DIVERSITY OF THE FUNGAL PATHOGEN *RHIZOCTONIA SOLANI* AG2-2

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology - Master of Science
Ecology, Evolutionary Biology and Behavior - Dual Major

2019

ABSTRACT

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Rhizoctonia solani AG2-2 is a diverse group of fungi that can cause disease on several economically important crops including sugar beet (*Beta vulgaris*) and dry bean (*Phaseolus vulgaris*). Three projects were conducted to help improve understanding of the diversity within the AG2-2 complex. The first project examined the virulence of 44 *R. solani* AG2-2 isolates on dry beans at both the seedling and adult growth stages. Disease severity ranged from 0.81 to 6.00 for seedlings and from 1.35 to 3.48 on adult plants where 0 = no disease and 6 = plant dead. Isolates in phylogenetic group 1 were, on average, more aggressive at both growth stages. The second project tested the ability of *R. solani* AG2-2 to cause disease on sugar beets at 11˚C. Our results indicate that some isolates can cause considerable disease at temperatures as low as 11˚C, which is well below the previously stated minimum of 15˚C. Disease severity at 11˚C varied from 0.47 to 3.92, where 0 = no disease and 5 = plant dead. The third project involved development of a set of microsatellite markers for *R. solani* AG2-2. Ten microsatellite loci were identified that were able to distinguish 20 unique genotypes among the 23 representative isolates tested. Groupings based on microsatellite distances largely agreed with the multigene phylogeny of Martin et al. (2014). Overall, *R. solani* AG2-2 is a highly diverse group and research that examines issues related to host response need to consider this variability. Additionally, knowledge of diversity may be useful in predicting the risk of disease in the field and assist in management decisions such as crop rotation.

To my Mom

ACKNOWLEDGMENTS

I would like to take this opportunity to thank my major professor, Dr. Linda Hanson, for her patience and support through this process. I would also like to thank the other members of my guidance committee, Dr. Martin Chilvers and Dr. Greg Bonito for their encouragement and willingness to help me through this process. I also appreciate the help and support I received from my fellow lab mates, Tom Goodwill, Qianwei (Jane) Jiang, Hailey Haist, and Celeste Dmytryszyn as well as many others. Finally, I want to express my appreciation to my family for their understanding and support the last several years as I pursue a major career change. I realize that this journey has not been easy and I am very grateful for your sacrifice.

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KEY TO ABBREVIATIONS

6-FAM	Fluorescein
AG	Anastomosis group
ANOVA	Analysis of variance statistical test
C0	Category ‘0’ anastomosis reaction (no reaction)
C1	Category ‘1’ anastomosis reaction (cell wall fusion only)
C2	Category ‘2’ anastomosis reaction (protoplasm fusion plus cell death)
C3	Category ‘3’ anastomosis reaction (protoplasm fusion with no cell death)
CWDE	Cell wall degrading enzymes
CVE	Coefficient of velocity of emergence
D	Simpson’s diversity index
DAPI	4’-6’-diamidino-2-phenylindole
DS	Disease severity score
ddH_2O	Double distilled water
dNTP	Deoxyribonucleotide triphosphate
HEX	Hexachlorofluorescein fluorescent dye
HSD	Honest significant difference (Tukey’s HSD)
ISG	Intraspecific groups
ITS	Internal transcribed spacer
LpBS	Lactophenol blue staining solution
MEB	Malt extract broth

$MgCl_2$	Magnesium Chloride
MSU	Michigan State University
N50	minimum contig length needed to cover 50% of the genome
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PIC	Polymorphism information content
PG	Phylogenetic group
rDNA	Ribosomal DNA
RRCR	Rhizoctonia root and crown rot
SMM	Stepwise mutational model
SSR	Simple sequence repeats
STR	Short tandem repeats
T_a	Annealing temperature
TPM	Two phase model
Tris-EDTA	Tris(hydroxymethyl)aminomethane - ethylenediaminetetraacetic acid
UV	Ultra violet
VCG	Vegetative compatibility group

CHAPTER 1:

REVIEW OF LITERATURE

Introduction

History of the Genus Rhizoctonia

The genus *Rhizoctonia,* meaning "root killer" (Baker, 1970), was established by de Candolle (1815) to accommodate the violet root rot pathogen, *R. crocorum* D.C. (Parmeter & Whitney, 1970). The basic characters that deCandolle used to define the genus were the production of sclerotia of uniform texture with hyphae originating from them, the association of the mycelium with plant roots, and a lack of conidia. These features were so general that nearly 100 species have since been assigned to the genus which has led to a mixture of unrelated species being classified as *Rhizoctonia* spp. (Parmeter & Whitney, 1970; Sneh et al., 1991). Many of these species have little in common with one another except for the lack of conidia.

The review of Ogoshi (1987) provided clarity on the characteristics of the *Rhizoctonia* genus. Ogoshi (1987) defined the genus *Rhizoctonia* as a group of imperfect fungi within the Basidiomycota and of the order Cantharellales with the following characteristics: "(a) branching near the distal septum in young, vegetative hyphae; (b) formation of a septum in the branch near the point of origin; (c) constriction of the branch; (d) dolipore septum; (e) no clamp connections; (f) no conidia, except moniliod cells; (g) sclerotia not differentiated into medulla and rind and (h) no rhizomorph." (Ogoshi, 1987 pg. 126). Furthermore, Ogoshi (1987) considered *Rhizoctonia* spp. to be sub-divided into three major groups based on the number of nuclei in each cell and the identity of the teleomorph. One group was the binucleate *Rhizoctonia*, with two nuclei per cell (rarely one or three) and teleomorphs in the genus *Ceratobasidium* D.P. Rogers. The second was the multinucleate *Rhizoctonia*, which have three

or more nuclei per cell, with teleomorphs in the genus *Waitea* Warcup and Talbot. The third group included the multinucleate *Rhizoctonia* with teleomorphs in the genus *Thanatephorus* Donk. Ogoshi's concept of the genus reduced legitimate *Rhizoctonia* spp. to forty-nine out of the approximately one-hundred species that had been reported at the time.

That same year, Moore (1987) proposed a new classification system for *Rhizoctonia*-like fungi. Moore (1987) argued that *Rhizoctonia*-like anamorphs represented four distinct groups of higher fungi that could be distinguished by their septal morphology. These included the "ascomycetes" (large septal pores and associated Woronin bodies), "ustomycetes" (simple septa with small pores), "homobasidiomycetes" (dolipore/parenthesome septal complexes with perforate parenthesomes), and "heterobasidiomycetes" (dolipore/parenthesome septal complexes with imperforate parenthesomes). *R. crocorum* (Pers.) DC., the type species of the *Rhizoctonia* genus at the time, had a simple pored septum and was therefore, an "ustomycete." Thus in his proposed system, Moore (1987) reserved the genus *Rhizoctonia* for anamorphs of ustomycetous fungi. The remainder of the *Rhizoctonia*-like "basidiomycetes" were then assigned to one of three genera, two of which were newly formed. The binucleate *Rhizoctonia* spp. with the teleomorph *Tulasnella* J. Schröt were assigned to the new genus *Epulorhiza* R.T. Moore. Those binucleates with the teleomorph *Ceratobasidium* D.P. Rogers were assigned to *Ceratorhiza* R.T. Moore. The anamorphs of *Thanatephorus* Donk and *Waitea* Warcup & P.H.B. Talbot with dolipore septal complexes with perforate parenthesomes (e.g. *Rhizoctonia solani)* were placed in the genus *Moniliopsis* Ruhland due to the already accepted synonymy of *R. solani* with *Moniliopsis aderholdii* Ruhland (Moore, 1987).

While Moore's system is taxonomically correct and properly justified, it was not well received nor was it followed in practice. Sneh et al. (1991) argued that due to the familiarity of the name *Rhizoctonia solani* and the extensive published literature, a name change would cause unnecessary confusion. Therefore in their monograph, Sneh et al. (1991) retained the name *Rhizoctonia* for Moore's *Epulorhiza* spp., *Ceratorhiza* spp., and *Moniliopsis* spp. Vilgalys and Cubeta (1994) followed Ogoshi (1987) and Sneh et al. (1991) by ascribing *Rhizoctonia* spp. to three groups: multinucleate species having a *Thanetphorus* teleomorph; binucleate species having a *Ceratobasidium* teleomorph; and multinucleate species having a *Waitea* teleomorph.

Stalpers et al. (1998) proposed that the name *Rhizoctonia* should be conserved and re-typified with *R. solani* as the type species. Stalpers et al. (1998) agreed with Sneh et al. (1991) that although Moore's proposed changes were in full agreement with the nomenclature code, the changes were not being followed in practice and the name *R. solani* was so familiar in the literature that it should not be changed. The proposal was approved unanimously by the International Association of Plant Taxonomy (IAPT) Committee for Fungi in 2001 (Gams, 2001) and *Rhizoctonia solani* established as the current type species of the genus *Rhizoctonia.*

Classification of Rhizoctonia solani

Julius Kühn first identified *Rhizoctonia solani* while viewing potato tubers under his microscope where he observed dark sclerotia adhered to the surface of the tuber that were connected with dark fungal hyphae (Menzies, 1970). Kühn's original description and illustrations appeared in his book on crop diseases in 1858 (Kühn, 1858; Menzies, 1970). Unfortunately, Kühn's illustrations included a few spores and structures from other fungi and his depictions of the hyphae and sclerotia were hardly diagnostic of what we consider *R. solani*

today. In addition, the disease symptoms that Kühn described were more characteristic of scab than the stolon and stem lesions that are actually caused by *R. solani* (Menzies, 1970).

The next major development in our understanding of the concept of *Rhizoctonia solani* came with the review of Duggar (1915). Despite Kühn's vague description and evident mistake of including structures from more than one fungus, Duggar (1915) agreed that the fungus he was studying in connection with damping off and other root diseases was the same fungus Kühn had described. This conclusion has been generally accepted by later workers and Kühn has been recognized as the first to publish *Rhizoctonia solani* as a valid name. Although Duggar (1915) provided a respectable description of *R. solani* and the diseases known at the time to be associated with the fungus, his review still lacked definitive diagnostic characteristics.

The lack of conidia and rarity of the sexual stage coupled with the highly variable nature of this group of fungi makes an adequate species concept difficult to formulate. No single character or feature is able to distinguish *R. solani* isolates from other similar fungi, except that the teleomorph is *Thanatephorus cucumeris* (Frank) Donk (Parmeter & Whitney, 1970). Instead, recognition of the species depends on the presence and absence of a combination of several characteristics (Parmeter & Whitney, 1970). Unfortunately, it can be difficult to describe mycelium in a sufficiently detailed way to assure that all workers will come to the same conclusion based on a written description (Parmeter & Whitney, 1970). In addition, because of the highly variable nature of the *R. solani* group, some characters are more reliable diagnostic features than others (Parmeter & Whitney, 1970). Parmeter and Whitney (1970) provided the description that forms our current species concept of *Rhizoctonia solani* and presented the following criteria as defining the species (adapted from Parmeter & Whitney, 1970):

Characters consistently present in *R. solani* isolates:

1. Actively growing hyphae are multinucleate with three or more nuclei per cell (Fig. 1.1A).
2. Presence of a prominent dolipore septa apparatus (Fig. 1.1B).
3. Branching near the distal septum in young vegetative hyphae (Fig. 1.1B)
4. Distinct constriction at the branch point and a septum that forms in the branch near the point of origin (Fig. 1.1B).
5. Some shade of brown. While young colonies may be white or nearly white, they will become brown as they age. There is, however, much variation in the shade of brown that is possible as some colonies may be pale brown and others, particularly the sclerotia, will be so dark brown as to be almost black.

Characters usually present in *R. solani* but one or more may be absent in some isolates:

6. Mycelium is fairly fast growing and large diameter (6 - 10µm).
7. Moniliod cells (Fig. 1.1C)
8. Sclerotia, when present, are not differentiated into rind and medulla (Fig. 1.1D).
9. Phytopathogenicity

Characteristics never present in *R. solani* isolates:

10. Clamp connections
11. Conidia

12. Rhizomorphs

13. Any pigment other than brown

14. Any perfect stage other than *Thanatephorus cucumeris*

(A.B. Frank) Donk

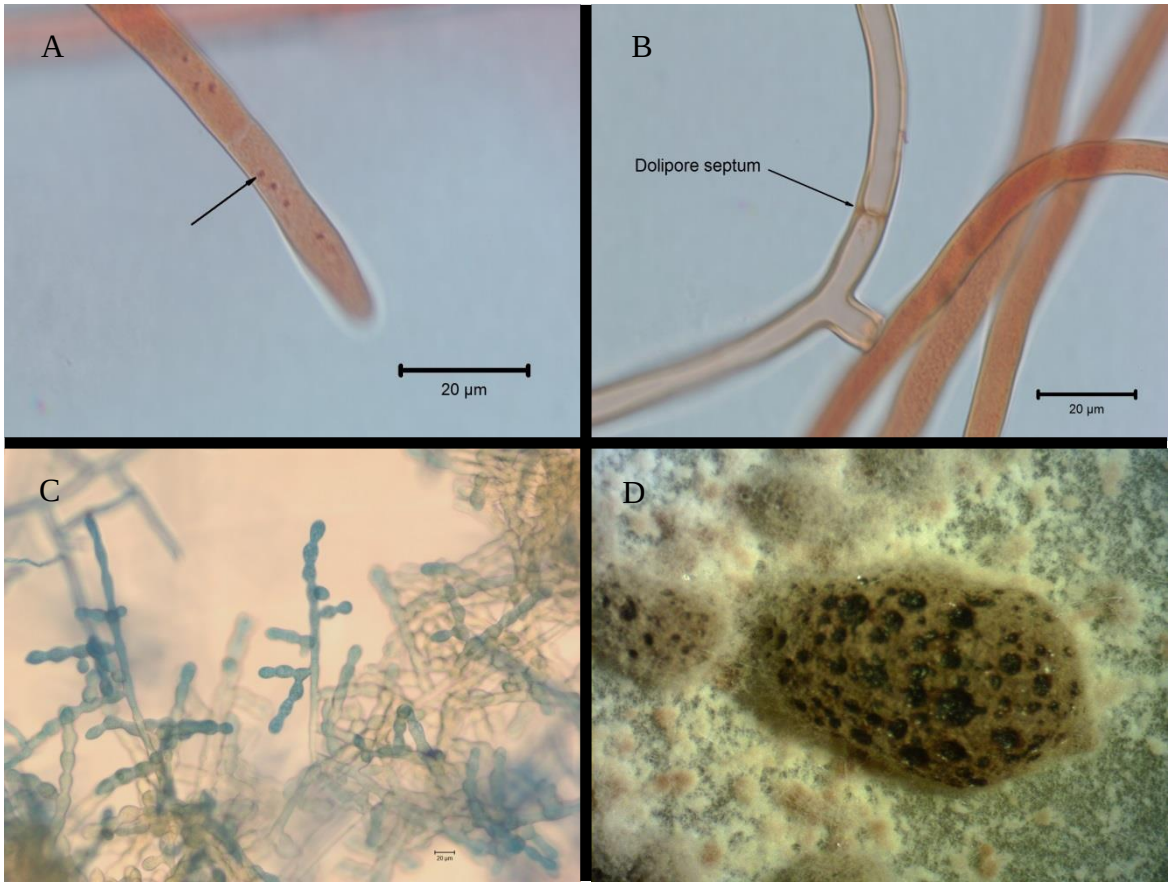

Figure 1.1 Micrographs of characteristic structures of *Rhizoctonia solani*. (A) Hyphum stained with safranin O showing multiple nuclei in a cell (400X). (B) Hyphae stained with Safranin O showing the dolipore septum, lack of clamp connection, branch close to the distal septum and conspicuous constriction at the branch (400X). (C) Moniliod cells stained with lactophenol blue solution (200X). (D) Sclerotum from a culture of AG1-1B grown on potato dextrose agar (40X).

Thus the review of Parmeter and Whitney (1970) provided a solid taxonomic foundation for the classification of *R. solani*. Nevertheless, difficulties with the species concept still remained. High levels of phenotypic variation and the lack of unequivocal characteristics in the

anamorph made conventional taxonomy difficult. The most certain way to avoid confusion in communicating the identity of an *R. solani* isolate is to associate the strain with its perfect state, *Thanatephorus cucumeris*. However, not only is this infeasible for many strains (Vilgalys & Cubeta, 1994) but it has also had the additional effect of adding confusion regarding the proper name to use.

Uncertainty surrounding the correct name to use for *R. solani* still appears to be abundant despite the new taxonomic rule of "One fungus, one name" effective as of 1 January 2013 (Hawksworth, et al., 2011; Article 59, International Association of Plant Taxonomists, http://www.iapt-taxon.org/nomen/main.php). According to the current rules, it should be fairly straightforward that the name *R. solani* has taxonomic priority given that *Rhizoctonia* was described by de Candolle in 1815 and *Rhizoctonia solani* was described by Kühn in 1858 while *Thanatephorus* was described much later by Donk (1956). Yet as recently as 2018, Ajayi-Oyetunde & Bradley (2018) reported that Index Fungorum (www.indexfungorum.org) had *Thanatephorus cucumeris* listed as the current name. However, when I referenced Index Fungorum in March of 2018, *R. solani* was properly listed as the current name. A search of Google Scholar (https://scholar.google.com/) revealed a sizable number of journal articles published between 2013 and 2018 that used *T. cucumeris* as the accepted name (e.g. Gonzalez et al., 2016). The continuing confusion regarding the taxonomy of the *Rhizoctonia* genus is not unexpected considering the history of its classification and the varying classification systems.

Separation of Rhizoctonia solani Subgroups by Anastomosis Reaction

It should be clear that the taxonomy of the *Rhizoctonia* genus is complex and controversial. It is now recognized that *R. solani* is a species complex composed of a number of

genetically distinct groups (Cubeta & Vilgalys, 1997). A species complex, also known as sibling species, is a closely related group of distinct, reproductively isolated organisms that cannot adequately be distinguished morphologically (Mayr, 1963). Within the group of fungi known as *Rhizoctonia solani*, there are a number of distinct groups with diverse life-histories (Cubeta & Vilgalys, 1997) but elucidating those relationships has been problematic for researchers.

Current classification of subgroups within the *R. solani* species complex relies on the concept of anastomosis groups (AG). Anastomosis groups are based on the premise that the hyphae of closely related isolates are able to recognize and fuse, or anastomose, with one another (Carling, 1996). Isolates that are able to anastomose are considered to be part of the same anastomosis group (AG) and isolates that are unable to fuse with one another are considered part of different AG. To date, at least 13 AG have been described in *R. solani*, designated as AG followed by a number, AG1 to AG13 (Sneh et al.,1991; Gonzalez et al., 2016). Anastomosis of compatible strains of *R. solani* was first reported more than 80 years ago by Matsumoto et al. (1932) who used the concept to differentiate strains of *Hypochnus sasakii* Shirai (syn. *Rhizoctonia solani*) from one another. Criteria for distinguishing between hyphal anastomosis reactions described by Matsumoto are shown in Table 1.1. Shultz (1936) first introduced the concept of grouping isolates based on anastomosis reactions, which he termed "Gruppe" and numbered from I to V (Table 1.2). Richter and Schneider (1953) further refined and added to the concept, using somewhat different terminology for their groupings, and identified six "Fusion gruppes" labeled A through F (Table 1.2).

Table 1.1 Summary of terminology and descriptions used to define categories of anastomosis reactions in *Rhizoctonia solani*. Terms towards the top of the table indicate close relationships and those towards the bottom indicate more distant relationships. Table adapted from Carling (1996) p. 40.

Matsumoto *et al.* (1932)	Flentje & Stretton (1964)	Parmeter *et al.* (1969)	Carling *et al.* (1988)
Perfect Hyphae are from same parental strain	S (Self reaction) Cell wall and membrane fusion; no cell death	2 (perfect) Cell wall and cytoplasmic fusion; cell death	'C3' Cell wall and membrane fusion; no cell death
Imperfect Membrane not completely fused; no mixing of cytoplasm	K (Killing reaction) Cell wall and membrane fusion; cell death	2 (imperfect) Cell wall but no cytoplasmic fusion; cell death	'C2' Cell wall fusion obvious; membrane fusion probable; cell death
Contact Hyphal contact but no fusion of cell wall or cytoplasm	WF (Wall fusion) Cell wall fusion but no fusion of membrane or cytoplasm	1 Hyphal contact but no fusion of cell wall or cytoplasm; no cell death	'C1' Hyphal contact and attachment; no membrane fusion; no cell death
No reaction No reaction	NR (No reaction) No reaction	0 No reaction	'C0' No reaction

These early reports on anastomosis groupings did not describe the cell death that commonly occurs after anastomosis (Carling, 1996). Flentje and Stretton (1964) were the first to report cell death as a criterion for the categorization of anastomosis reactions and termed this category the 'K' or killing reaction. Although this reaction is similar to the imperfect fusion of Matsumoto et al. (1932), it is not completely equivalent as cell death was not reported by Matsumoto et al. (1932). When microscopic observation suggested that cell wall fusion had occurred, Flentje and Stretton (1964) punctured one of the two anastomosed cells using a glass needle to determine if a cytoplasmic connection had occurred. Reactions where both the punctured cell and the neighboring cell collapsed were considered evidence that membrane

fusion had occurred. Those reactions where only the punctured cell collapsed but the connection between the hyphae was not easily separated were considered to be category 'WF' or wall fusion in their system (Table 1.1).

