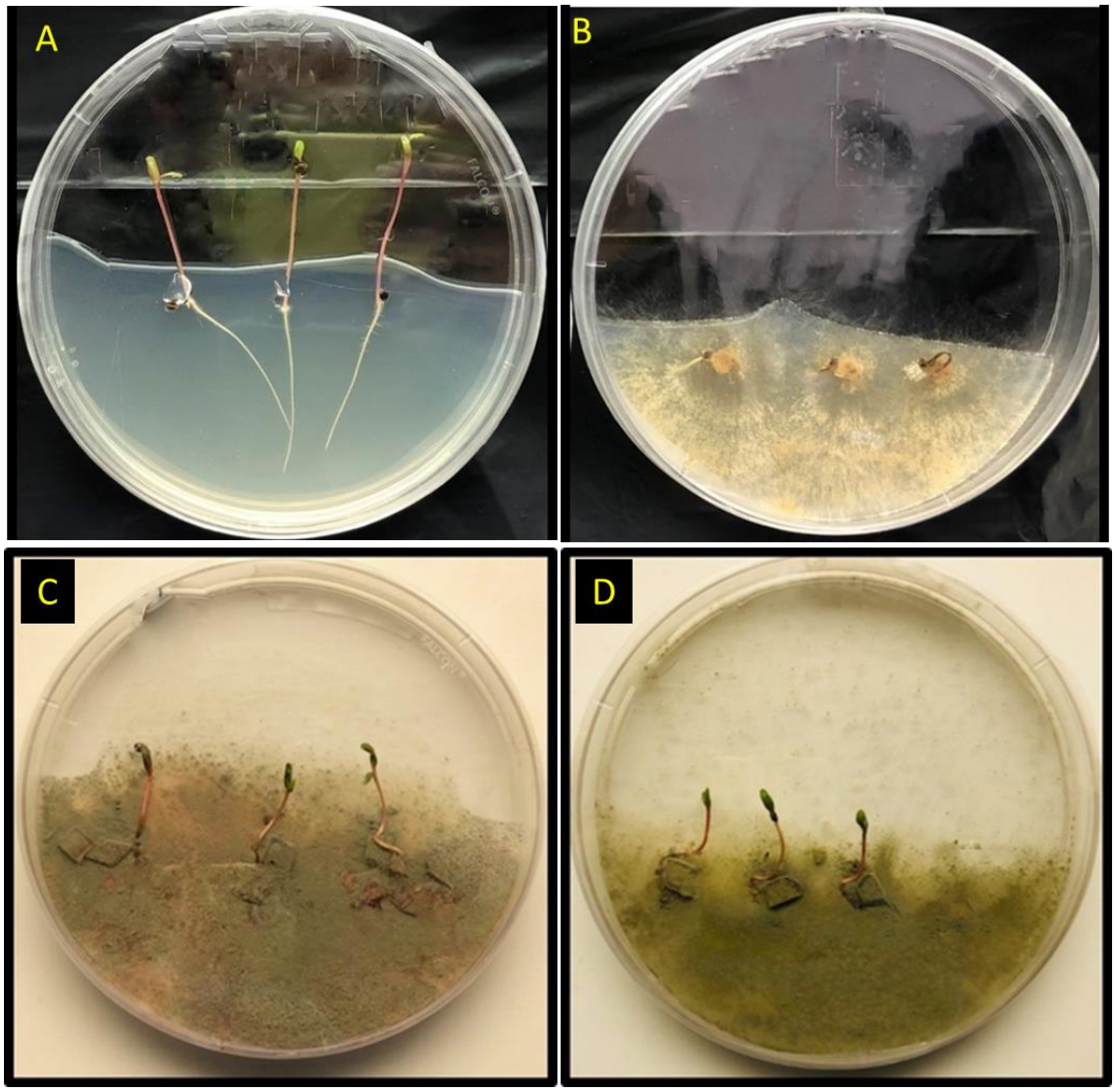

Figure E.3. *In vitro* inoculation of sugar beet seed on 50%PDA media using three groups of inocula and a control at 7 dpi- A. Non-inoculated check hard healthy seedlings with no damping-off, B. Plate with seeds and mycelial plugs of *R. solani* showed 100% damping-off, C. Plate with seeds and mycelial plugs of *R. solani* + propagules of *P. pinophilum*-showed no damping-off, D. Plate with seeds and propagules of *P. pinophilum*-showed no damping-off of seedlings but with reduced seedling growth.