Table 1.2 Historic groupings of *Rhizoctonia solani* based on hyphal anastomosis reactions. Table adapted from Carling (1996).

Year	Author	Proposed groups	Present day equivalent
1936	Schultz	Gruppe I (*hortensis*) Gruppe II (*brassicae*) Gruppe III (*typica*) Gruppe IV (*chicorii-endiviae*) Group V (*fuchsiae*)	AG 1 AG 2 AG 3 AG 4 binucleate
1953	Richter & Schneider	Fusion gruppe A Fusion gruppe B Fusion gruppe C Fusion gruppe D "*cruciferen*" Fusion gruppe E Fusion gruppe F "*kartoffel*"	AG 1 AG 5 AG 4 AG 2 binucleate AG 3
1969	Parmeter *et al.*	AG 1 AG 2 AG 3 AG 4	AG 1 AG 2 AG 3 AG 4

The next major advancement in anastomosis grouping was made by Parmeter et al. (1969) when their work began the formalized use of the term 'anastomosis group'. Parmeter et al. (1969) made use of the terminology of Matsumoto et al. (1932), which created some confusion since descriptions of the criteria for each category were not equivalent; particularly with the inclusion of cell death as a condition (Table 1.1). Parmeter et al. (1969) defined three categories of anastomosis reactions: '0', or no reaction; '1' where there was hyphal contact but no fusion and '2' where cell wall and cytoplasmic fusion occurred often followed by cell death.

They also identified the anastomosis groups AG1, AG2, AG3 and AG4, which are still in use today.

Categorization of Anastomosis Reactions

By the late 1970's, the concept of using anastomosis to delineate *R. solani* into more consistent groups was gaining some acceptance but there were still doubts as to how meaningful the groupings were. Isolates that were grouped by anastomosis reactions did not always correspond with groupings based on morphology or pathogenicity (Parmeter & Whitney, 1970). Furthermore, many isolates would not anastomose with members of any of the anastomosis groupings recognized at the time (Carling, 1996). These uncertainties were further amplified because the process of anastomosis was not well understood and even less was known about the genetic mechanisms that regulated anastomosis success.

In an attempt to overcome the confusion associated with the varied definitions and terminologies related to anastomosis groupings, Carling et al. (1988) developed a system of four categories for describing anastomosis reactions. This system combined information from the previous three systems and clarified some definitions and descriptions (Table 1.1). In a "Category 0" reaction (C0), no reaction occurs and the hyphae generally grow right past one another without recognition. This indicates that the isolates are in separate AG. In a "Category 1" reaction (C1), hyphal contact occurs and hyphal attachment is apparent, but no membrane fusion occurs. A C1 reaction indicates that isolates are only distantly related perhaps within highly heterogeneous AG or in AG with bridging capability. A "Category 2" reaction (C2) occurs when cell wall fusion is obvious, membrane fusion is probable and anastomosing cells frequently die. This reaction category is an indication that isolates are in the same AG but are in

different vegetative compatibility groups (VCG). A “Category 3” reaction (C3) consists of fusion of the cell wall and of the membrane and the anastomosing cells frequently remain alive. This reaction is an indication that isolates are very closely related and not only in the same AG but also in the same VCG. Self-anastomosis reactions are normally a “Category 3” reaction.

Although it is tempting to consider the categories defined by Matsumoto et al. (1932), Flentje and Stretton (1964), Parmeter et al. (1969), and Carling et al. (1988) to be analogous to one another, a more careful examination of Table 1.1 reveals this to not be the case. Significant differences between the definitions make direct comparison difficult and reveal that the categories presented by the four authors are not equivalent. The most obvious difference is the use of cell death as a descriptive criterion. Matsumoto et al. (1932) did not mention cell death in any of his descriptions while Parmeter et al. (1969) includes cell death as part of his description of ‘perfect’ fusion. In contrast, Flentje and Stretton (1964) and Carling et al. (1988) both consider anastomosis between the most closely related isolates to be ‘perfect fusion’ and to include cell wall and membrane fusion with no cell death.

The terminology used to describe anastomosis reactions can cause some confusion among researchers unfamiliar with the classification system. It is not uncommon to hear the terms ‘perfect’, ‘imperfect’ and ‘contact’ applied to the categories defined by Carling et al. (1988) but this has the potential of being imprecise since Carling et al. (1988) made no use of those terms and the descriptions of Carling et al. (1988) are not exactly equivalent to those presented by Matsumoto et al. (1932) or Parmeter et al. (1969). ‘Perfect’ fusion roughly corresponds to category ’C3’ of Carling et al. (1988) and typically refers to anastomosis between hyphae of the same isolate or very closely related isolates. ‘Imperfect’ fusion refers to

the anastomosis of isolates that is similar to the 'C2' category of Carling et al. (1988) where paired isolates are in the same AG but not the same vegetative compatibility group (VCG). The term 'Contact' fusion refers to the anastomosis reaction where cell wall fusion occurs but there is no mixing of cytoplasm. This category is sometimes referred to as a 'bridging reaction' although that terminology could also be imprecise. An isolate pairing with a high proportion of 'C1' reactions may be said to have a bridging relationship or considered to be a bridging isolate, but the anastomosis reaction itself should be referred to as 'contact fusion' or a 'C1' reaction. The categories as described by Carling et al. (1988) are currently the most widely used method of evaluating anastomosis reactions and authors should be careful that they understand the appropriate way to use the terminology. Probably the most accurate and least confusing way to describe anastomosis reactions is to use the terms 'perfect', 'imperfect', and 'contact' informally and employ the terminology of Carling et al. (1988) for more formal reporting.

Since 1969, when Parmeter et al. (1969) characterized AGs 1 through 4, as many as 10 other AGs have been described (Table 1.3) including AG5 (Ogoshi, 1975), AG6 and AGBI (Kuninaga et al., 1979), AG7 (Homma et al., 1983), AG8 (Neate & Warcup, 1985; Rovira et al., 1986), AG9 (Carling et al., 1987), AG10 (MacNish et al., 1995), AG11 (Carling et al., 1994), AG12 (Carling et al., 1999) and AG13 (Carling et al., 2002a). These groups have diverse host ranges, cultural characteristics, thiamine requirements and temperature optima. Since their hyphae do not fuse, there is limited opportunity for exchange of genetic material. Thus, these anastomosis groups basically represent reproductively isolated lineages and therefore, can be thought of as separate species. Whether the groups that are currently recognized as anastomosis groups will eventually be elevated to independent species remains to be seen.

Table 1.3 Currently recognized AG and cultural types. Table provides a summary of information related to anastomosis groups (AG) and the associated cultural types. Data was taken from literature cited in the references column and from Sneh et al. (1991). Anastomosis groups are differentiated by hyphal anastomosis whereas cultural types are differentiated by the characteristics listed in the applicable column. Tester strains are used for anastomosis testing and not for differentiating cultural types by hyphal fusion.

AG	Cultural Types	Tester strain	Characteristics of cultural types	References
AG 1	IA IB IC	ATCC 76121 ATCC 76122 ATCC 76123	sclerotial form, cultural characteristics, DNA base sequence homology	Parmeter et al., 1969 Kuninaga & Yokosawa, 1985
AG 2-1	- 2t Nt	ATCC 76168	fusion frequency ITS1 sequence similarity	Ogoshi, 1975 Schneider et al., 1997 Kuninaga et al., 2000b
AG 2-2	IIIB IV LP WB	ATCC 76124 ATCC 76125	growth at 35˚C, cultural and morphological characteristics host range, symptoms	Sneh, et al., 1991 Hykumachi et al., 1998 Godoy-Lutz et al., 2008
AG 2-3				Naito and Kanematsu, 1994
AG 2-4				Carling et al., 2002b
AG 3	TB PT	ATCC 76167		Parmeter et al., 1969 Kuninaga et al., 2000a
AG 4	HGI HGII HGIII	ATCC 76126	DNA base sequence homology, sclerotia form	Parmeter et al., 1969 Kuninaga & Yokosawa, 1985
AG 5		ATCC 76128		Ogoshi, 1975
AG 6	HG-1 GV	ATCC 76129 ATCC 76130	DNA base sequence homology	Kuninaga et al., 1979 Kuninaga & Yokosawa, 1985
AG 7		ATCC 76131		Homma et al., 1983
AG 8	ZG1 ZG2 ZG3 ZG4 ZG5	ATCC 76106		Neate & Warcup, 1985 Rovira et al., 1986 Neate et al., 1988
AG 9	TP TX	ATCC 62804 ATCC 62804	Thiamine requirement, DNA base sequence homology	Carling et al, 1987 Carling & Kuninaga, 1990
AG 10		ATCC 76107		MacNish et al., 1995
AG 11				Carling et al., 1994
AG 12				Carling et al., 1999
AG 13				Carling et al., 2002a
AG BI		ATCC 76132		Kuninaga et al., 1979

Classification of AG2

Rhizoctonia solani AG2 has been further separated into two major subgroups, type 1 and type 2, and designated as AG2-1 and AG2-2 (Ogoshi, 1976). These subgroups were differentiated by hyphal fusion frequency, where members of AG2-1 anastomose at low frequencies with members of AG2-2 (Sneh et al.,1991). Ogoshi (1976) considered AG2-1 and AG2-2 to be independent anastomosis groups even though they anastomose at low rates. Adams (1988) argued that based on DNA homology, AG2-1, AG2-2 and AGBI should be merged into a single group designated AG2. Carling et al. (2002b) also argued that AGBI should be included in AG2 based on strong hyphal anastomosis reactions with AG2-1 and AG2-2 and proposed it be designated as AG2BI.

Additional subsets of AG2 were characterized by Naito and Kanematsu (1994), who described AG2-3, and Carling et al. (2002b), who described AG2-4. A neighbor joining tree illustrated in Carling et al. (2002b), showed subsets 2-1, 2-2, 2-3, 2-4 and 2-BI as well supported, separate groups (Fig. A1.1) and was intended to support the concept that AG2 was a unified group with distinct subsets. Similar results were found by Salazar et al. (1999). However, without including representative AG outside of the AG2 complex, it is difficult to determine if these groups truly represent subsets within a larger, monophyletic group or if they are actually entirely separate groups. When phylogenetic analysis included a larger number of AG, both Kuninaga et al. (1997) and Vilgalys and Cubeta (1994) showed that AG2-1 is genetically closer to AG9 and AGBI than it is to AG2-2. Likewise, AG2-2 is genetically closer to AG5 than it is to AG2-1 lending support to the hypothesis that AG2-1 and AG2-2 should be considered separate AG.

I agree with the argument that AG2-1 and AG2-2 should be considered separate anastomosis groups. First is the fact that they are genetically distinct and more closely related to other AG than to each other (Kuninaga, et al., 1997; Vilgalys & Cubeta, 1994). Secondly, while the two groups do have some overlap in host range, the primary hosts are diverse. Members of AG2-1 are considered to be slow growing isolates that are pathogens of winter crops, primarily the in *Brassicaceae* while AG2-2 are faster growing and primarily infect members of the *Chenopodiaceae*, *Fabaceae* and *Poaceae* (Ogoshi, 1987; Salazar et al., 2000a; Sneh, 1991). Another issue is that several AG are capable of fusing with other AG at low levels (Table 1.4) and are still considered separate groups. As discussed below, low levels of anastomosis is common within *R. solani* and does not signify isolates are of the same anastomosis group. Finally, considering AG2-1 and AG2-2 to be subgroups of AG2 simply adds another level of confusion, since both groups have additional subgroups or cultural types. There is sufficient evidence that AG2-1 and AG2-2 are genetically diverse (Carling et al., 2000; Salazar et al, 1999; Vilgalys & Cubeta, 1994) and there is not a satisfactory reason to add to an already confusing situation.

Bridging Reactions

Isolates within some established AG are able to fuse at low levels with members of other AG and are considered to be bridging isolates (Carling, 1996). The most well-known group of bridging isolates is AGBI (alternately AG2BI) but several other groups, such as AG6, AG8 and AG11 can also fuse with other AG at low levels (Table 1.4). Bridging reactions are generally 'Category 1' (C1) reactions but they can make placing an isolate into the proper AG a difficult task. The fate of isolates that undergo a bridging reaction, whether they exchange genetic

material or create a hybrid state, is uncertain. However, since most bridging reactions involve cell wall fusion and not membrane fusion, the exchange of genetic material seems unlikely.

Table 1.4 Anastomosis groups with bridging capability. Isolates within these bridging groups are known to be able to fuse with isolates in some other groups at low levels.

AG	Capable of bridging with these groups
AG BI	AG 2, AG 3, AG 6, AG 8, AG 11
AG 2	AG 3, AG 8, AG 11, AG BI
AG 3	AG 2, AG 8; AG BI
AG 6	AG 8, AG BI
AG 8	AG 2, AG 3, AG 6, AG 11, AG BI
AG 11	AG 2, AG 8, AG BI

Anastomosis Group Cultural Types

Adding to the complexity of *Rhizoctonia solani* classification, many of the recognized AG have identifiable cultural types for which Ogoshi (1996) proposed the term intraspecific groups (ISG). The use of the term in the literature resulted in some confusion; probably due to a misunderstanding of what was meant by subgroup since the AG themselves could be considered subgroups. So, even though the term 'ISG' is frequently used in the literature, in this review, I will refer to the subgroups of the AG as 'cultural types' rather than ISGs.

Rhizoctonia solani cultural types are not distinguishable from other cultural types within the same AG on the basis of hyphal anastomosis reactions. Instead they are identified by cultural or physiological properties such as colony morphology, pathogenicity, nutrient utilization, sclerotia size and shape, DNA-DNA complementarity, zymogram patterns, DNA sequence and temperature tolerance (Sneh, et al., 1991; Vilgalys & Cubeta, 1994). Currently recognized AG cultural types are listed in Table 1.3.

While these cultural types do have relevance to the study and management of *Rhizoctonia*-induced diseases, they can create additional confusion in an already complex classification scheme. Nomenclature associated with cultural types can be a source of some confusion. As already mentioned, there can be misunderstanding as to what should be considered a subgroup. For example, AG2-1 and AG2-2 both have several cultural types and if they are considered to be subgroups of AG2 then the cultural types would be subgroups of subgroups. In addition, some of the cultural types have designations that are similar to names of other groups within *R. solani*. For instance, there is the group AG4 and there is also a cultural type AG2-2IV (Table 1.3). It would be beneficial if standardized terminology were developed for cultural type designation within *R. solani*. Part of the problem lies in the fact that anastomosis groups are not a taxonomically recognized ranking although some have suggested that the AG should be accorded species status (Carling, 1996).

Cultural Types of AG2-2

Rhizoctonia solani AG2-2 has three widely recognized cultural types: AG2-2IIIB (mat rush type), AG2-2IV (root rot type) and AG2-2LP (large patch type) (Hyakumachi et al., 1998). A fourth cultural type, AG2-2WB (web blight type) has been described by Godoy-Lutz et al. (2008), but reports are limited and acceptance is uncertain. Cultural types AG2-2IIIB and AG2-2IV were separated based on pathogenicity and cultural morphology (Ogoshi, 1987), but cannot be distinguished based on hyphal fusion. The traditionally accepted criterion used for differentiating these cultural types is their ability to grow at 35˚C; where AG2-2IIIB isolates grow well at 35˚C and AG2-2IV isolates do not (Sneh et al., 1991).

Pathogenicity and Host Range

Although *Rhizoctonia solani* is commonly described as having a broad host range (Brooks, 2007; Carling et al., 2002a; Liu & Sinclair, 1992), this attribution tends to be somewhat exaggerated. If anastomosis groups are thought of as independent evolutionary units, the host range of these individual units is much more restricted (Sneh et al., 1991). For example, the host range of *R. solani* AG3 is primarily confined to the *Solanaceae* (Sneh et al., 1991). Isolates in AG8 are primarily isolated from cereal roots (MacNish et al., 1988; Neate & Warcup, 1985) while the groups AG10 and AG13 are considered non-pathogenic (Carling et al., 2002; MacNish et al., 1995), and those in AG12 are mycorrhizal on orchids (Carling et al., 1999). Although technically it is correct to suggest that the *R. solani* species complex, as a whole, has a broad host range, researchers should be mindful that doing so could be, to some extent, misleading. Instead, discussion of host range should be confined to the anastomosis group, or groups, under consideration.

Rhizoctonia solani AG2-2, being a soilborne pathogen, primarily causes 'root rots' or diseases of plant parts in contact with the ground or close to the soil surface (Ogoshi, 1987; Sneh, 1991), although there are diseases that affect above ground parts as well (Godoy-Lutz et al., 2008; Herr, 1996). In addition to 'sheath blight' of mat rush, *R. solani* AG2-2 causes 'sheath blight' of rice (*Oryza sativa*) (Watanabe & Matsuda, 1966), 'Rhizoctonia blight' of turfgrass (Burpee & Martin, 1996), and 'web blight' of common bean (Godoy-Lutz et al., 2008). *R. solani* AG2-2 isolates also cause 'large patch' of *Zoysia* grass, but these isolates are distinct from the main subgroups and have been designated as AG2-2LP (Hyakumachi et al., 1998).

Root rots and diseases of plant parts in contact with the ground are the most common and widespread result of *R. solani* AG2-2 infection. *R. solani* AG2-2 causes 'root and crown rot' of sugar beet (Herr, 1996; Windels & Nabben, 1989); 'root rot' of soybean (Liu & Sinclair, 1991) and common bean (Sumner, 1985); 'crown and brace root rot' of corn (Sumner & Bell, 1982; Sumner & Minton, 1989); 'stem rot' of tobacco (Shew, 1991) and 'belly rot' of cucumber (Goodwill & Hanson, 2011). Other crops reported to be affected by *R. solani* AG2-2 are carrot (Anderson, 1982); spinach (Naiki & Kanoh, 1978); cauliflower, lettuce and radish (Carling et al., 2002b); and alfalfa (Rush & Winter, 1990).

Methods of Identification

Introduction to Identification

This section is provided as a brief overview for the process of identifying *Rhizoctonia solani* isolates. It is not intended to be an exhaustive examination of all such techniques nor is it a detailed description of the methodologies involved. Rather, it is meant to give the reader some guidance in identifying *Rhizoctonia solani* isolates using techniques that have worked well for this author. I will briefly describe these techniques and discuss potential problems and difficulties in their application. For more information on different techniques see Sneh et al. (1991).

Obtaining Pure Cultures

Pure cultures of *Rhizoctonia* are best obtained by growing the culture on a low nutrient medium such as 2% water agar and examining for hyphae with characteristic branching. *Rhizoctonia*-like hyphae are isolated by hyphal tip transfer (Nelson et al., 1983; Whitney &

Parmeter, 1963), where single growing tips are excised at the first branch point and transferred to a nutrient rich medium such as potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO). Cultures are grown for 5-10 days at room temperature and examined for pigment; any color other than brown is an indication that the culture is not *Rhizoctonia*. A small amount of mycelium can be teased apart on a slide and examined microscopically to confirm *Rhizoctonia*-like branching, dolipore septa and that there are no clamp connections or conidia present (Fig. 1.1).

Nuclear Staining

Rhizoctonia-like cultures are examined to determine nuclear condition (binucleate or multinucleate). The easiest way to accomplish this is to coat a sterile slide with 1% water agar by placing five to seven drops of molten (>80˚C) agar in the center of the slide (Windels & Nabben, 1989; Jones & Belmar, 1989). When the agar hardens, a small piece of actively growing hyphae is placed on one end of the slide. The inoculated slides are kept in a humid chamber and the hyphae are allowed to grow across the slide for 2-4 days. The hyphae are stained (see below), covered with a cover slip and observed at 400x. An oil immersion lens should not be used since the surface of the agar will not be flat and it may result in the objective touching the surface of the agar or the cover slip.

There are several staining protocols recommended for nuclear staining (Sneh et al., 1991). The simplest method uses a protocol modified from Herr (1979). Lactophenol blue staining solution (LpBS) is prepared by diluting with a wetting agent [47ml ddH_2O, 25µl lactic acid, 25µl Tween20, 3ml lactophenol blue solution (Sigma-Aldrich, St. Louis, MO, USA)]. Several drops of LpBS are placed on the hyphae and allowed to sit for one minute. Excess solution is

carefully removed with an absorbent tissue. The lactophenol blue stain is rather indiscriminate in the structures it stains and it can be difficult to distinguish nuclei from other cytoplasmic bodies. This protocol does work but performs better as a general hyphal stain than a nuclei specific stain.

Safranin O stains nuclei much more selectively than lactophenol blue. One drop of safranin O solution (6 ml of 0.5% (w/v) safranin O, 10 ml 3.0% (w/v) KOH, 5 ml glycerine, 79 ml distilled water) and one drop of 3.0% (w/v) KOH are placed on the slide and a cover slip is applied (Sneh et al., 1991). The trick to making this technique work well is to create a gradient of staining intensity where the safranin O solution and the KOH solutions intermix. This gradient should be in the zone of hyphal interaction or where young growing hyphae are present. Nuclei will be stained red /orange and are readily visible at 400x magnification (Fig. 1.2A).

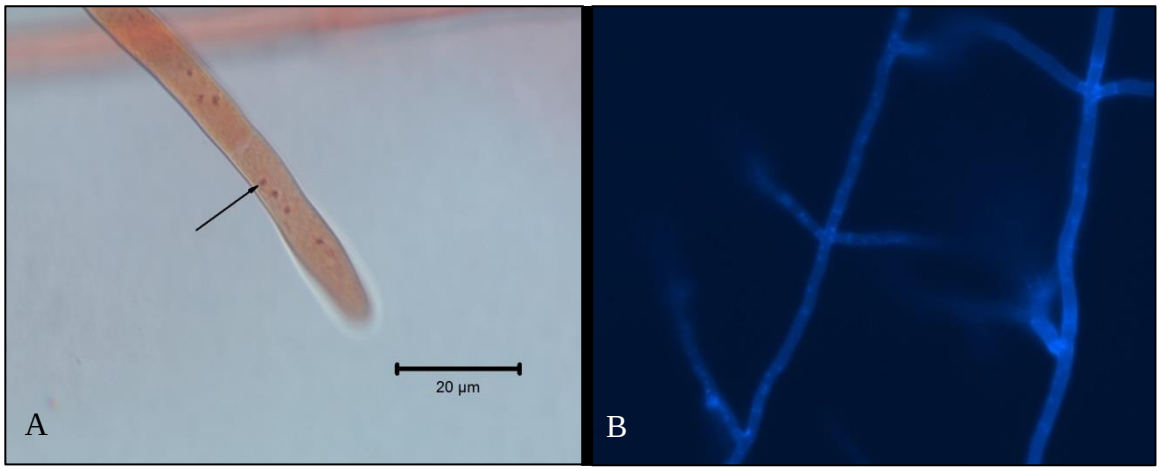

Figure 1.2 Micrographs of *Rhizoctonia solani* hyphae showing stained nuclei. (A) Hyphae stained with Safranin O, nuclei appear red-orange (400X). (B) Hyphae stained with DAPI fluorescent stain. Nuclei appear as bright spots against a darker background (200X). Excitation/emission maximum: 358/461.

A more selective method for staining nuclei uses the DNA binding probe 4'-6'-diamidino-2-phenylindole (DAPI). Hyphae growing on a water agar slide are fixed by flooding with 3% formaldehyde for 2 min. and then rinsing with distilled water for 1 minute. Hyphae are then

flooded with 1µg/ml DAPI solution for 5 - 10 minutes and destained with distilled water for 3 minutes (Sneh et al., 1991). Excess water is blotted dry and a drop of glycerol and a cover slip are added. Nuclei are viewed using fluorescence microscopy [excitation/emission maximum: 358/461] and nuclei are highly visible and easy to distinguish from other cellular structures (Fig. 1.2B). The major disadvantage of this method is that both formaldehyde and DAPI are hazardous chemicals that require proper disposal practices. The other difficulty with this method is that since DAPI is so selective for the nucleus, it can be difficult to identify the extents of each cell, which can make it complicated to count the number of nuclei per cell. Alternating between fluorescent and standard light will usually help one identify the septa and establish the boundaries of the cell.

Anastomosis Testing

In order to place an isolate in one or another anastomosis group, it must be paired with a recognized tester strain and evaluated for interactions between the confronted hyphae (Carling et al., 1988; Ogoshi, 1987). Several methods have been described that allow the hyphae to interact in such a way that the anastomosis reactions can be visualized and assessed (Sneh et al., 1991) including water agar in petri plates (Parmeter, 1969; Rovira et al, 1986; Ogoshi, 1975), cellophane overlaying agar media (Parmeter, 1969; Carling & Sumner, 1992), agar coated slides (Windels & Nabben, 1989; Jones & Belmar, 1989), and bare slides (Kronland & Stanghellini, 1988). Thin cultures provide for a shallow depth of field during microscopic examination and allow for easier visualization of hyphal interactions and therefore, techniques that produce thin cross sections are the most effective. The techniques that have worked best for this author are the water agar in petri plates and agar coated slides, with the petri plate

method being slightly more favorable as it is often easier to trace the interacting hyphae to the parent colony source.

Agar coated slides are prepared as above for nuclear staining by applying five to seven drops of molten 1% agar to a sterile slide. Small pieces of hyphal tissue from the growing margins of cultures of a known anastomosis tester strain and an unknown sample are placed on opposite ends of the agar coating and incubated until the hyphae of the two strains come into contact with one another. The hyphae are stained with a drop or two of LpBS and covered with a cover slip. Excess stain can be removed with an absorbent wipe. Hyphae are observed under 200x or 400x magnification and hyphal interactions are noted. The drawback to this method is that since the slide is rather narrow, the hyphae growing towards the edge of the slide will tend to turn back towards the center of the slide and it can be difficult to trace the hyphae back to the source.

Performing the reaction in a petri dish can help overcome this problem by allowing the hyphae more room to grow laterally and limiting the tendency to double back upon itself. The bottom of a 60 mm petri dish is coated with about 1.5 ml of 1% water agar to form a thin layer (Parmeter, 1969; Rovira et al, 1986). Dropping the molten agar onto the plate and swirling the plate rapidly helps to distribute the agar onto the bottom of the plate. Small pieces of a tester strain and a sample are placed on opposite side of the dish and incubated until the hyphae of the two strains come into contact with one another. The surface of the plate is then flooded with LpBS and allowed to sit for 1 - 2 minutes. Excess stain is removed with an absorbent tissue. Stained plates can be sealed with Parafilm (Bemis NA, Neenah, WI) and kept for several days

until they can be analyzed. Plates are placed on the microscope stage upside down or on an inverted microscope and viewed under 200x or 400x magnification.

Regardless of the method used for confronting isolates, evaluation and interpretation of the reactions is necessary. Contact points between hyphae of opposing isolates are counted and categorized according to the scheme of Carling et al. (1988). Any place where opposing hyphae contact one another and have the opportunity to anastomose should be counted as a contact point. It is important that interacting hyphae are traced back to their source to ensure they originate from opposing isolates.

The system of Carling et al. (1988) is the recommended method for categorizing anastomosis reactions. Four categories of reactions are identified as: 'C0'= no reaction, 'C1'= wall fusion only, 'C2'= membrane fusion with cell death, and 'C3'= membrane fusion with no cell death (Chapter 1, pg. 12, this thesis). Reactions in categories 'C2' and 'C3' indicate isolates are in the same anastomosis group (AG), while reactions in categories 'C0' and 'C1' indicate isolates are in different anastomosis groups. Therefore, when conducting AG testing, the categories could be reduced to just two categories; positive reactions (+) and negative reactions (-). However, since category 'C1' reactions can indicate a bridging relationship (Carling, 1996), it may be useful to note 'C1' reactions in addition to positive reactions.

For each microscopic field of view, the total number of contact points, the number of 'C1' reactions and the number of positive (+) reactions should be counted and recorded. Ten to fifteen microscopic fields of view are examined and counted as described above and a fusion

frequency (Ogoshi, 1975; Carling & Sumner, 1992) is calculated using the following formula:

Equation 1.1 % fusion frequency = $\frac{total\ number\ of\ positive\ reactions}{total\ number\ of\ contact\ points}$ x 100

A fusion frequency of greater than 50% is consider to be high frequency, 30-50% moderate frequency and less than 30% is low frequency (Sneh et al., 1991). Thus, isolates that are paired with a known AG tester that have a high frequency of fusion with that tester strain would be considered to be in the same anastomosis group as the tester. Those with moderate fusion frequency could be considered as probable members of the same anastomosis group as the tester strain. Low fusion frequency generally indicates that the isolate is in a different anastomosis group than the tester strain but also could signify a bridging relationship. The presence of additional 'C1' reactions could help reinforce this conclusion.

rDNA-ITS Sequencing

Interpretation of anastomosis reactions can be complicated and time consuming (Sharon et al., 2006). Furthermore, classifying anastomosis reactions into discrete categories can be rather subjective, especially considering that anastomosis reactions represent a continuum of hyphal interactions (Vilgalys & Cubeta, 1994). In contrast, molecular methods have the advantage of being somewhat more objective and reproducible (Vilgalys & Cubeta, 1994). Ribosomal DNA (rDNA) sequences have been widely used for investigating evolutionary relationships of *R. solani* and have added support for the classical anastomosis classification system (Carling et al., 2001; Godoy-Lutz et al., 2006; Salazar et al., 1999; Sharon et al., 2007; Strausbaugh et al., 2011).

Ribosomal genes are located in the mitochondria or the nuclei and contain regions of highly conserved sequences and regions of highly variable sequences (White et al., 1990). Nuclear ribosomal genes are arranged in tandemly repeated units of up to several hundred copies (Buckner et al., 1988; Rogers & Bendich, 1987). Each unit contains the genes for the 18S (small subunit), 5.8S and 28S (large subunit) ribosomal subunits (Fig. 1.3) which are highly conserved across closely related individuals (Hamby & Zimmer, 1992; Salazar et al., 2000a; Vilgalys & Gonzlez, 1990). The subunits are separated by internal transcribed spacers (ITS) that are transcribed but not translated and because of this, display a high level of sequence variation (Gonzalez et al., 2001; Kuninaga et al., 1997). This combination of features (large copy number

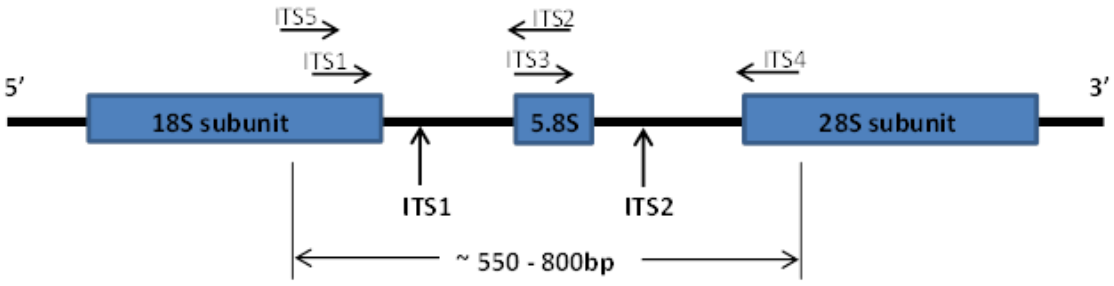

Figure 1.3 Schematic illustrating the organization of ribosomal RNA genes in fungi. Primer sequences are from White et al., (1990) and are drawn as arrows indicating the direction of orientation. ITS1 and ITS2 are internal transcribed spacers that are transcribed into mRNA but excised before translation. Subunits are highly conserved between groups while the ITS regions are highly variable (White et al., 1990)

and highly variable sequences interspersed between highly conserved sequences) makes rDNA-ITS genes desirable for many types of molecular studies (Schoch et al., 2012).

Diversity of the Anastomosis Group 2-2

Problems with Traditional AG2-2 Subgroup Designations

The traditional subgroups AG2-2IIIB and AG2-2IV were first separated by host range, but it was later found that the host range of both types was much broader than originally understood (Ogoshi, 1987). The groups were then distinguished based on ability to grow at 35˚C with AG2-2IIIB able to grow well at that temperature and AG2-2IV unable to grow (Sneh, 1991). Researchers found that there were a number of isolates that could grow at 35˚C, but did so at a much slower rate than those that were identified as AG2-2IIIB (Engelkes & Windels, 1996; Hanson, unpublished data). These have been designated as 'intermediates' and their inclusion in groups AG2-2IIIB or AG2-2IV is uncertain. PCR based methods have been developed to help differentiate AG2-2IIIB and AG2-2IV (Bolton et al., 2010; Carling et al., 2002b; Salazar et al., 2000b). Primers specific to AG2-2 (Carling et al., 2002b) have worked well but those that were intended to differentiate the cultural types have shown inconsistencies (Brantner, unpublished data; Fenille et al., 2003; Hanson, unpublished data; Martin et al., 2014).

Classification Proposed by Martin et al. (2014)

In order to clarify the relationships within *Rhizoctonia solani* AG2-2, Martin et al. (2014) sequenced four nuclear genes using markers that had been developed for the classification of *Rhizoctonia* fungi (Gonzalez et al., 2016). Sixty-three *R. solani* isolates from diverse regions were analyzed and their phylogenetic relationships determined. Their work confirmed that the traditional subgroups AG2-2IIIB and AG2-2IV were not phylogenetically supported (Fig. A1.2). Instead, at least two major clades were observed with one containing two well supported sub-clades. They referred to these groups as clade 1, 2A and 2B and each contained a mix of AG2-

2IIIB and AG2-2IV isolates. These results agree with previous findings that, based on ITS sequences, AG2-2IIIB and AG2-2IV were polyphyletic (Carling et al., 2002b; Strausbaugh et al., 2011).

Objectives

The main objective of this thesis is to examine the diversity within *Rhizoctonia solani* AG2-2 and examine the potential relationship of that variability to the newly identified phylogenetic groups of Martin et al. (2014). A more accurate and comprehensive understanding of the variability within this important group of pathogens can better inform management decisions. For this I have conducted three separate projects that will help to address these issues and provide a better understanding regarding the diversity within the AG2-2 complex.

The first project examines the virulence of *R. solani* AG2-2 isolates on dry beans (*Phaseolus vulgaris*) and contrasts the three phylogenetic groups. To the best of my knowledge, this is the largest screening of AG2-2 isolates on dry beans to date and examines disease severity at both the seedling and adult life stages. It provides an alternative assessment of virulence diversity and distribution as compared to the traditional subgroups of AG2-2IIIB and AG2-2IV.

The second project evaluates the ability of AG2-2 isolates to cause disease at low temperatures (11˚C). Previous reports stated that the minimum temperature for infection was 15˚C and that the risk of disease below that temperature was minimal to non-existent. Since sugar beet (*Beta vulgaris*) is typically planted very early in the spring when soil temperatures are well below 15˚C, it is important to confirm that infection does not occur below the accepted

norm. My approach differs from previous studies in that it uses a broader range of isolates that are representative of the three phylogenetic groups.

The third project involves the development of a set of microsatellite markers specifically for *R. solani* AG2-2. These markers can be used as a tool to address questions that involve the extent of genotype flow between regions, the effect of crop rotations on *R. solani* AG2-2 populations, and the presence of host preferences. The approach used for identifying potential markers involved NextGen sequencing of representative isolates and *in silico* selection of prospective loci. This approach made screening simpler and more efficient compared to more traditional methods of microsatellite isolation (Glenn & Schable, 2005; Selkoe & Toonen, 2006). The project is still ongoing and only preliminary results are reported here.

APPENDIX

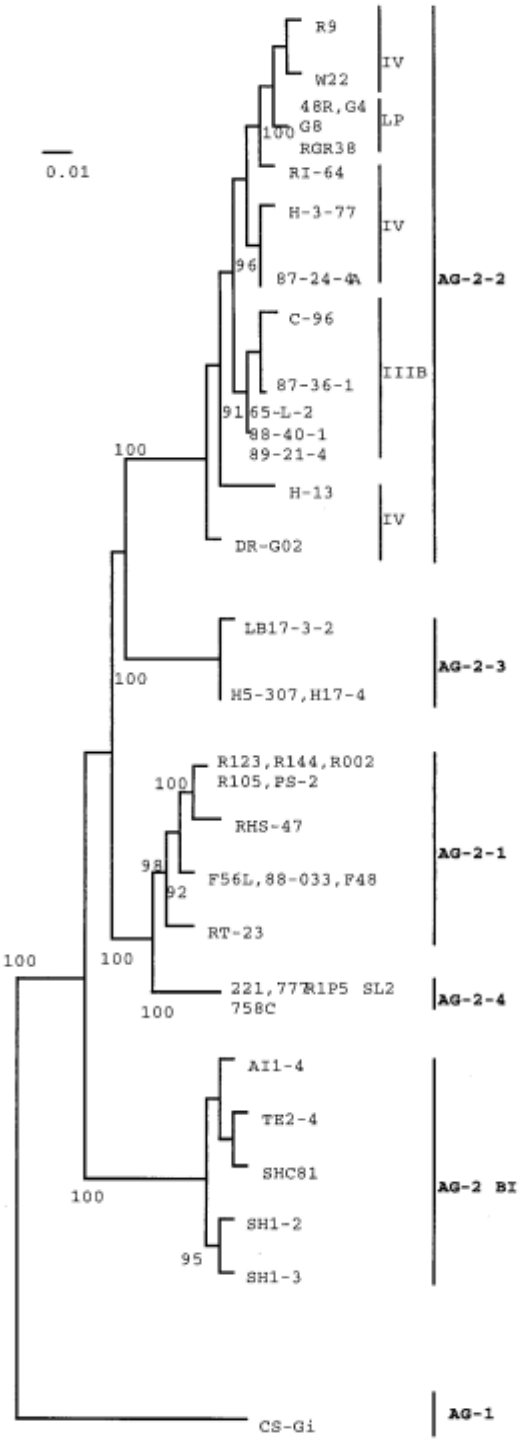

Figure A1.1 Neighbor-joining tree from Carling et al. (2002b). The tree illustrates estimated relationships of *Rhizoctonia solani* AG2 groups based on rDNA-ITS sequences. Bar indicates 1 base change per 100 nucleotide positions. Numbers at branches indicate the percentages greater than 90% of congruent clusters in 1,000 bootstrap trials.

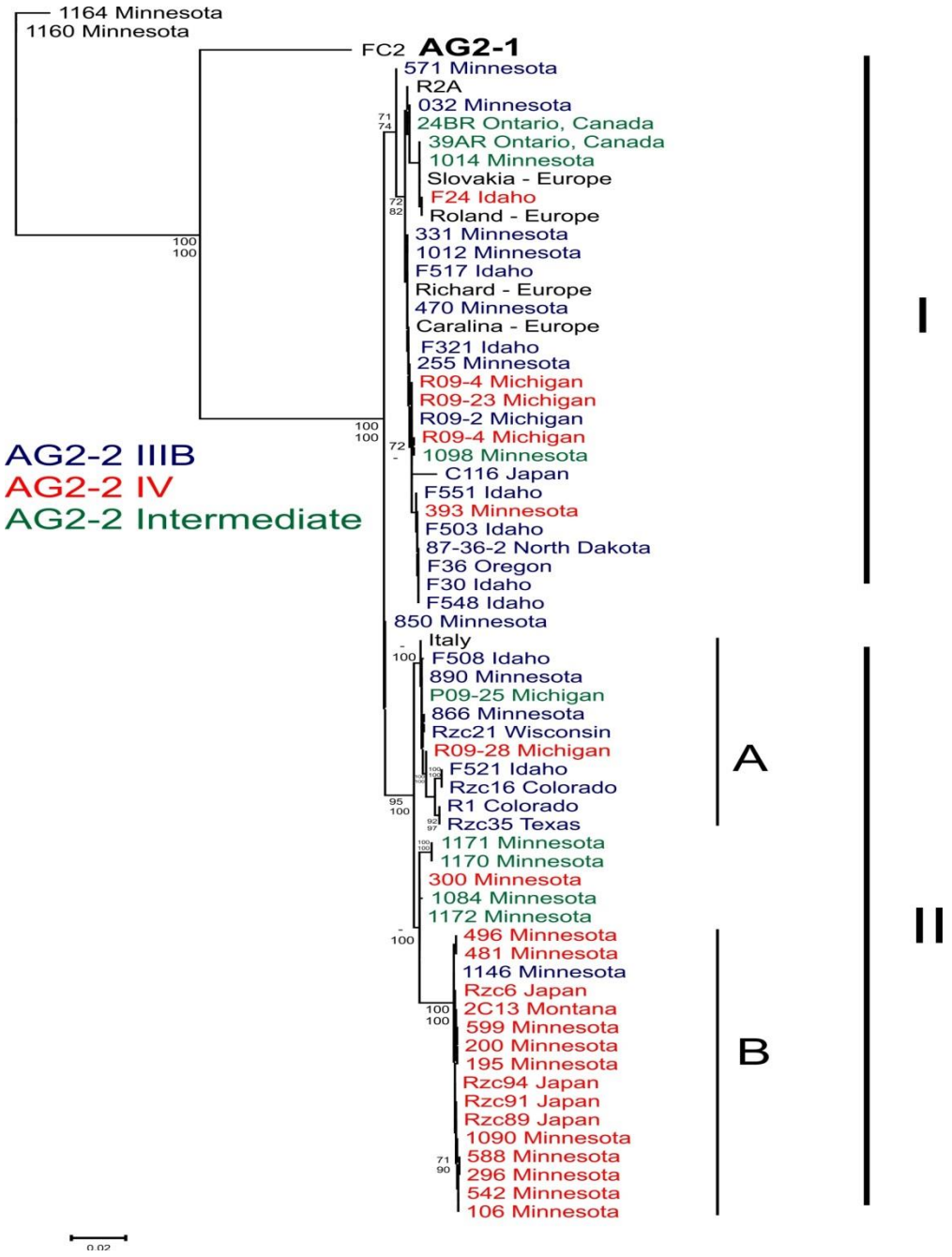

Figure A1.2 Multigene phylogeny of 63 *Rhizoctonia solani* AG2-2 isolates according to Martin et al. (2014). Genes sequenced included *rpb2*, tef1*, ITS,* and *LSU* as reported in Gonzalez et al. (2016) with minor modifications to improve reliability and specificity for AG2-2 (unpublished data). Isolates in blue were originally identified as AG2-2IIIB, those in red identified as AG2-2IV, and those in green were intermediates based on growth at 35˚C - where AG2-2IIIB grows well at 35˚C and AG2-2IV do not. An AG2-1 isolate was used for the outgroup. Phylogram used curtesy of Martin et al. (2014).

REFERENCES

REFERENCES

Adams, G.C. jr. (1988). *Thanatephorus cucumeris* (*Rhizoctonia solani*) a species complex of wide host range. Advances in Plant Pathology, 6: 535-552.

Ajayi-Oyetunde, O.O. and Bradley, C.A. (2018). *Rhizoctonia solani*: taxonomy, population biology and management of Rhizoctonia seedling disease of soybean. Plant Pathology, 67: 3-17.

Anderson, N.A. (1982). The genetics and pathology of *Rhizoctonia solani.* Annual Review of Phytopathology, 20: 329-347.

Andersen, T.F. and Stalpers, J.A. (1994). A check-list of *Rhizoctonia* epithets. Mycotaxon, 51: 437-457.

Baker, K.F. (1970). Types of *Rhizoctonia* diseases and their occurrence. Pages 125-148 in *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr., University of California Press, Berkeley, CA.

Bolton, M.D., Panella, L., Campbell, L. and Khan, M.F.R. (2010). Temperature, moisture and fungicide effects in managing Rhizoctonia root and crown rot of sugar beet. Phytopathology, 100: 689-697.

Brooks, S.A. (2007). Sensitivity to a phytotoxin from *Rhizoctonia solani* correlates with sheath blight susceptibility in rice. Phytopathology, 97: 1207-1212.

Buckner, B., Novotny, C.P. and Ullrich, R.C. (1988). Organization of the ribosomal RNA gene of *Schizophyllum commune*. Current Genetics, 13: 417-424.

Burpee, L.L. and Martin, S.B. (1996). Biology of turfgrass diseases incited by *Rhizoctonia* species. Pages 359-368 in *Rhizoctonia*: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Edited by Sneh, B., Jabaji-Hare, S., Neate, S. and Dijst, G., Kluwer Academic Publishers, Dordrecht, the Netherlands.

de Candolle, A.P. (1815). *Mémoire sur les Rhizoctones, noveau genera de champignons qui attaque les raciness, des plantes et en particulier de la Luzerne cultivée.* Memorias del Museo de Historia Natura, 2: 209-216.

Carling, D.E. (1996). Grouping in *Rhizoctonia solani* by hyphal anastomosis reaction. Pages 37-47 in *Rhizoctonia*: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Edited by Sneh, B., Jabaji-Hare, S., Neate, S. and Dijst, G., Kluwer Academic Publishers, Dordrecht, the Netherlands.

Carling, D.E., Baird, R.E., Gitaitis, R.D., Brainard, K.A. and Kuninaga, S. (2002a). Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. Phytopathology, 92: 893-899.

Carling, D.E. and Kuninaga, S. (1990). DNA base sequence homology in *Rhizoctonia solani* Kühn: Inter- and intragroup relatedness of anastomosis group-9. Phytopathology, 80: 1362-1364.

Carling, D.E., Kuninaga, S. and Brainard, K.A. (2002b). Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence level among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. Phytopathology, 92: 43-50.

Carling, D.E., Kuninaga, S. and Leiner, R.H. (1988). Relatedness within and among intraspecific groups of *Rhizoctonia solani*: a comparison of groupings by anastomosis and by DNA hybridization. Phytoparasitica, 16: 209-210. (Abstr.)

Carling, D.E., Leiner, R.H. and Kebler, K.M. (1987). Characterization of a new anastomosis group (AG 9) of *Rhizoctonia solani*. Phytopathology, 77: 1609-1612.

Carling, D.E., Pope, E.J., Brainard, K.A. and Carter, D.A. (1999). Characterization of mycorrhizal isolates of *Rhizoctonia solani* from an orchid, including AG-12, a new anastomosis group. Phytopathology, 89: 942-946.

Carling, D.E., Rothrock, C.S., MacNish, G.C., Sweetingham, M.W., Brainard, K.A. and Winter, S.A. (1994). Characterization of anastomosis group-11 (AG 11) of *Rhizoctonia solani.* Phytopathology, 84: 1387-1393.

Carling, D.E., and Sumner, D.R. (1992). *Rhizoctonia*. Pages 157-165 in Methods for Research on Soilborne Phytopathogenic Fungi. Edited by Singleton, L.L., Mihail, J.D. and Rush, C.M. APS Press, St. Paul, MN, USA.

Cubeta, M.A. and Vilgalys, R. (1997). Population biology of the *Rhizoctonia solani* complex. Phytopathology, 87: 480-484.

Duggar B.M. (1915). *Rhizoctonia crocorum* (Pers.) D.C. and *R. solani* Kühn (*Corticum vagum* B. & C.), with notes on other species. Annals of the Missouri Botanical Garden, 2: 403-458.

Engelkes, C.A. and Windels, C.E. (1996). Susceptibility of sugar beet and beans to *Rhizoctonia solani* AG2-2 IIIB and AG2-2 IV. Plant Disease, 80: 1413-1417.

Flentje, N.T. and Stretton, H.M. (1964). Mechanisms of variation in *Thanatephorus cucumeris* and *T. praticolus*. Australian Journal of Biological Sciences, 17: 686-704.

Gams, W. (2001). Report of the committee for fungi: 9. Taxon, 50: 269-272. Retrieved from http://www.jstor.org/stable/1224527 on Feb 15, 2018.

Glenn, T.C. and Schable, N.A. (2005). Isolating microsatellite DNA loci. Methods in Enzymology, 395: 202-222.

Godoy-Lutz, G., Kuninaga, S., Steadman, J.R. and Powers, K. (2008). Phylogenetic analysis of *Rhizoctonia solani* subgroups associated with web blight symptoms on common bean based on ITS-5.8S rDNA. Journal of General Plant Pathology, 74: 32-40.

Gonzalez, D., Rodriguez-Carres, M., Boekhout, T., Stalpers, J., Kuramae, E.E., Nakatani, A.K. ... and Cubeta, M.A. (2016). Phylogenetic relationships of *Rhizoctonia* fungi within the Cantharellales. Fungal Biology, 120: 603-619.

Goodwill, T.R., Hanson, L.E. 2011. Rhizoctonia belly rot in cucumber fruit using *Rhizoctonia solani* isolated from sugar beet. Proceedings of the American Society of Sugar Beet Technologists. 36th Biennial Meeting, March 2-5, 2011, Albuquerque, New Mexico. 2011 CDROM.

Hamby, R.K. and Zimmer, E.A. (1992). Ribosomal RNA as a phylogenetic tool in plant systematics. Pages 50-91 in Molecular Systematics of Plants. Edited by Soltis, P.S. Chapamn & Hall, New York, NY.

Hawksworth, D.L., Crous, P.W., Redhead, S.A., Reynolds, D.R., Samson, R.A., Siefert, K.A. ... Zhang, N. (2011) The Amsterdam declaration on fungal nomenclature. IMA Fungus, 2: 105-112.

Herr, L.J. (1996). Sugar beet disease incited by *Rhizoctonia* spp. Pages 341-349 in *Rhizoctonia*: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Edited by Sneh, B, Jabaji-Hare, S, Neate, S. and Dijst, G. Kluwer Academic Publishers, The Netherlands.

Homma, Y., Yamashita, Y. and Ishii, M. (1983). A new anastomosis group (AG 7) of *Rhizoctonia solani* Kühn from Japanese radish fields. Annals of the Phytopathological Society of Japan, 49: 184-190.

Hykumachi, M., Mushika, T. Ogiso, Y, Tdoa, T. Kageyama, K. and Tsuge, T. (1998). Characterization of a new cultural type (LP) of *Rhizoctonia solani* AG2-2 isolated from warm-season turfgrasses, and its genetic differentiation from other cultural types. Plant Pathology, 47: 1-9.

Jones, R.K. and Belmar, S.B. (1989). Characterization and pathogenicity of *Rhizoctonia* spp. isolated from rice, soybean, and other crops grown in rotation with rice in Texas. Plant Disease, 73: 1004-1010.

Kronland, W.C. and Stanghellini, M.E. (1988). Clean slide technique for the observation of anastomosis and nuclear condition of *Rhizoctonia solani*. Phytopathology, 78: 820-822.

Kühn, J.G. (1858). Die Krankheiten der Kulturegewächse, ihre ursachen und ihre Verhütung. Gustav Bosselmann, Berlin. pg. 312.

Kuninaga, S., Carling, D.E., Takeuchi, T. and Yokosawa, R. (2000a). Comparison of rDNA-ITS sequences between potato and tobacco strains in *Rhizoctonia solani* AG3. Journal of General Plant Pathology, 66: 2-11.

Kuninaga, S., Natsuaki, T., Takeuchi, T. and Yokosawa, R. (1997). Sequence variation of the rDNA ITS regions between anastomosis groups in *Rhizoctonia solani*. Current Genetics, 32: 237-243.

Kuninaga, S., Nicoletti, R., Lahoz, E. and Naito, S. (2000b). Ascription of Nt-isolates of *Rhizoctonia solani* to anastomosis group 2-1 (AG2-1) on account of rDNA-ITS sequence similarity. Journal of Plant Pathology, 82: 61-64.

Kuninaga, S. and Yokosawa, R. (1985). DNA base sequence homology in *Rhizoctonia solani* Kühn. VI. Genetic relatedness among seven anastomosis groups. Annals of the Phytopathological Society of Japan, 51: 127-132.

Kuninaga, S., Yokosawa, R. and Ogoshi, A. (1979). Some properties of anastomosis group 6 and BI in *Rhizoctonia solani* Kühn. Annals of the Phytopathological Society of Japan, 45: 207-217.

Liu, Z.L. and Sinclair, J.B. (1991). Isolates of *Rhizoctonia solani* anastomosis group 2-2 pathogenic to soybean. Plant Disease, 75: 682-687.

Liu, Z.L. and Sinclair, J.B. (1992). Genetic diversity of *Rhizoctonia solani* anastomosis group 2. Phytopathology, 82: 778-787.

MacNish, G.C., Carling, D.E., Sweetingham, M.W., Ogoshi, A. and Brainard, K.A. (1995). Characterization of anastomosis group-10 (AG 10) of *Rhizoctonia solani*. Australasian Journal of Plant Pathology, 25: 252-260.

Martin, F., Windels, C., Hanson, L. and Brantner, J. (2014). Analysis of population structure and pathogenicity of *Rhizoctonia solani* AG2-2 (ISG IIIB and IV) isolates from Michigan, Minnesota and North Dakota. Sugarbeet Research Reports. Beet Sugar Development Foundation, Denver, CO. Published on CD.

Matsumoto, T., Yamamoto, W. and Hirane, S. (1932). Physiology and parasitology of the fungi generally referred to as *Hypochnus sasakii* Shirai. I. Differentiation of the strains by means of hyphal fusion and culture in differential media. Journal of the Society of Tropical Agriculture, 4:370-388.

Mayr,E. (1963). Populations, species, and evolution: an abridgement of animal species and evolution. The Belknap Press of Harvard University, Cambridge MA.

Menzies, J.D. (1970). Introduction: The first century of *Rhizoctonia solani*. Pages 3-5 in: *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr., University of California Press, Berkeley, CA.

Moore, R.T. (1987). The genera of *Rhizoctonia*-like fungi: *Ascorhizoctonia, Ceratorhiza* gen. nov., *Epulorhiza* gen. nov., *Moniliopsis*, and *Rhizoctonia*. Mycotaxon, 29: 91-99.

Naiki, T. and Kanoh, M. (1978). Grouping of *Rhizoctonia solani* Kuehn causing root disease of spinach in plastic house cropping. Annals of the Phytopathological Society of Japan, 44: 554-560.

Naito, S. and Kanematsu, S. (1994). Characterization and pathogenicity of a new anastomosis subgroup AG2-3 of *Rhizoctonia solani* Kühn isolated from leaves of soybean. Annals of the Phytopathological Society of Japan, 60: 681-690.

Neate, S.M., Cruikshank, R.H. and Rovira, A.D. (1988). Pectic enzyme patterns of *Rhizoctonia solani* from agricultural soils in South Australia. Transactions of the British Mycological Society, 90: 37-42.

Neate, S.M. and Warcup, J.H. (1985). Anastomosis grouping of some isolates of *Thanatephorus cucumeris* from agricultural soils in South Australia. Transactions of the British Mycological Society, 85: 615-620.

Nelson, P.E., Tousoun, T.A. and Marasas, W.F.O. (1983). *Fusarium* species: An illustrtated manual for identification. The Pennsylvania State University Press. University Park, PA.

Ogoshi, A. (1976). Studies on the grouping of *Rhizoctonia solani* Kühn with hyphal anastomosis, and on the perfect stage of groups. Bulletin of the National Institute on Agricultural Sciences Series C, 30: 1-63.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annual Review of Phytopathology, 25:124-143.

Parmeter, J.R. jr., Sherwood, R.T. and Platt, W.D. (1969). Anastomosis grouping among isolates of *Thanatephorus cucumeris.* Phytopathology, 59: 1270-1278.

Parmeter, J.R jr. and Whitney, H.S. (1970). Taxonomy and nomenclature of the imperfect state. Pages 7-19 in: *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R., Jr., University of California Press, Berkeley, CA.

Richter, H. and Schneider, R. (1953). Untersuchungen zur Morphologischen und biologischen differenzierung von *Rhizoctonia solani* K. Phytopathologische Zeitschrift, 20: 167-226. (as reported in Carling, 1996).

Rogers, S.O. and Bendich, A.J. (1987). Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Molecular Biology, 9: 509-520.

Rovira, A.D., Ogoshi, A. and McDonald, H.J. (1986). Characterization of isolates of *Rhizoctonia solani* from cereal roots in South Australia and New South Wales. Phytopathology, 76: 1245-1248.

Salazar, O., Julián, M.C., Hyakumachi, M. and Rubio, V. (2000a). Phylogenetic grouping of cultural types of *Rhizoctonia solani* AG 2-2 based on ribosomal ITS sequences. Mycologia, 92: 505-509.

Salazar, O., Julian, M.C. and Rubio, V. (2000b). Primers based on specific rDNA-ITS sequences for PCR detection of *Rhizoctonia solani*, *R. solani* AG2 subgroups and ecological types, and binucleate *Rhizoctonia*. Mycological Research, 104: 281-285.

Salazar, O., Schneider, J.H.M., Julián, M.C., Keijer, J. and Rubio, V. (1999). Phylogenetic subgrouping of *Rhizoctonia solani* AG 2 isolates based on ribosomal ITS sequences. Mycologia, 91: 459-467.

Schneider, J.H.M., Schilder, M.T. and Dijst, G. (1997). Characterization of *Rhizoctonia solani* AG2 isolates causing bare patch in field grown tulips in the Netherlands. European Journal of Plant Pathology, 103: 265-279.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Vincent, R., Spouge, J.L., Levesque, C.A., Chen, Wen and Fungal Barcoding Consortium (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. PNAS, 109: 6241-6246. https://doi.org/10.1073/pnas.1117018109

Selkoe, K.A. and Toonen, R.J. (2006). Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters, 9: 615-629.

Sharon, M., Kuninaga, S., Hyakumachi, M. and Sneh, B. (2006). The advancing identification and classification of *Rhizoctonia* spp. using molecular and biotechnological methods compared with the classical anastomosis grouping. Mycoscience, 47: 299-316.

Shew, H.D. (1991) Sore shin and damping-off. Pages 24-25 in Compendium of Tobacco Diseases. Edited by Shew, H.D and Lucas G.B., APS Press, St. Paul, MN.

Shultz, H. (1936). Vergleichende Undersuchungen zur Okologic, Morphologie und Systematik des Vermchrungspilzes. Abr. Biol. Reichsanst. Land-u Forstwirtsch., Berl. 22:1-41. (as reported in Carling, 1996).

Sneh, B., Burpee, L. and Ogoshi, A. (1991). Identification of *Rhizoctonia* species. APS Press, St. Paul, MN.

Stalpers, J.A., Andersen, T.F. and Gams, W. (1998). Two proposals to conserve the names *Rhizoctonia* and *R. solani* (*Hyphomycetes*). Taxon, 47: 725-726.

Sumner, D.R. (1985). Virulence of anastomosis groups of *Rhizoctonia solani* and *Rhizoctonia*-like fungi on selected germplasm of snap bean, lima bean, and cowpea. Plant Disease, 69: 25-27.

Sumner, D.R. and Bell, D.K. (1982). Root diseases of corn induced by *Rhizoctonia solani* and *Rhizoctonia zeae*. Phytopathology, 72: 86-91.

Sumner, D.R. and Minton, N.A. (1989). Crop losses in corn induced by *Rhizoctonia solani* AG2-2 and nematodes. Phytopathology, 79: 934-941.

Vilgalys, R. and Cubeta, M.A. (1994). Molecular systematics and population biology of *Rhizoctonia*. Annual Review of Phytopathology, 32: 135-155.

Vilgalys, R. and Gonzalez, D. (1990). Ribosomal DNA restriction fragment length polymorphisms in *Rhizoctonia solani*. Phytopathology, 80: 151-158.

Watanabe, B. and Matsuda, A. (1966). Studies on the grouping of *Rhizoctonia solani* Kühn pathogenic to upland crops. Bulletin for Appointment Experiments (Plant Disease and Insect : Pests), 7: 1-131.

Windels, C.E. and Nabben, D.J. (1989). Characterization and pathogenicity of anastomosis groups of *Rhizoctonia solani* from *Beta vulgaris*. Phytopathology, 79: 83-88.

Whitney, H.S. and Parmeter, J.R. Jr. (1963). Synthesis of heterokaryons in Rhizoctonia solani Kühn. Canadian Journal of Botany, 41: 879-886.

CHAPTER 2:

VIRULENCE OF *RHIZOCTONIA SOLANI* AG2-2 ISOLATES

ON DRY BEAN (*PHASEOLUS VULGARIS*)

Introduction

Rhizoctonia solani Kühn is a ubiquitous soilborne fungus that causes disease on many economically important crops throughout the world (Anderson, 1982; Ogoshi, 1987; Sneh et al., 1991). It is one of the most prevalent and damaging root pests of sugar beet (*Beta vulgaris* L.) in Michigan, Minnesota and North Dakota (Windels & Nabben, 1989; Poindexter, 2014) causing yield loss, reduced sucrose content, and increased susceptibility to storage rots (Strausbaugh et al., 2011b; Windels et al., 2009). Furthermore, many of the crops that are grown in rotation with sugar beet are also susceptible to the same strains of *R. solani* (Ruppel, 1985) making cultural management challenging. Understanding how different crops are affected by *R. solani* can help inform management decisions and improve the effectiveness of disease control measures.

One of the most common and important strategies in managing disease caused by *R. solani* is crop rotation (Buhre et al., 2009). Increased disease pressure as a result of continuous monocultures has been shown for several cropping systems, including potato (*Solanum tuberosum*) (Gilligan et al., 1996, Larkin & Honeycut, 2006), wheat (*Triticum aestivum*) (Schillinger & Paulitz, 2006) and sugar beet (Maxson, 1938; Schuster & Harris, 1960). Since the primary objective of crop rotation is to reduce pathogen impact over successive growing seasons, it is important to understand how rotational crops influence population structure and persistence of the pathogen (Boine et al., 2014). Crop types that are hosts to the same virulent strains are likely to cause increased damage when grown in close rotation (Buhre et al., 2009, Engelkes & Windels, 1996).

The effect of crop rotation on disease can be difficult to interpret and is highly dependent on the pathogen/host system (Sumner et al., 1981). *R. solani*-induced diseases and the effect of rotational crops have been examined for several systems including potato (Emmond & Ledingham, 1972), wheat (Rovira, 1986), corn (Sumner & Bell, 1986), soybean (Nelson et al., 1996), and sugar beet (Buhre et al., 2009; Ruppel, 1985; Rush & Winter, 1990). However, conclusions regarding the contribution of rotational crops to disease severity have not always been consistent (Ruppel, 1985; Windels & Brantner, 2004). For example, Ruppel (1985) found that alfalfa (*Medicago sativa*) was not a host of strains of *R. solani* that had been isolated fom sugar beet, which was in contrast to previous findings by Maxson (1938). Maxson (1938) also found that a rotation including small grains decreased disease severity in a subsequent sugar beet crop. In contrast, Ruppel (1985) determined that although barley reduced disease on a subsequent beet crop, it was a host of *R. solani* AG-2. Coons and Kotila (1935) showed corn decreased disease severity while Windels and Brantner (2004) showed that corn increased disease severity on a following beet crop. Sumner & Minton (1989) confirmed corn was a host for *Rhizoctonia solani* AG2-2.

One possible reason for these discrepancies is that there is more than just susceptibility to be considered when selecting a suitable rotational crop. For example, *R. solani* can survive saprophytically on crop residues (Papavizas, 1970). Persistence in the soil may depend on how well a particular crop residue contributes to pathogen survival (Frank & Murphy, 1977; Ruppel, 1985). There may also be a connection between residual NO_3-N and disease incidence (Rush & Winter, 1990). Crops that deplete residual NO_3-N could increase vulnerability of the following crop by slowing plant development. Fertilizer applications reduced disease severity in

continuous sugar beet cropping in trials and this and may be due, at least in part, to the seedlings maturing faster and thus escaping early stages of infection (Schuster & Harris, 1960). However, the connection between soil nitrogen and *Rhizoctonia*-induced disease has not been fully explored.

Another possible reason for some of the discrepancies in the assessment of rotational crops is the variability of the pathogen. *R. solani* is a heterogeneous species complex consisting of at least 14 subgroups (Carling et al., 2002a) which are distinguished based on the ability of the hyphae to fuse or anastomose (Ogoshi, 1987; Sneh et al., 1991). Although *R. solani* as a whole has a very broad host range, affecting more than 200 plant species (Baker, 1970; Salazar et al., 2000), the various anastomosis groups (AG) represent genetically isolated groups and each AG has a more restricted host range (Cubeta & Vilgalys, 1997; Ogoshi, 1987; Parmeter et al, 1969). Still, some individual AGs can affect a large number of plant species (Ogoshi, 1987) and make crop rotation choices difficult. The anastomosis group 2-2 (AG2-2) is the primary AG that attacks adult sugar beet and typically causes a rot of the crown and root, known as Rhizoctonia root and crown rot (RRCR) (Ogoshi, 1987; Windels & Nabben, 1989). Moreover, many of the crops commonly grown in rotation with sugar beet are also susceptible to *R. solani* AG2-2. These rotational crops include corn (*Zea mays*) (Sumner & Minton, 1989; Windels & Brantner, 2006), soybean (*Glycine max*) (Fenille et al., 2002; Nelson et al., 1996) and common bean (*Phaseolus vulgaris*) (Muyolo et al., 1993; Peña et al., 2013).

In addition to the divisions based on anastomosis grouping, several AG within *R. solani* are further divided into cultural types, commonly known as intraspecific groups (ISGs). These cultural types have been identified using physiological or genetic characteristics such as host

range, sclerotial form, differential auxotrophy or DNA homology (Ogoshi, 1987; Sneh et al., 1991). *R. solani* AG2-2 is one of the anastomosis groups that have been subdivided into several cultural types and these include AG2-2IIIB ('mat-rush' type), AG2-2IV ('root rot' type) and AG2-2LP ('large patch' type) (Ogoshi, 1987; Watanabe & Matsuda 1966; Hyakumachi et al., 1998). AG2-2IIIB and AG2-2IV are the primary groups that are responsible for economic losses in sugar beet, while AG2-2LP causes disease primarily on warm season turf grasses (Hyakumachi et al., 1998; Ogoshi, 1987). Originally, AG2-2IIIB and AG2-2IV were separated based on host range with AG2-2IIIB causing sheath blight of mat rush (*Juncus effuses*) and AG2-2IV causing root rot of sugar beet (Ogoshi, 1987). It is now recognized that both types have a wider host range than originally described and both types have been reported to cause disease on sugar beet (Engelkes & Windels, 1996; Nelson et al., 1996; Strausbaugh et al., 2011a), although they are still reported to show some variability in host range and virulence (Carling et al., 2002b; Engelkes & Windels, 1996; Strausbaugh et al., 2011a). These cultural types are now distinguished by the ability of the cultures to grow at 35˚C; where the IIIB type grows at 35˚C and the IV type does not (Sneh et al., 1991).

Despite the lack of host range differentiation originally used to define them, the subgroups of AG2-2 have remained as important divisions. Differences in virulence have been noted by several workers where AG2-2IIIB was found to be more aggressive on sugar beet roots than AG2-2IV (Carling et al., 2002b; Kuninaga et al., 1997; Strausbaugh et al., 2011a). Windels and Brantner (2006) showed that corn increased the prevalence of AG2-2IIIB (but not AG2-2IV), which resulted in greater disease severity on a following sugar beet crop compared to soybeans or wheat. Similar variability in aggressiveness and host preference are also recognized in other

R. solani AG subgroups with other hosts, such as AG1-1A, AG1-1B and AG1-1C (Ogoshi, 1987; Priyatmojo et al., 2001). This ability to at least somewhat predict aggressiveness from cultural type and the tendency for certain crop rotations to increase the more virulent groups helped maintain interest in separating AG2-2IIIB and AG2-2IV.

In work by Carling et al. (2002b) and Strausbaugh et al. (2011a), the genetic relationship of AG2-2IIIB and AG2-2IV was found to be questionable. In order to clarify these relationships, Martin et al. (2014) analyzed the phylogenetic relationships of 63 AG2-2 isolates using four nuclear gene regions and confirmed that the cultural types AG2-2IIIB and AG2-2IV are not phylogenetically supported. Instead, at least two major clades were observed with one of them containing two well supported sub-clades. These phylogenetic groups were referred to as clades 1, 2A and 2B and contained a mix of AG2-2IIIB and AG2-2IV isolates. Therefore, inferences based on the subgroups AG2-2IIIB and AG2-2IV need to be reexamined.

Reports on the susceptibility of rotational crops prior to the late 1980's were done without the current understanding of anastomosis groupings and it is not always clear what AG were present. Recent analysis often relied on a small number of isolates to test for pathogenicity on rotational crops and may not have adequately considered the variability of the pathogen. With these considerations, studies that examine the variation in virulence within AG2-2 are fairly limited. Those that have been reported show considerable variability among isolates. For example, the severity of root rot on adult sugar beet varied from 19% to 100% among 47 AG2-2IIIB isolates and from 34% to 71% among 4 AG2-2IV isolates in a study by Strausbaugh et al. (2011a). Nelson et al. (1996) tested 28 isolates of *R. solani* AG4 on soybean seedlings and disease severity in two separate experiments varied from 1.77 to 3.37 and 2.09 to

3.46 (1 to 5 scale where 1 = no disease, 5 = 75% leaves wilted or plant dead). Ohkura et al. (2009) also showed variability in disease severity on corn caused by AG2-2 isolates with median scores ranging from 1 to 4 on a scale of 0 to 5 (0 = no disease, 5 = plant dead), although no indication of cultural type was given. From these results it can be concluded that the specific isolate, or isolates, selected for screening can have an effect on the determination of host susceptibility. Clarification of the range in variability on common rotation crops and the association of that variability to phylogenetic group could promote more informed management decisions.

In the current study, we examined the virulence of AG2-2 isolates from sugar beet on common bean (*Phaseolus vulgaris* L.), which is often grown in rotation with sugar beet (Buhre et al., 2009). Symptoms of Rhizoctonia root rot on common bean include reddish brown sunken lesions on the hypocotyl beginning below the soil line and extending downward (Hagedorn & Hanson, 2005). Above ground symptoms resemble drought stress and can be difficult to distinguish from other root diseases and abiotic conditions. Yellowing, wilting, stunting and leaf drop are typical above ground symptoms and are indicative of root tissue damage that has resulted in reduced water and nutrient uptake (Hagedorn & Hanson, 2005). Field level symptoms appear as patchy areas of dead plants or bare soil that tends to spread up the rows.

Objectives

Crop rotation is an integral part of *Rhizoctonia*-induced disease control in sugar beet as well as in other crops. Variability in virulence among isolates can confound studies of host susceptibility and produce inconsistent conclusions (Strausbaugh et al., 2013). The current

study examined the variability in virulence of 36 *R. solani* AG2-2 isolates on common bean. Virulence was assessed at both the seedling stage and 14 days after planting to determine age related effects. The relationship of that virulence to phylogenetic group was also analyzed to determine if subgroup association might give an indication of aggressiveness.

Methods

Thirty-six *Rhizoctonia solani* AG 2-2 isolates from North America (Table 2-1) and eight isolates from Europe and Japan (Table 2-2) were selected from those included in the phylogenetic analysis of Martin et al. (2014) and grown on potato dextrose agar (PDA) (Sigma-Aldrich Corp., St Louis, MO). Inoculum was prepared in 100 x 20 mm plastic petri dishes using the whole grain method (Sneh et al., 1991). Hull-less barley grains (Bob's Red Mill, Milwaukie, OR) were soaked overnight in distilled water and autoclaved for 40 minutes. Sterile barley was inoculated with 6 mm plugs cut with a #2 cork borer from actively growing cultures and incubated at room temperature until all grains were infested. Infested barley was air dried overnight in a biosafety cabinet and ground in a Waring grinder (Conair Corp., Stamford, CT) prior to use. Uninfested sterile barley was ground and used as a mock-inoculated control.

Screening of U.S. isolates was conducted in a greenhouse with ambient temperatures between 21˚ and 25˚C and 14 hour supplemental lighting. The experiment was arranged in an incomplete block design with each isolate replicated four times per block and repeated once for a total of at least eight experimental units per isolate. Eight isolates collected from Europe and Japan were screened in a growth chamber (Conviron PGW36; Controlled Environments Inc., Pembina, ND, USA) set to 21˚C with a 14 hour photoperiod using the same experimental

methodology as above except the experimental units were arranged in a completely randomized design.

Seed from the dry bean variety 'RedHawk' (Kelly et al., 1998) was surface disinfested by soaking in 0.6% sodium hypochlorite plus 0.1% Tween 20 for 15 minutes and then rinsed twice with sterile distilled water. Disinfested seed was treated with a 2% solution of metalaxyl (Allegiance-FL; Bayer Crop Science, Research Triangle Park, NC) prior to planting to protect against Pythium seed rot. Plants were grown in 1.75 liter (15 cm diameter) plastic pots filled with commercial potting mix (SureMix Pearlite; Michigan Grower Products, Inc., Galesburg, MI). Prior to planting, potting mix was drenched with Gnatrol ® WDG (Valent USA Corp., Walnut Creek, CA) according to label instructions for control of fungal gnats. Plants were watered when the surface of the potting mix was dry to the touch.

To test the virulence of the *R. solani* isolates on dry bean when inoculated at planting, pots were filled approximately 3/4 full with potting mix and three seeds placed on the surface. Ground inoculum was combined with additional potting mix at a rate of 1:500 (inoculum: potting mix; v/v) and the mix was used to finish filling pots so that seeds were covered to a depth of 1.5 - 2 cm. Plants were grown as above and experiments were harvested 21 days after planting/inoculation.

Table 2.1 Disease severity of 36 *Rhizoctonia solani* AG2-2 isolates on the dry bean variety RedHawk showing state or providence where originally collected, original collector, interspecific group (ISG) as determined by growth at 35˚C, and phylogenetic group (PG) as determined by Martin et al. (2014). Plants inoculated at planting were rated for root rot 21 days after inoculation. Plants inoculated 14 days after planting were rated for root rot 14 days after inoculation. All plants were rated on a scale of 0 to 6 where 0 = no disease and 6 = plant dead. Control was mock-inoculated with sterile barley. Experiment was conducted in a greenhouse at 20 - 24˚C with 14 h. supplemental lighting.

					Mean Disease Severity	
Isolate	**Origin**	**Collector**	**ISG[4]**	**PG**	**Inoculate at planting[3]**	**Inoculated 14 days after planting[3]**
Rs1146	Minnesota, USA	C. Windels	IIIB	2B[2]	6.00[1] a	3.17 ab
Rs890	Minnesota, USA	C. Windels	IIIB	2A	6.00[1] ab	2.27 c-f
F30	Idaho, USA	C. Strausbaugh	IIIB	1	6.00[1] ab	3.08 ab
Rs393	Minnesota, USA	C. Windels	IV	1	6.00[1] ab	3.45 a
F503	Idaho, USA	C. Strausbaugh	IIIB	1	6.00 ab	3.16 ab
F548	Idaho, USA	C. Strausbaugh	IIIB	1	5.96 ab	3.48 a
R09-23	Michigan, USA	L. Hanson	IV	1	5.92 ab	2.28 c-f
R09-25	Michigan, USA	L. Hanson	Int	2A	5.92 ab	2.23 c-f
Rs1012	Minnesota, USA	C. Windels	IIIB	1	5.91 ab	3.08 ab
F551	Idaho, USA	C. Strausbaugh	IIIB	1	5.88 ab	2.77 a-c
87-36-2	N. Dakota, USA	C. Windels	IIIB	1	5.70 a-c	3.11 ab
F36	Oregon, USA	C. Strausbaugh	IIIB	1	5.68 a-c	2.05 c-g
R09-2	Michigan, USA	L. Hanson	IIIB	1	5.64 a-c	3.19 ab
Rs255	Minnesota, USA	C. Windels	IIIB	1	5.58 a-c	2.77 a-c
F517	Idaho, USA	C. Strausbaugh	IIIB	1	5.47 a-c	3.32 ab
F321	Idaho, USA	C. Strausbaugh	IIIB	1	5.45 a-c	2.69 bc
Rs1090	Minnesota, USA	C. Windels	IV	2B	5.37 a-c	2.27 c-f
Rzc35 (R4)	Texas, USA	C. Rush	IIIB	2A	5.34 a-c	1.86 d-g
39AR	Ontario, CAN	C. Truman	Int	1	5.12 a-c	2.76 a-c
24BR	Ontario, CAN	C. Truman	Int	1	4.93 a-c	2.28 c-f
Rs866	Minnesota, USA	C. Windels	IIIB	2A	4.91 a-d	1.72 e-g
Rs106	Minnesota, USA	C. Windels	IV	2B	4.79 a-e	1.72 e-g
Rzc16 (R9)	Colorado, USA	E. Ruppel	IIIB	2A	4.65 b-e	2.02 c-g
F508	Idaho, USA	C. Strausbaugh	IIIB	2A	4.55 b-e	2.24 c-f
R09-28	Michigan, USA	L. Hanson	IV	2A	4.38 b-e	2.70 a-c
R1	Colorado, USA	E. Ruppel	IIIB	2A	4.18 c-e	1.64 fg
2C13	Montana, USA	B. Bugbee	IV	2B	4.14 c-f	1.94 c-g
F521	Idaho, USA	C. Strausbaugh	IIIB	2A	3.50 d-g	1.88 c-g
Rs200	Minnesota, USA	C. Windels	IV	2B	3.29 e-h	2.23 c-f
Rs496	Minnesota, USA	C. Windels	IV	2B	2.75 f-i	2.52 b-d
Rs599	Minnesota, USA	C. Windels	IV	2B	2.54 f-i	2.34 c-e
Rzc21 (W-22)	Colorado, USA	R.T. Sherwood	IIIB	2A	2.29 g-i	1.57 fg
Rs481	Minnesota, USA	C. Windels	IV	2B	1.79 h-j	2.33 c-e
Rs296	Minnesota, USA	C. Windels	IV	2B	1.65 h-k	1.88 c-g
Rs542	Minnesota, USA	C. Windels	IV	2B	1.38 i-k	1.35 g
Rs588	Minnesota, USA	C. Windels	IV	2B	0.81 jk	1.67 e-g
Control	-	-	-	-	0.22 k	0.55 h

Table 2.1 Disease severity of 36 *Rhizoctonia solani* AG2-2 isolates on the dry bean variety RedHawk (cont'd)
Footnotes: (1) Values shown were adjusted to reflect the maximum value of the 0 - 6 rating scale used. Actual least square estimate values were as follows: Rs1146 = 6.17; Rs890 = 6.15; F30 = 6.04; and Rs393 = 6.04. (2) Martin et al. (2014) identified isolate Rs1146 as being in group 2B. Evidence from the current study and from microsatellite markers (this thesis, chapter 4) indicate that Rs1146 is probably in group 1 and will need to be reexamined. (3) Means within the same inoculation timing with the same letter are not significantly different from one another (Tukey, α =.05). (4) Isolates labeled as 'Int' had growth intermediate between type IIIB and type IV.

Table 2.2 Disease severity of 8 international *Rhizoctonia solani* AG2-2 isolates on the dry bean variety RedHawk showing region where originally collected, original collector, interspecific group (ISG) as determined by growth at 35˚C, and phylogenetic group (PG) as determined by Martin et al. (2014). Plants inoculated at planting were rated for root rot 21 days after inoculation. Plants inoculated 14 days after planting were rated for root rot 14 days after inoculation. All plants were rated on a scale of 0 to 6 where 0 = no disease and 6 = plant dead. Control was mock-inoculated with sterile barley. Isolate 'R1' was used as a positive control. Experiment was conducted in a growth chamber at 23˚C with 14 h. diurnal lighting.

					Mean Disease Severity	
Isolate	**Origin**	**Collector**	**ISG**	**PG**	**Inoculated at planting[(2)]**	**Inoculated 14 days after planting[(2)]**
Rickard	Europe	B. Holtschulte	IIIB[(1)]	1	5.42 a	2.40 b
Italian	Europe	B. Holtschulte	IIIB[(1)]	2A	4.67 ab	3.33 a
Cavallie	Europe	B. Holtschulte	IIIB[(1)]	1	4.58 ab	3.17 ab
Slovakia	Europe	B. Holtschulte	IIIB[(1)]	1	4.08 ab	3.36 a
R1	Colorado, USA	E. Ruppel	IIIB	2A	3.50 b	2.50 b
Rzc89 (RH193)	Japan	H. Uchino	IV	2B	1.75 c	3.30 a
Rzc91 (RH65)	Japan	H. Uchino	IV	2B	1.50 cd	2.64 ab
Rzc6 (R-164s))	Japan	A. Ogoshi	IV	2B	1.08 cd	2.45 b
Rzc94 (RH188)	Japan	H. Uchino	IV	2B	0.92 cd	2.45 b
Control	-	-	-	-	0.08 d	0.08 c

(1) The Europeans claim to have only AG2-2IIIB but these isolates were not specifically tested for growth at 35˚C (2) Means within the same inoculation timing with the same letter are not significantly different from one another (Tukey, α =.05).

The virulence of AG2-2 isolates on dry bean when inoculated 14 days after planting was tested by sowing three seeds to a depth of 1.5 - 2 cm in pots filled with potting mix. Fourteen days after planting, plants were inoculated by pulling the potting mix back from the stem and adding 0.62 cc of ground inoculum using a 1/8 tsp. measuring spoon. After adding inoculum, potting mix was filled in around the stem. Plants were grown as above and experiments were harvested 14 days after inoculation.

Upon harvesting, roots were washed with tap water and rated for root rot on a scale of 0 to 6 where 0 = no disease; 1 = small lesions covering < 20% root tissue area; 2 = larger lesions covering 20-50% of the root tissue area; 3 = 50-90 % root tissue area affected; 4 = more than 90% root tissue area affected but pith still solid; 5 = more than 90% root tissue area affected and pith rotted; and 6 = plant dead. Disease severity (DS) score was calculated as the average of all disease severity ratings for a given isolate.

Data was analyzed using the Proc GLIMMIX procedure in SAS 9.4 (SAS Institute, Inc., Cary, NC.) with replication and block considered random effects and the model set for unequal variance using the Kenward-Roger degrees of freedom approximation. Pairwise comparisons of isolates were made using Tukey's HSD as were pairwise comparisons for the average disease severity of the phylogenetic groups. Linear regression analysis and box plot graphics were generated using R Version 3.4.0 (R Core Team, 2017).

Results

A total of 36 *R. solani* AG2-2 isolates, including 15 isolates from group 1, 10 from group 2A and 11 from group 2B, were inoculated at planting on dry beans and screened in a greenhouse. Disease severity scores ranged from 0.81 to 6.00 with a mean score of 4.61 and a median score of 5.23 (Table 2.1). When inoculated at planting, isolates in phylogenetic group 1 (mean DS = 5.64) were, on average, more aggressive (Tukey, $p < 0.001$) than isolates in group 2A (mean DS = 4.36) and isolates in group 2A were more aggressive (Tukey, $p < 0.001$) than isolates in group 2B (mean DS = 3.01) (Fig. 2.1). Disease severity scores for isolates in group 1 ranged from 4.93 to 6.17 (Fig. 2.2). Disease severity scores for group 2A isolates ranged from 2.29 to 6.15 and group 2B isolates ranged from 0.81 to 5.37 (Fig. 2.2). Group 2B isolates had the most variable disease severity scores with a range of 4.56 while group 1 isolates had the least variable disease severity scores with a range of 1.24. Fourteen of the 15 isolates (93%) in group 1 had disease severity scores greater than 5.00 whereas group 2A and 2B had only 3 isolates out of 10 (30%) and 2 isolates out of 11 (18%), respectively, with disease scores over 5.00 (Fig. 2.2). Side-by-side boxplot comparison (Fig. 2.3) shows the three phylogenetic groups clearly differentiated from one another with group 1 having the highest median disease severity score and the smallest interquartile range. Isolate Rzc21 (W-22) was identified as an outlier in group 2A with a DS value of 2.29.

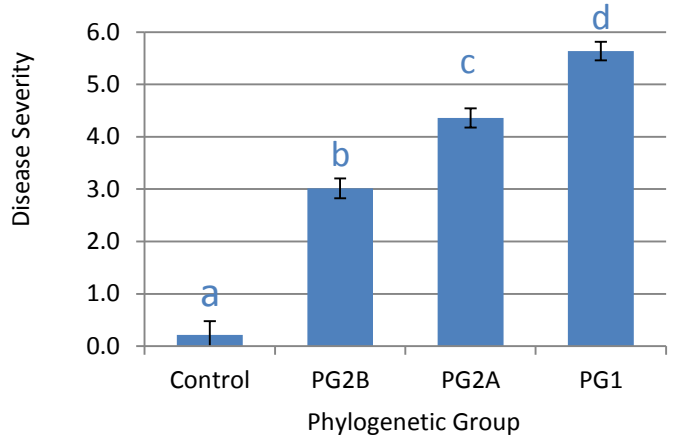

Figure 2.1 Comparison of phylogenetic groups when inoculated at planting. Average disease severity by phylogenetic group (PG) of 36 R*hizoctonia solani* AG2-2 isolates inoculated at planting on the dry bean variety RedHawk. Disease severity scores range from 0 to 6, where 0 = no disease and 6 = plant dead. Means with the same letter are not significantly different (Tukey, α = 0.05). Control was mock-inoculated with sterile barley. Phylogenetic group was determined according to Martin et al. (2014). Error bar indicate standard error.

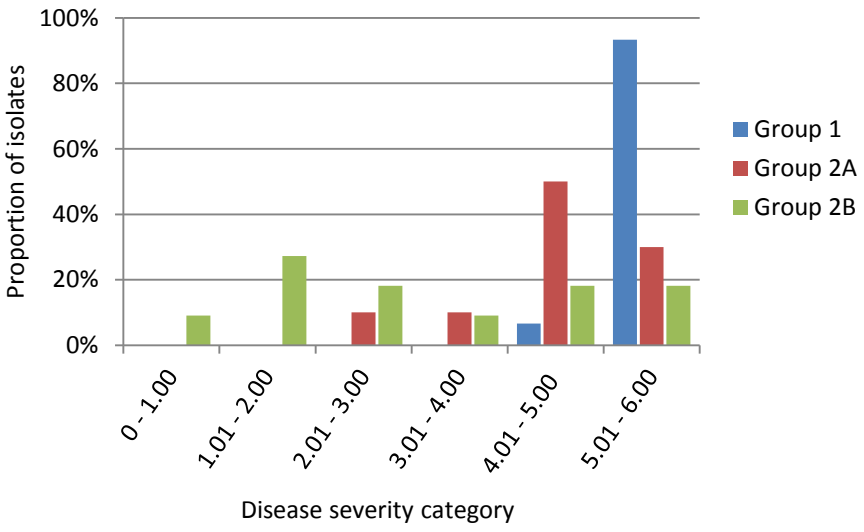

Figure 2.2 Distribution of disease severity scores when inoculated at planting. Thirty-six isolates of R*hizoctonia solani* AG2-2 were inoculated at planting on the dry bean variety RedHawk. Disease severity scale ranged from 0 to 6 where 0 = no disease and 6 = plant dead. Disease severity category designation was arbitrary and intended only to permit visualization of the distribution patterns. Group designation is the phylogenetic group according to Martin et al. (2014).

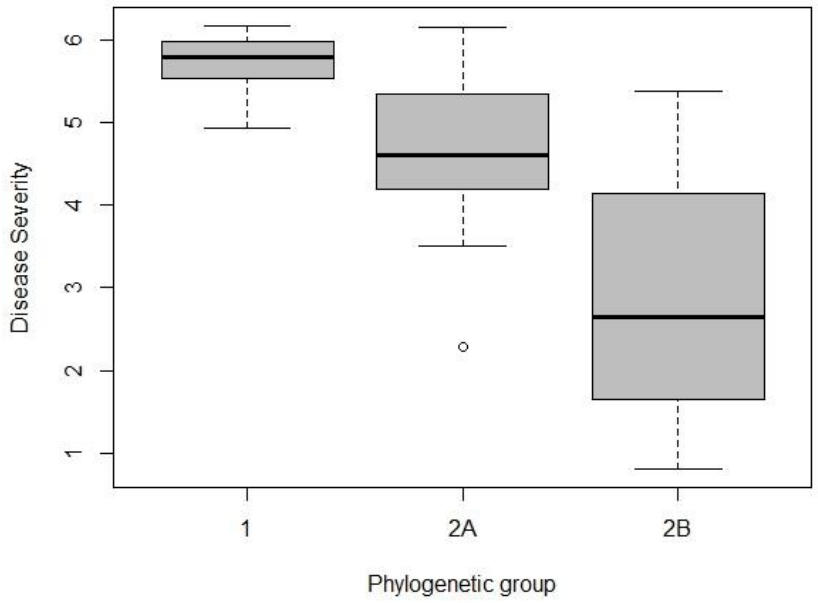

Figure 2.3 Side-by-side boxplot comparison of phylogenetic groups when inoculated at planting. Thirty-six isolates of *Rhizoctonia solani* AG2-2 were inoculated on the dry bean variety RedHawk with group 1: n = 15; group 2A: n = 10; group 2B: n = 11. Disease severity scale ranged from 0 to 6 where 0 = no disease and 6 = plant dead. Phylogenetic group is according to Martin et al. (2014).

The same 36 isolates as above were inoculated on dry beans 14 days after planting and screened in the same greenhouse. Disease severity (DS) scores ranged from 1.35 to 3.48 with a mean score of 2.42 and median score of 2.28 (Table 2.1). Isolates in phylogenetic group 1 (mean DS = 2.83) were, on average, more aggressive (Tukey, $p < 0.001$) than isolates in groups 2A (mean DS = 1.97) or 2B (mean DS = 2.02) (Fig. 2.4). Disease severity scores for isolates in group 1 inoculated 14 days after planting ranged from 2.05 to 3.48 (Fig. 2.5). Disease severity scores for isolates in group 2A inoculated 14 days after planting ranged from 1.57 to 2.70 and group 2B isolates ranged from 1.35 to 2.52 (Fig. 2.5). Phylogenetic group 2A and group 2B had

similar median disease severity scores and range. Side-by-side boxplot comparison (Fig. 2.6) indicated that group 1 was clearly differentiated from groups 2A and 2B, but had a similar range of disease severity values. There were no outliers identified in the isolates inoculated 14 days after planting.

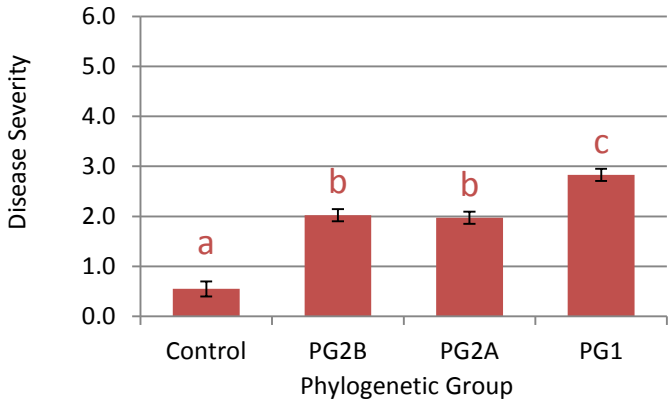

Figure 2.4 Comparison of phylogenetic groups when inoculated 14 days after planting. Average disease severity by phylogenetic group (PG) of 36 R*hizoctonia solani* AG2-2 isolates inoculated 14 days after planting on the dry bean variety RedHawk. Disease severity scale ranged from 0 to 6, where 0 = no disease and 6 = plant dead. Means with the same letter are not significantly different (Tukey, α = 0.05). Control was mock-inoculated with sterile barley. Phylogenetic group is according to Martin et al. (2014). Error bars indicate standard error.

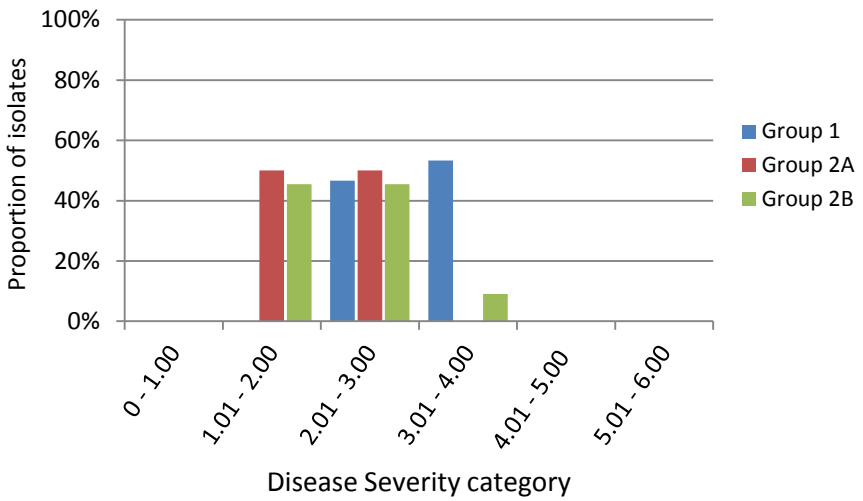

Figure 2.5 Distribution of disease severity scores when inoculated 14 days after planting. Thirty-six isolates of R*hizoctonia solani* AG2-2 isolates were inoculated 14 days after planting on the dry bean variety RedHawk. Disease severity scale ranged from 0 to 6 where 0 = no disease and 6 = plant dead. Disease severity category designation was arbitrary and intended only to permit visualization of the distribution patterns. Group designation is the phylogenetic group according to Martin et al. (2014).

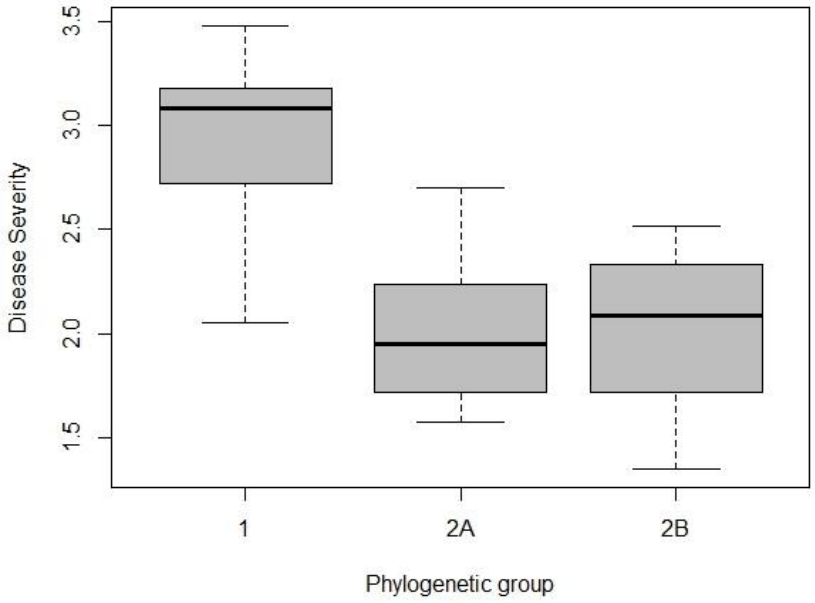

Figure 2.6 Side-by-side boxplot comparison of phylogenetic groups when inoculated 14 days after planting. Thirty-six isolates of *Rhizoctonia solani* AG2-2 were inoculated on the dry bean variety RedHawk with group 1: n = 15; group 2A: n = 10; group 2B: n = 11. Disease severity scale ranged from 0 to 6 where 0 = no disease and 6 = plant dead. Phylogenetic group is according to Martin et al. (2014).

Four isolates each from Europe and Japan had similar results with disease severity scores ranging from 0.92 to 5.42 for isolates inoculated at planting and from 2.40 to 3.36 for isolates inoculated 14 days after planting (Table 2.2). Isolates in group 1 were, on average, more aggressive (Tukey, $p < 0.001$) than isolates in group 2B when inoculated at planting (Fig. 2.7).

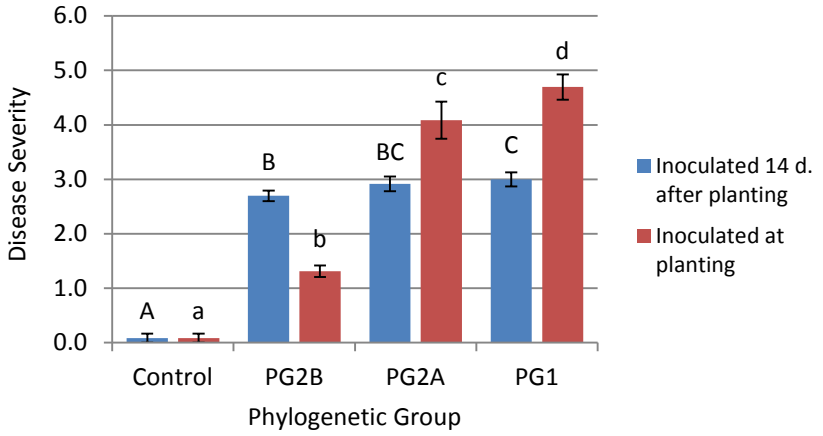

Figure 2.7 Comparison of phylogenetic groups for international isolates. Eight international isolates of *Rhizoctonia solani* AG2-2 from Europe and Japan were inoculated on the dry bean variety RedHawk at planting or 14 days after planting. Disease severity scale ranged from 0 to 6 where 0 = no disease and 6 = plant dead. Means with the same letter were not significantly different (Tukey, $\alpha = 0.05$); uppercase letters are for inoculations 14 days after planting and lowercase letters are for inoculations at planting. Control was mock-inoculated with sterile barley. Isolate R1 was used as a positive control. Phylogenetic group is according to Martin et al. (2014). Error bars indicate standard error.

The correlation between disease severity on plants inoculated at planting and disease severity on plants inoculated 14 days after planting had a significant linear component with a correlation coefficient of 0.618 ($F = 20.97$, $p < 0.001$) and an R^2 value of 0.382 (Fig. 2.8). The coefficient for disease severity on plants inoculated at planting was 1.63 which indicates that disease severity on plants inoculated at planting increases 1.63 points for every 1.0 point increase in disease severity on plants inoculated 14 days after planting.

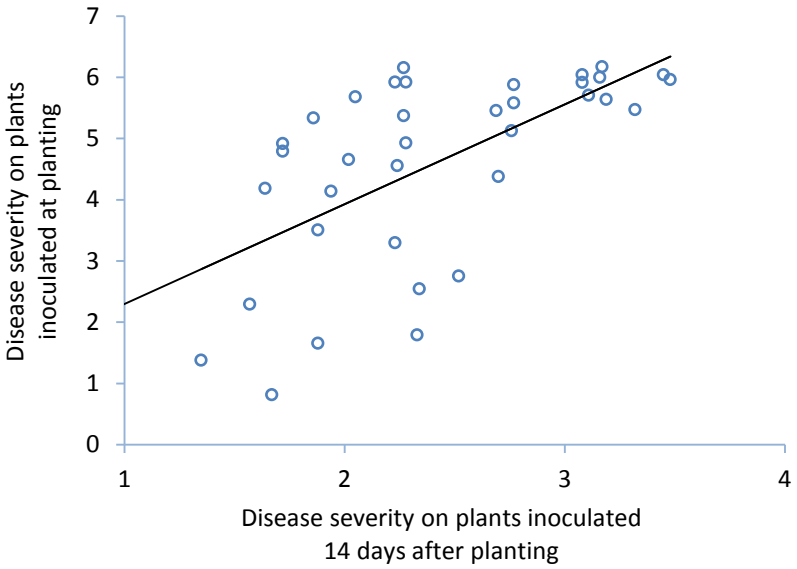

Figure 2.8 Scatterplot comparing disease severity on dry bean when inoculated at planting and at 14 days after planting. Thirty-six isolates of *Rhizoctonia solani* AG2-2 were inoculated on the dry bean variety RedHawk. Disease severity scale ranged from 0 to 6 where 0 = no disease and 6 = plant dead. Trendline is a linear regression ($p < 0.001$) with a slope of 1.63 and an R^2 of 0.38.

Comparison of the traditional subgroups AG2-2IIIB and AG2-2IV produced different results for plants inoculated at planting and for those inoculated 14 days after planting (Fig. 2.9). Plants inoculated at planting had significant differences between the groups ($p = 0.004$) with average disease severity scores of 5.23 for AG2-2IIIB isolates and 3.44 for AG2-2IV isolates. Plants inoculated 14 days after planting showed no significant differences between the groups ($p = 0.100$) with average disease severity scores of 2.55 for AG2-2IIIB isolates and 2.21 for AG2-2IV isolates.

Discussion

There was considerable variation in virulence among the *R. solani* AG2-2 isolates tested, which was consistent with what other researchers have found (Carling et al., 2002b; Kuninaga et al., 1997; Strausbaugh et al., 2011). Disease severity scores for plants inoculated at planting ranged from 0.81 to 6.00 with three of thirty-six (8%) isolates not significantly different than the mock-inoculated control and five (14%) isolates that killed all plants they were tested on. Although disease severity scores were lower for plants inoculated 14 days after planting there was less variability. Disease severity scores ranged from 1.35 to 3.48 with all isolates significantly different than the mock-inoculated control. These results are consistent with other studies of variability in virulence such as Strausbaugh et al. (2011a) that reported disease severity from 19% to 100% on sugar beet roots.

Isolates in phylogenetic group 1 were, on average, significantly more aggressive than group 2A or group 2B isolates on both beans inoculated at planting and those inoculated 14 days after planting (Fig. 2.1 & Fig. 2.4). Group 2A isolates were, on average, more aggressive than isolates in group 2B but only on plants inoculated at planting. In general, isolates in group 2B were less aggressive than isolates in the other two groups, although they were statistically similar to isolates in group 2A when inoculated 14 days after planting. Since group 2B isolates are primarily type IV and group 1 isolates are primarily type IIIB, these results are consistent with previous reports that identified AG2-2IIIB isolates as being more aggressive than AG2-2IV isolates (Engelkes & Windels, 1996; Strausbaugh et al., 2011a; Windels & Brantner, 2006).

In the current study, there were significant differences in virulence between isolates identified as AG2-2IIIB and those identified as AG2-2IV by temperature response, but only when

inoculated at planting (Fig. 2.9). On seedlings, disease severity scores of isolates identified as AG2-2IIIB were clustered towards the high end of the scale with 70% having disease severity scores greater than 5.00 (Table 2.1). Disease severity scores of isolates identified as AG2-2IV were much more evenly distributed among the severity classes with some isolates being very weak and some very aggressive (Table 2.1). For example, isolates Rs393, Rs1090 and R09-23 were identified as type IV, but had disease severity scores that were not significantly different than the type IIIB isolate with the highest disease severity score on plants that were inoculated at planting. Conversely, the only isolates that did not cause significantly more disease symptoms than the mock-inoculated controls were type AG2-2IV. Phylogenetic group appears to provide a potential prediction of virulence with group 1 being consistently more virulent than groups 2A and 2B when inoculated both at planting and at 14 days after planting. However, in this study, average virulence of groups 2A and 2B was only significantly different when inoculated at planting. Identification of the phylogenetic group for isolates present in a specific field could aid in management decisions by predicting risk associated with planting a particular crop. For example, the presence of group 1 isolates in a field could indicate that planting dry beans would be risky and high levels of disease are likely to develop. Unfortunately, since several isolates in groups 2A and 2B had disease severity scores greater than 5.00 when inoculated at planting, the presence of particular isolates in group 2A or 2B would still pose an elevated risk. Therefore, phylogenetic group is only marginally better at predicting virulence than the original designations of AG2-2IIIB and AG2-2IV. Ultimately, risk depends on the individual isolates present and the identification of specific virulence factors would be recommended in order to accurately predict disease risk.

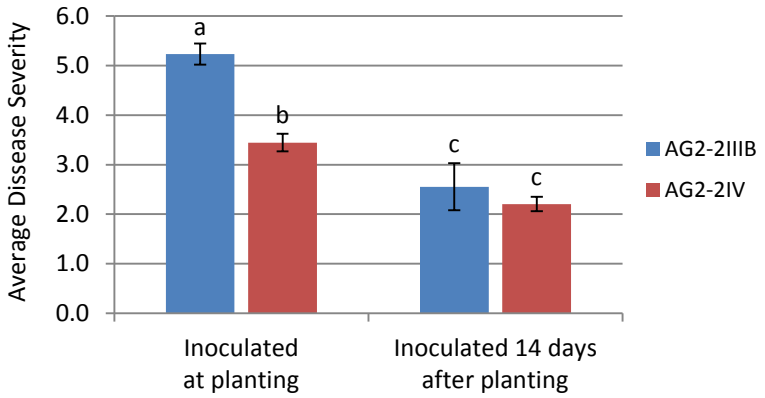

Figure 2.9 Comparison of disease severity by traditional subgroups AG2-2IIIB and AG2-2IV. Thirty-six isolates of *Rhizoctonia solani* were inoculated on the dry bean variety RedHawk. Disease severity scale ranged from 0 to 6, where 0 = no disease and 6 = plant dead. Traditional subgroups, also referred to as intraspecific groups (ISG), were determined by growth at 35˚C. Means with the same letter are not significantly different. Error bars indicate standard error.

Regardless of the risk associated with phylogenetic group, most of the isolates tested in the current study caused considerable disease symptoms on the dry bean variety RedHawk. The current study is certainly not the first to report the susceptibility of dry beans to *Rhizoctonia solani* AG2-2 (Engelkes & Windels, 1996; Galindo et al., 1982; Muyolo et al., 1993). However, most studies only examined a limited number of isolates and so did not represent the full range of variability present in *R. solani* AG2-2. Our data includes representatives of different genetic groups and provides a more comprehensive assessment of the variability within the AG2-2 group.

Other researchers have concluded that beans and sugar beet should not be grown in close rotation (Engelkes & Windels, 1996; Ruppel, 1985; Windels & Brantner, 2004) because both crops were susceptible to AG2-2IIIB and AG2-2IV isolates. The results of the current study generally agree with that conclusion since many of the isolates recovered from sugar beet were also aggressive on dry beans. Some isolates were non-virulent or caused low-levels of disease

but unfortunately, these weak strains do not correlate well enough with a particular group to allow the designation of a low risk group. Overall, groups 2A and 2B may not be as aggressive as group 1 on dry beans, but, individual isolates can still pose a substantial risk to dry beans.

In addition, more testing is needed on additional rotational crops. The intent would be to identify rotational crops that increase the prevalence of highly aggressive strains of *R. solani* AG2-2 and to avoid using those crops in rotation. For example, including corn in the rotation has been shown to increase the prevalence of AG2-2IIIB strains, which have been considered more aggressive on sugar beet (Windels & Brantner, 2004; Windels & Brantner, 2006). However, these conclusions need to be reassessed in order to accommodate the revised genetic groups of Martin et al. (2014). Moreover, the role that rotational crops play in influencing inoculum levels and strain prevalence is complex. Host susceptibility alone is an incomplete indicator of how crop rotation will affect populations of *R. solani* (Ruppel, 1985). Instead, there are several factors that need to be considered when determining suitable crops for rotation including: how well residues are colonized; soil conditions, such as nitrogen levels; and regional differences in climate, soil type, and variety choices (Ruppel, 1985; Rush & Winter, 1990). For these reasons, it is not prudent to make management decisions based on susceptibility data alone. Field experiments that assess the effects of rotation crops on subsequent crops need to be conducted in each growing region. To my knowledge, comprehensive testing of crop rotations in relation to RRCR of sugar beet has not been conducted in Michigan.

The virulence of isolates when inoculated at planting was significantly correlated to virulence when inoculated 14 days after planting indicating that those isolates that were

aggressive on young plants were also aggressive on older plants. However, the R^2 value of this relationship was only 0.382 indicating that only 38% of the variance is due to general aggressiveness of the isolate under these conditions. The majority of the variation in virulence may be explainable as a differential response to host age. Age-related resistance has been described for several pathosystems (Develey-Rivière & Galiana, 2007; Panter & Jones, 2002; Kus et al., 2002) including common beans (Bateman & Lumsden, 1964). The cuticle appears to play an important role in age-related resistance of common bean, becoming more resistant to invasion as the plant ages (Stockwell & Hanchey, 1984). However, since seedling bean plants are especially susceptible to *R. solani* AG2-2, management practices that delay or slow disease progress, such as fungicide treatments, may be needed to allow the plants to develop to the stage where they have a greater resistance to infection.

Another potential explanation for the differential response between inoculation at planting and inoculation 14 days after planting may be that the specific combination of cell wall degrading enzymes (CWDE) produced by a particular isolate could affect the ability of the isolate to penetrate the cell wall at different growth stages (Bellincampi et al., 2014; Scala et al., 1980). The *R. solani* AG2-2IIIB genome has been predicted to have over 1,000 putative CWDE (Wibberg et al., 2016) providing substantial enzymatic variations with which to overcome physical barriers. Variation in the structure or regulation of these enzymes could affect the ability of the pathogen to overcome variations in plant defensive strategies such as polygalacturonase-inhibitor proteins (Bergmann et al., 1994; Matteo et al., 2006). Thus, identification and characterization of the interactions between pathogen cell wall degrading enzymes and host defenses could provide insight into host resistance.

Conclusions

Rhizoctonia solani AG2-2 is highly variable in its virulence on dry beans (*Phaseolus vulgaris*), with strains ranging from very aggressive to non-pathogenic. Although most strains cause substantial disease symptoms on the dry bean variety RedHawk, phylogenetic group 1 was significantly more aggressive on plants inoculated both at planting and 14 days after planting than were groups 2A and 2B. There was little difference in virulence between groups 2A and 2B in regard to virulence on dry beans. Because both beans and sugar beet are susceptible to *R. solani* AG2-2, care should be taken when growing them in rotation in fields with a history of *Rhizoctonia* disease.

REFERENCES

REFERENCES

Anderson, N.A. (1982). The genetics and pathology of *Rhizoctonia solani*. Annual Review of Phytopathology, 20: 329-347.

Baker, K.F. (1970). Types of *Rhizoctonia* diseases and their occurrence. Pg. 125-148 in *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr., University of California Press, Berkeley, CA.

Bateman, D.F. and Lumsden, R.D. (1964). Relation of calcium content and nature of the pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of *Rhizoctonia solani*. Phytopathology, 55: 734-738.

Bellincampi, D., Cervone, F. and Lionetti, V. (2014). Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. Frontiers in Plant Science, 5: 228.

Bergmann, C.W., Ito, Y., Singer, D., Albersheim, P. and Darvill, A.G. (1994). Polygalacturonase-inibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. The Plant Journal, 5: 625-634.

Boine, B., Renner, A., Zellner, M. and Necheatal, J. (2014). Quantitative methods for assessment of the impact of different crops on the inoculum density of *Rhizoctonia solani* AG2-2IIIB in soil. European Journal of Plant Pathology, 140: 745-756.

Buhre, C.K., Kluth, C., Bürchy, K., Märländer, B., and Varrelmann, M. (2009). Integrated control of root and crown rot in sugar beet: Combined effects of cultivar, crop rotation, and soil tillage. Plant Disease, 93: 155-161.

Carling, D.E., Baird, R.E., Gitaitis, R.D., Brainard, K.A., and Kuninaga, S. (2002a). Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. Phytopathology, 92: 893-899.

Carling, D.E., Kuninaga, S. and Brainard, K.A. (2002b). Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. Phytopathology, 92: 43-50.

Coons, G.H. and Kotila, J.E. (1935). Influence of preceding crops on damping off of sugar beets. Phytopathology, 25:13. (Abstr.)

Cubeta, M.A. and Vilgalys, R. (1997). Population biology of the *Rhizoctonia solani* complex. Phytopathology, 87: 480-484.

Develey-Rivière, M. and Galiana, E. (2007). Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom. New Phytologist, 175: 405-416.

Emmond, G.S. and Ledingham, R.J. (1972). Effects of crop rotation on some soil-borne pathogens of potato. Canadian Journal of Plant Science, 52: 605-611.

Engelkes, C.A. and Windels, C.E. (1996). Susceptibility of sugar beet and beans to *Rhizoctonia solani* AG-2-2 IIIB and AG-2-2 IV. Plant Disease, 80: 1413-1417.

Fenille, R.C., de Souza, N.L. and Kuramae, E.E. (2002). Characterization of *Rhizoctonia solani* associated with soybean in Brazil. European Journal of Plant Pathology, 108: 783-792.

Frank, J.A. and Murphy, H.J. (1977). The effect of crop rotations on Rhizoctonia disease of potato. American Potato Journal, 54: 315-322.

Galindo, J.J., Abawi, G.S. and Thurston, H.D. (1982). Variability among isolates of *Rhizoctonia solani* associated with snap bean hypocotyls and soils in New York. Plant Disease, 66: 390-394.

Gilligan, C.A., Simons, S.A. and Hide, G.A. (1996). Inoculum density and spatial pattern of *Rhizoctonia solani* in field plots of *Solanum tuberosum*: effects of cropping frequency. Plant Pathology, 45: 232-244.

Hagedorn, D.J. and Hanson, L.E. (2005). Rhizoctonia root rot. Pages 19-20 in Compendium of Bean Diseases, 2nd ed., Edited by Schwartz, H.F., Steadman, J.R., Hall, R. and Forster, R L. APS Press, St. Paul, MN.

Hykumachi, M., Mushika, T. Ogiso, Y, Tdoa, T. Kageyama, K. and Tsuge, T. (1998). Characterization of a new cultural type (LP) of *Rhizoctonia solani* AG2-2 isolated from warm-season turfgrasses, and its genetic differentiation from other cultural types. Plant Pathology, 47: 1-9.

Kelly, J.D., Hosfield, G.L., Varner, G.V., Uebersax, M.A., Long, R.A. and Taylor, J. (1998). Registration of 'Red Hawk' dark red kidney bean. Crop Sciences 38: 280-281.

Larkin, R.P. and Honeycut, C.W. (2006). Effects of different 3-year cropping systems on soil microbial communities and Rhizoctonia diseases of potato. Phytopathology, 96: 68-79.

Kuninaga, S., Natsuaki, T., Takeuchi, T. and Yokosawa, R. (1997). Sequence variation of the rDNA ITS regions within and between anastomosis groups in *Rhizoctonia solani*. Current Genetics, 32: 237-243.

Kus, J.V., Zaton, K., Sarkar, R. and Cameron, R.K. (2002). Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. The Plant Cell, 14: 479-490.

Martin, F., Windels, C., Hanson, L. and Brantner, J. (2014). Analysis of population structure and pathogenicity of *Rhizoctonia solani* AG2-2 (ISG IIIB and IV) isolates from Michigan, Minnesota and North Dakota. Sugarbeet Research Reports. Beet Sugar Development Foundation, Denver, CO.

Matteo, A.D., Bonivento, D., Tsernoglou, D., Federici, L. and Cervone, F. (2006). Polygalacturonase-inhibiting protein (PGIP) in plant defense: a structural view. Phytochemistry, 67: 528-533.

Maxson, A.C. (1938). Root-rots of the sugar beet. Proceedings of the American Society of Sugar Beet Technologists, 1938: 60-64.

Muyolo, N.G., Lipps, P.E. and Schmitthenner, A.F. (1993). Anastomosis grouping and variation in virulence among isolates of *Rhizoctonia solani* associated with dry bean and soybean in Ohio and Zaire. Phytopathology, 83: 438-444.

Nelson, B., Helms, T., Christianson, T. and Kural, I. (1996). Characterization and pathogenicity of *Rhizoctonia* from soybean. Plant Disease, 80: 74-80.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annual Review of Phytopathology, 25: 124-143.

Okura, M., Abawi, G.S., Smart, C.D. and Hodge, K.T. (2009). Diversity and aggressiveness of *Rhizoctonia solani* and *Rhizoctonia*-like fungi on vegetables in New York. Plant Disease, 93: 615-624.

Panter, S.N. and Jones, D.A. (2002). Age-related resistance to plant pathogens. Pages 251-280 in Advances in Botanical Research, 38. Edited by Callow, J. A. Academic Press, San Diego, CA.

Papavizas, G.C. (1970). Colonization and growth of *Rhizoctonia solani* in soil. Pg. 108-122 in: *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr., University of California Press, Berkeley, CA.

Parmeter, J.R. Jr., Sherwood, R.T. and Platt, W.D. (1969). Anastomosis grouping among isolates of *Thanatephorus cucumeris*. Phytopathology, 59: 1270-1278.

Peña, P.A., Steadman, J.R., Eskridge, K.M. and Urrea, C.A. (2013). Identification of sources of resistance to damping-off and early root/hypocotyl damage from *Rhizoctonia solani* in common bean (*Phaseolus vulgaris* L.). Crop Protection, 54: 92-99.

Priyatmojo, A., Escopalao, V.E., Tangonan, N.G., Pascual, C.B., Suga, H., Kageyama, K. and Hyakumachi, M. (2001). Characterization of a new subgroup of *Rhizoctonia solani* anastomosis group 1 (AG-1-ID), causal agent of a necrotic leaf spot on coffee. Phytopathology, 91: 1054-1061.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Poindexter, S. (2014). Evaluate the effectiveness of Rhizoctonia control in sugar beets. The Sugar Beet Grower. July/August: 16-17.

Rovira, A.D. (1986). Influence of crop rotation and tillage on Rhizoctonia bare patch of wheat. Phytopathology, 76: 669-673.

Rupple, E.G. (1985). Susceptibility of rotation crops to a root rot isolate of *Rhizoctonia solani* from sugar beet and survival of the pathogen in crop residues. Plant Disease, 69: 871-873.

Rush, C.M. and Winter, S.R. (1990). Influence of previous crops on Rhizoctonia root and crown rot of sugar beet. Plant Disease, 74: 421-425.

Salazar, O., Julián, M.C., Hyakumachi, M. and Rubio, V. (2000). Phylogenetic grouping of cultural types of *Rhizoctonia solani* AG 2-2 based on ribosomal ITS sequences. Mycologia, 92: 505-509.

Scala, A. ,Camardella, L., Scala, F. and Cervone, F. (1980). Multiple forms of polygalacturonase in two strains of *Rhizoctonia solani*. Journal of General Microbiology, 116: 207-211.

Schillinger, W.F. and Paulitz, T.C. (2006). Reduction of Rhizoctonia bare patch in wheat with barley rotations. Plant Disease, 90: 302-306.

Schuster, M.L. and Harris, L. (1960). Incidence of Rhizoctonia crown rot on sugar beet in irrigated crop rotation. Journal of the American Society of Sugar Beet Technologists, 11: 128-136.

Sneh, B., Burpee, L. and Ogoshi, A. (1991). Identification of *Rhizoctonia* species. APS Press, St. Paul, MN, USA.

Strausbaugh, C.A., Eujayl, I.A. and Panella, L.W. (2013). Interaction of sugar beet host resistance and *Rhizoctonia solani* AG2-2IIIB strains. Plant Disease, 97: 1175-1180.

Strausbaugh, C.A., Eujayl, I.A., Panella, L.W. and Hanson, L.E. (2011a). Virulence, distribution and diversity of *Rhizoctonia solani* from sugar beet in Idaho and Oregon. Canadian Journal of Plant Pathology, 33: 210-226.

Strausbaugh, C.A., Rearick, E., Eujayl, I. and Foote, P. (2011b). Influence of Rhizoctonia-bacterial root rot complex on storability of sugar beet. Journal of Sugar Beet Research, 48: 155-180.

Stockwell, V. and Hanchey, P. (1984). The role of the cuticle in resistance of beans to *Rhizoctonia solani*. Phytopathology, 74:1640-1642.

Sumner, D.R. and Bell D.K. (1986). Influence of crop rotation on severity of crown and brace root rot caused in corn by *Rhizoctonia solani*. Phytopathology 76: 248-252.

Sumner, D.R. and Minton, N.A. (1989). Crop losses in corn induced by *Rhizoctonia solani* AG-2-2 and nematodes. Phytopathology, 79: 934-941.

Sumner, D.R., Doupnik, B. Jr., and Boosalis, M.G. (1981). Effects of reduced tillage and multiple cropping systems on plant diseases. Annual Review of Phytopathology, 19: 167-187.

Taheri, P. and Tarighi, S. (2012). Genetic and virulence analysis of *Rhizoctonia* spp. associated with sugar beet root and crown rot in the northeast region of Iran. Plant Disease, 96:398-408.

Watanabe, B. and Matsuda, A. (1966). Studies on the grouping of *Rhizoctonia solani* Kühn pathogenic to uplandcrops. Bulletin for Appointment Experiments (Plant Disease and Insect : Pests), 7: 1-131.

Wibberg, D., Andersson, L., Tzelepis, G., Rupp, O., Blom, J., Jelonek, L., ... Dixelius, C. (2016). Genome analysis of the sugar beet pathogen *Rhizoctonia solani* AG2-2IIIB revealed high numbers in secreted proteins and cell wall degrading enzymes. BMC Genomics, 17: 245.

Windels, C. and Brantner, J. (2004). Previous crop influences *Rhizoctonia* on sugarbeet. Sugarbeet Research and Extension Reports, 35: 227-231.

Windels, C. and Brantner, J. (2006). *Rhizoctonia* inoculum and rotation crop effects on a following sugarbeet crop. Sugarbeet Research and Education Board of Minnesota and North Dakota. 37: 182

Windels, C.E., Jacobsen, B.J. and Harveson, R.M. (2009). Rhizoctonia root and crown rot. Pages 33-36 in Compendium of Beet Diseases and Pests. Edited by Harveson, R.M., Hanson, L.E. and Hein, G.L., APS Press, St. Paul, MN.

Windels, C. and Nabben, D. (1989). Characterization and pathogenicity of anastomosis group or *Rhizoctonia solani* isolated from *Beta vulgaris*. Phytopathology, 79: 83-88.

CHAPTER 3:

VARIABILITY IN THE VIRULENCE OF *RHIZOCTONIA SOLANI* AG2-2 ISOLATES ON SUGAR BEET SEEDLINGS IN RESPONSE TO LOW TEMPERATURE

Introduction

Rhizoctonia solani Kühn is a ubiquitous, soilborne fungus that can cause disease on several economically important crops (Anderson, 1982; Ogoshi, 1987; Sneh, 1991). Traditionally, strains of *R. solani* have been categorized into anastomosis groups (AG) based on the ability of the hyphae to fuse (Ogoshi, 1987; Sneh, 1991) with at least 13 AG presently recognized (Carling et al., 2002b; Cubeta & Vigalys, 1997). *R. solani* AG2-2 is the primary causal agent of Rhizoctonia root and crown rot (Windels, et al. 2009) in sugar beet (*Beta vulgaris* L.) but can also cause significant seedling disease, typically referred to as damping-off (Harveson, 2009; Kirk et al., 2008; Windels & Brantner, 2005). Substantial economic losses due to damping-off can occur in all regions where sugar beets are grown (Harveson, 2009; Herr, 1996).

Damping-off can present itself as a seed or pre-emergence decay which is usually recognized by poor stand establishment (Baker, 1970). More commonly, *R. solani* is associated with post-emergent damping-off, which can occur at any time after emergence until the seedling is past the juvenile stage (Baker, 1970; Harveson, 2009; Herr, 1996). Post-emergent decay caused by *R. solani* usually begins at or near the soil line and primarily affects the area of the hypocotyl just below the soil surface, although the disease can progress further down into the root (Fig. 3.1). Lesions are dark brown to black and can weaken the stem, causing the seedling to wilt and collapse, often resulting in death of the plant. Field level symptoms tend to occur in irregular patches or down the rows as the pathogen spreads (Herr, 1996; Windels et al., 2009).

Figure 3.1 Sugar beet seedlings in various stages of decay from *Rhizoctonia solani* AG2-2 infection. Dark brown to black lesion extends downward from the soil surface. As the disease progresses, it can cause leaves to wilt and yellow, the stem to become weakened and the plant to collapse, and finally die. Plants were rated on a scale of 0 to 5 where '0' = no disease (plant on left) and '5' = plant dead (plant on right). Above ground symptoms were not readily visible on plants rated '3' or below (first four plants from left).

One of the recommendations for managing losses from *Rhizoctonia* is to plant early when there is a reduced risk of infection (http://cropwatch.unl.edu/plantdisease/sugarbeet /rhizoctonia-root-crown-rot; Leach, 1986; Leach & Garber, 1970; Windels & Brantner, 2005). However, there are diverse reports as to what soil temperature presents a reduced risk. The most widely cited temperature below which *R. solani* becomes inactive is 15˚C (American Crystal Sugar Company, 2016; Harveson, 2009; Neher & Gallian, 2011) but other sources have identified 12˚C as the lower limit of *R. solani* activity (Harveson, 2008; Windels & Brantner, 2005). Sugar beet is typically planted when soil temperatures are between 5 and 10˚C (Kirk et al., 2008) making it important to understand when risk of infection begins so that appropriate management practices can be implemented. For example, the American Crystal Sugar Company (2016) recommends fungicide application after the soil warms to between 18˚ to 21˚C. This

could be a concern because if infection can occur at temperatures as low as 12˚C, seedlings could be vulnerable to infection for three to four weeks before initial fungicide application.

In general, *R. solani* prefers warm, moist soil with temperatures between 20˚ and 30˚C being optimal for disease progression (Baker & Martinson, 1970; Bolton et al., 2010; Kirk et al., 2008; Windels et al., 2009). However, *R. solani* AG2-2 is traditionally subdivided into two main subgroups that are distinguished by temperature tolerance. AG2-2IIIB is recognized as a high-temperature group and cultures can grow at 35˚C while isolates in the subgroup *R. solani* AG2-2IV do not grow at 35˚C (Ogoshi, 1987; Sneh et al., 1991). This separation of groups based on temperature suggests the possibility that there could be some differential response to low temperatures as well and that the group AG2-2IV may have a lower temperature range than the group AG2-2IIIB.

Experiments conducted by Bolton et al., (2010) indicate that *R. solani* AG2-2 does not cause disease when growing conditions were set to 14.4˚C day / 8.9˚C night. Disease symptoms did occur when conditions were set to temperatures of 15.6˚C day / 10˚C night, although there was no significant difference in disease severity between isolates of AG2-2IIIB and AG2-2IV under those conditions. Based on these experiments, it appears the minimum temperature for infection of AG2-2 isolates in either sub-group is 15˚C, which is the value most commonly cited as below which *R. solani* is inactive. It should be noted, however, that these experiments were conducted with a single isolate of AG2-2IIIB and a single isolate of AG2-2IV, both collected in the Minnesota beet growing region. Since *R. solani* is such a diverse group (Chapter 2, this thesis; Ohkura et al., 2009; Strausbaugh et al., 2011), conclusions based on tests using a single isolate may be questionable.

In contrast to the above report, some work out of Ireland showed that there were differential responses to low temperatures in AG2 isolates, with one of the AG2 isolates they tested being more aggressive at 10 and 15˚C than at 20 or 25˚C (O'Sullivan and Kavanagh, 1991). The authors did not classify the isolates as to whether they belonged to the subgroups AG2-1 or AG2-2, and so the relationship of the isolates they tested to those tested by Bolton et al. (2010) is uncertain. AG2-1 isolates are reported to be pathogens of "winter crops" (Sneh et al., 1991) and have been isolated from sugar beet seedlings (Naito et al., 1975 as referenced by Sneh et al., 1991; Windels & Nabben, 1989). Kaminski & Verma (1985) reported that optimal temperature for growth of AG2-1 isolates from rapeseed (*Brassica napus* L. and *B. campestrus* L.) was lower than for AG4 isolates and that AG2-1 isolates exhibited growth at temperatures as low as 2˚C. Windels and Nabben (1989) reported that an isolate of AG2-1 caused a significant reduction in stand counts on sugar beet seedlings, although it was a weak pathogen on adult plants. Therefore, it is possible that the low temperature AG2 isolate identified by O'Sullivan and Kavanagh (1991) was an AG2-1 strain, rather than an AG2-2, but this is uncertain.

Studies on the virulence of AG2-2 isolates generally compare ratings between subgroups (Carling et al., 2002a; Kuninaga et al., 1997; Strausbaugh et al., 2011) with broad agreement that AG2-2IIIB, as a whole, is more virulent than AG2-2IV. Although studies that examine variation in virulence within AG2-2 are limited, those studies that have been reported show considerable variation among AG2-2 isolates. Strausbaugh et al. (2011) reported that root rot on adult sugar beet varied from 19% to 100% among 47 AG2-2IIIB isolates and from 34% to 71% among 4 AG2-2IV isolates. Virulence trials on corn also showed variability in disease severity, with median scores ranging from 1 to 4 on a scale of 0 to 5 (Ohkura et al., 2009). Minier

(Chapter 2, this thesis) found significant differences in virulence among AG2-2 isolates on dry beans (*Phaseolus vulgaris*). Isolates classified as AG2-2IIIB ranged in virulence from 26% to 58% on adult plants and 38% to 100% on seedlings; whereas AG2-2IV isolates ranged from 22% to 58% on adult plants and 14% to 100% on seedlings.

Sequence analysis of the internal transcribed spacer (ITS1 and ITS2) and 5.8S subunit of the ribosomal DNA (rDNA) region has also demonstrated variability within as well as between AG2-2 subgroups (Gonzalez et al., 2001; Salazar *et al*., 2000; Strausbaugh *et al*., 2011). In general, classification using rDNA sequences supports the classic groups based on anastomosis (Gonzalez et al., 2001; Sharon et al., 2008), although the relationships within the group AG2-2 are not as clear, particularly when based solely on rDNA-ITS analysis (Carling et al., 2002b; Liu & Sinclair, 1992; Strausbaugh et al., 2011).

The phylogenetic analysis of the anastomosis group AG2[1] presented by Carling et al. (2002a) showed that subgroup AG2-2IV was polyphyletic with at least two clusters of AG2-2IV isolates surrounding a cluster of AG2-2IIIB isolates. Strausbaugh et al. (2011) had similar results, with AG2-2IIIB isolates mixed in with and surrounding a cluster of AG2-2IV isolates. A multi-gene phylogenetic analysis by Martin et al. (2014) confirmed that the traditional sub-groups known as AG2-2IIIB and AG2-2IV were not phylogenetically supported (Fig. A3.1). Instead, there are at least three genetic groups within AG2-2 that contain a mix of AG2-2IIIB and AG2-2IV isolates. This finding raises questions regarding studies on virulence and other screenings that ascribe phenotypic characteristics to the traditional sub-groups.

[1] Whether AG2-1 and AG2-2 are sub-types of a single anastomosis group, AG2, or separate and independent anastomosis groups has been controversial since they can anastomose with each other at low rates. Current evidence indicates that they are, in fact, separate and independent groups (Gonzalez et al., 2016; Veldre et al., 2013).

Objectives

Little has been reported on the variability in virulence within *Rhizoctonia solani* AG2-2, especially regarding the effect of low temperature on virulence and sugar beet seedling diseases. In addition, the work by Martin et al. (2014) indicates that traditional sub-group designations within AG2-2 are not phylogenetically supported (Fig. A3.1) and therefore, inferences regarding group characteristics need to be reexamined. In the current study, we evaluate the virulence of 35 AG2-2 isolates in response to low temperature and the relationship of that response to the phylogenetic groupings.

Methods

Selection of AG2-2 Isolates

Isolates were chosen from the culture collection of Linda Hanson (USDA-ARS, East Lansing, MI) as representatives of the three phylogenetic groups identified by Martin et al. (2014). All isolates screened in this study (Table 3.1) had been isolated previously from sugar beet (*Beta vulgaris*) from diverse growing regions and stored on whole barley grains (Sneh et al., 1991) at -20˚C. Isolates were recovered from storage and grown on potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO) for 10 - 14 days to verify culture characteristics.

Table 3.1 *Rhizoctonia solani* AG 2-2 isolates used in the current study. Phylogenetic group shown is as determined by Martin et al. (2014). Previous ISG designation is based on growth at 35˚C. Locale indicates state or region of isolation. All isolates were originally isolated from sugar beet roots.

Isolate	Phylogenetic group	ISG designation	Origin	Original collector
24BR	1	Intermediate	Ontario	C. Truman
C116	1	IIIB	Japan	A. Ogoshi
F30	1	IIIB	ID	C. Strausbaugh
F36	1	IIIB	OR	C. Strausbaugh
F503	1	IIIB	ID	C. Strausbaugh
F548	1	IIIB	ID	C. Strausbaugh
F551	1	IIIB	ID	C. Strausbaugh
R09-2	1	IIIB	MI	L. Hanson
R09-23	1	IV	MI	L. Hanson
Rs1012	1	IIIB	MN	C. Windels
Rs331	1	IIIB	MN	C. Windels
Rs393	1	IV	MN	C. Windels
Rs470	1	IIIB	MN	C. Windels
Rs571	1	IIIB	MN	C. Windels
Slovakia	1	IIIB (1)	Europe	B. Holtschulte
F508	2A	IIIB	ID	C. Strausbaugh
F521	2A	IIIB	ID	C. Strausbaugh
Italian	2A	IIIB (1)	Europe	B. Holtschulte
R09-25	2A	Intermediate	MI	L. Hanson
R09-28	2A	IV	MI	L. Hanson
R1	2A	IIIB	CO	E. Ruppel
Rs866	2A	IIIB	MN	C. Windels
Rzc16 (R9)	2A	IIIB	CO	E. Ruppel
Rzc21 (W-22)	2A	IIIB	WI	R.T. Sherwood
2C13	2B	IV	MT	B. Bugbee
Rs106	2B	IV	MN	C. Windels
Rs1090	2B	IV	MN	C. Windels
Rs1146	2B	IIIB	MN	C. Windels
Rs200	2B	IV	MN	C. Windels
Rs481	2B	IV	MN	C. Windels
Rs496	2B	IV	MN	C. Windels
Rs588	2B	IV	MN	C. Windels
Rs599	2B	IV	MN	C. Windels
Rzc6 (R164S)	2B	IV	Japan	A. Ogoshi
Rzc94 (RH188)	2B	IV	Japan	H. Uchino

(1) European isolates have not been tested for growth at 35˚C, however, only type IIIB has been reported from Europe.

Preparation of Inoculum

Inoculum was prepared in 100 x 20 mm plastic petri dishes using the whole grain method (Sneh, et. al., 1991). Hull-less barley grains (Bob's Red Mill, Milwaukie, OR) were soaked overnight in distilled water and autoclaved for 40 minutes. Sterile barley was added to a petri dish so that the dish was about half full. Barley was inoculated with four 6mm plugs cut from actively growing cultures with a #2 cork borer and incubated at 21˚C for 5-7 days until all grains were infested. Infested barley was air dried overnight in a biosafety cabinet and ground in a Waring grinder (Conair Corp., Stamford, CT) prior to use. Uninfested, sterile barley was ground as above to be used as a mock-inoculated control.

Analysis of the Virulence of AG2-2 *Isolates on Sugar Beet Seedlings*

The virulence of thirty-five *Rhizoctonia solani* AG2-2 isolates (Table 3.1) was evaluated on sugar beet seedlings at 11˚C and 21˚C using the sugar beet line 'C869' (Lewellen, 2004), a monogerm, *R. solani*-susceptible germplasm. Experiments were conducted in a growth chamber (Conviron PGW 36, Controlled Environments Inc., Pembina, ND) set to a 14 hour photoperiod and were arranged in an incomplete block design. Each isolate was replicated three times per block and repeated once for a total of at least six experimental units per isolate. Each block included a mock-inoculated control using uninfested, sterile barley as the inoculum.

Sugar beet seed was surface sanitized by soaking in 0.6% sodium hypochlorite plus 0.1% Tween 20 solution for 15 minutes and then rinsed twice in sterile water. To enhance germination, sanitized seed was soaked overnight in a 0.3% hydrogen peroxide solution (McGrath et al., 2000) with shaking at 120 rpm. Disinfested seed was treated with a 2% solution of metalaxyl (Allegiance-FL; Bayer Crop Science, Research Triangle Park, NC) prior to planting to

protect against Pythium seed rot. Seed was sown, in excess, in 1.75 L (15 cm diameter) plastic pots partially filled with potting mix (Suremix Pearlite; Michigan Grower Products, Galesburg, MI) and covered with about 1.0 to 1.5 cm of additional potting mix. Initial experiments where seeds were germinated at 11˚C resulted in excessively uneven stands (unpublished data) and thus all seeds were germinated at 21˚C to ensure even aged stands. When plants reached the two leaf stage (about 7-10 days after planting), pots were thinned or transplanted as needed to five plants per pot.

For the low temperature test (11˚C), the growth chamber temperature was reduced from 21˚C to 11˚C over an 8 hour period 1 day after pots had been thinned. For the high temperature test (21˚C), the temperature was maintained at 21˚C throughout the experiment. When the chamber was at the desired temperature, 0.62 cc of ground inoculum was sprinkled evenly in each pot using a 1/8 tsp. measuring spoon. Plants were kept at 11˚C for 21 days or at 21˚C for 7 days and then removed from pots and the potting mix was rinsed from roots using tap water. The root and hypocotyl were examined for disease symptoms, as described above (Fig. 3.1), and rated on a scale of 0 to 5 where 0 = no disease, 1 = lesion covered less than 20% of the tissue, 2 = lesion covered about 20 - 60% of the tissue, 3 = lesion covered 60 - 90% of the tissue but tops still appear healthy, 4 = lesions covered more than 90% of the tissue and the plant was wilted but not completely dead, 5 = plant was completely dead. Representative plants with characteristic infection symptoms were surface sanitized and plated on 2% water agar plates to confirm the presence of *R. solani*.

Data was analyzed using PROC MIXED (SAS 9.4; SAS Institute Inc., Cary, NC) with block and replicate considered random effects. Dunnett's one-tailed test ($\alpha = 0.05$) was used to

identify isolates that caused disease as compared to the mock-inoculated controls. Average disease severity of the phylogenetic groups was compared using Tukey's HSD test. A student t-test assuming unequal variances was used to compare means based on previous sub-group designations of IIIB and IV of isolates as determined by growth rate at 35˚C (Ogoshi, 1987).

Growth Rate of AG2-2 Isolates in vitro

To determine if the virulence of *R. solani* isolates is related to fungal growth rate, particularly at low temperatures, 12 isolates were selected from those that had been screened for virulence at 11˚C by ordering them from highest average disease severity rating to lowest and selecting every third isolate. A second set of 12 isolates were selected in the same manner starting with the isolate that had the second highest average disease severity rating. Two isolates used on the first run (R1 and F503) representing moderate and fast growth rates at 11˚C were repeated in the second run to ensure consistency of the two runs.

Fungal isolates were grown on PDA for 5-7 days at 21˚C. A 6 mm plug cut with a #2 cork borer from near the margin of the growing cultures was transferred to a fresh 100 x 15 mm PDA plate. Each isolate was replicated three times. Plates were sealed with Parafilm (Bemis Flexible Packaging, Oshkosh, WI) and maintained in an incubator (New Brunswick Innova 44R; Eppendorf, Hauppauge, NY) in the dark, at either 11˚ or 21˚C. Plates at 21˚C were assessed after 4 days by measuring with calipers from the edge of the plug to the margin of the growing mycelium at two locations 180˚ apart. Plates at 11˚C were allowed to incubate for three days and the margin of the growing mycelium was marked on the plate with an ultra-fine point marker (Sharpie, Chicago, IL). Measurements were taken from this reference mark to the growing margin at two locations approximately 180˚ apart after 4 days and again after 8 days.

Data was analyzed using PROC MIXED (SAS 9.4; SAS Institute Inc., Cary, NC) with block and replication considered random effects. Regression analysis was performed using PROC REG (SAS 9.4; SAS Institute Inc., Cary, NC).

Results

Selection of Isolates

The phylogenetic analysis of Martin et al. (2014) identified two well supported clades, one of which consisted of two sub-clades. These clades were labeled as group 1, group 2A and group 2B (Fig. A3.1). Fifteen of the 29 (52%) isolates identified as group 1, nine of the 11 (82%) identified as group 2A, and 11 of the 16 (69%) in group 2B were selected for this study for a total of 35 isolates (Table 3.1). Eighteen (51%) were previously designated as AG2-2IIIB and 13 (37%) as AG2-2IV. Two isolates (6%) were previously designated as intermediate, with a growth rate at 35˚C that made designation of AG2-2IIIB and AG2-2IV uncertain and two isolates (6%) had not been tested for growth at 35˚C and so subgroup was undesignated.[2]

Virulence of AG2-2 Isolates on Sugar Beet Seedlings at 21˚ and 11˚C

Mean disease scores at 11˚C ranged from 0.47 to 3.92 (Table 3.2) with 27 isolates (77%) having disease severity (DS) significantly higher than the mock-inoculated control (Dunnett's, $\alpha = 0.05$). Average disease severity at 11˚C was 2.01 with a median score of 1.78. The category with 'low' virulence scores (DS = 1.2 to 2.0) had the greatest number of isolates (34%) while there were no isolates in the 'very high' virulence category (DS = 4.0 to 5.0) (Fig. 3.2).

[2] Both isolates were from Europe where it is stated they have only isolates from cultural type AG2-2IIIB; but they were not tested for growth at 35˚C and so were treated as undesignated for the purposes of this study.

Table 3.2 Mean disease severity scores of 35 *Rhizoctonia solani* AG2-2 isolates on sugar beet seedlings at 11˚ and 21˚C. Bolding indicates means that are not significantly different than the mock-inoculated control (α = 0.05). Phylogenetic group (PG) designation is according to Martin et al. (2014). Previous sub-group is the traditional designation of IIIB and IV based on growth at 35˚C. N is the sample size for each isolate. Reduction in rate indicates the percent reduction in disease severity at 11˚C compared to 21˚C when the length of time of exposure to inoculum is considered and is calculated by the formula: (R21 - R11) / R21 where Ry = rate of disease progress at temperature y (see text; equations 3.1 & 3.2).

Isolate	PG	Previous sub-group	N	11C		21C		Reduction in rate
				Mean	Std Err	Mean	Std Err	
Control	-	-	60	**0.17**	0.239	**0.22**	0.205	-
Rzc21	2A	IIIB	30	**0.47**	0.306	2.18	0.229	93%
Rs588	2B	IV	30	**0.48**	0.306	1.13	0.267	86%
F551	1	IIIB	30	**0.71**	0.306	3.83	0.272	94%
Slovakia	1	-	30	**0.88**	0.264	3.51	0.267	92%
Rs331	1	IIIB	30	**0.94**	0.306	1.20	0.272	74%
R09-25	2A	Intermediate	15	**0.98**	0.406	2.84	0.272	88%
C116	1	IIIB	45	**1.04**	0.264	2.36	0.272	85%
Rzc6	2B	IV	30	**1.17**	0.306	**1.10**	0.272	85%
R09-23	1	IV	30	1.24	0.306	3.77	0.272	65%
Rs106	2B	IV	30	1.27	0.306	3.97	0.272	89%
Rs866	2A	IIIB	30	1.30	0.306	4.24	0.272	90%
Rzc94	2B	IV	30	1.30	0.306	3.66	0.272	88%
Rs599	2B	IV	30	1.36	0.304	3.71	0.267	88%
2C13	2B	IV	30	1.52	0.306	4.04	0.272	87%
Rs481	2B	IV	30	1.53	0.304	3.55	0.267	86%
Rs1012	1	IIIB	30	1.73	0.306	4.26	0.272	86%
Rs571	1	IIIB	30	1.76	0.304	3.63	0.272	84%
Rs200	2B	IV	15	1.78	0.406	3.93	0.267	85%
R1	2A	IIIB	60	1.90	0.239	4.12	0.205	85%
24BR	1	Intermediate	30	1.93	0.306	4.17	0.272	85%
F548	1	IIIB	30	2.10	0.306	3.87	0.272	82%
F503	1	IIIB	30	2.42	0.306	4.17	0.272	81%
R09-2	1	IIIB	30	2.59	0.306	2.87	0.272	70%
Rs1146	2B	IIIB	30	2.77	0.306	2.91	0.267	68%
Rs496	2B	IV	30	2.77	0.306	3.25	0.267	72%
R09-28	2A	IV	30	2.80	0.306	4.17	0.272	78%
F508	2A	IIIB	30	2.82	0.306	4.31	0.267	78%
Rs470	1	IIIB	30	3.01	0.306	5.00[(1)]	0.272	80%
Rzc16	2A	IIIB	30	3.12	0.306	4.50	0.272	77%
Italian	2A	-	30	3.14	0.306	4.50	0.272	77%
F30	1	IIIB	30	3.30	0.306	3.77	0.272	71%
F36	1	IIIB	30	3.33	0.306	1.90	0.272	42%
Rs1090	2B	IV	30	3.44	0.306	4.05	0.267	72%
F521	2A	IIIB	30	3.46	0.306	2.61	0.362	56%
Rs393	1	IV	30	3.92	0.306	3.90	0.272	66%

Note: [(1)] Value was adjusted to reflect the maximum value of the 0 - 5 rating system used. Least square means estimate for isolate Rs470 at 21˚C was actually 5.03.

There were 174 plants out of a total of 1110 observations (16%) that were scored as a '5' (plant dead), indicating that 'very high' damage does occur at 11˚C. Those plants that were scored as a '5' were fairly evenly distributed among the phylogenetic groups with group 1 at 37%, group 2A at 26% and group 2B at 37%.

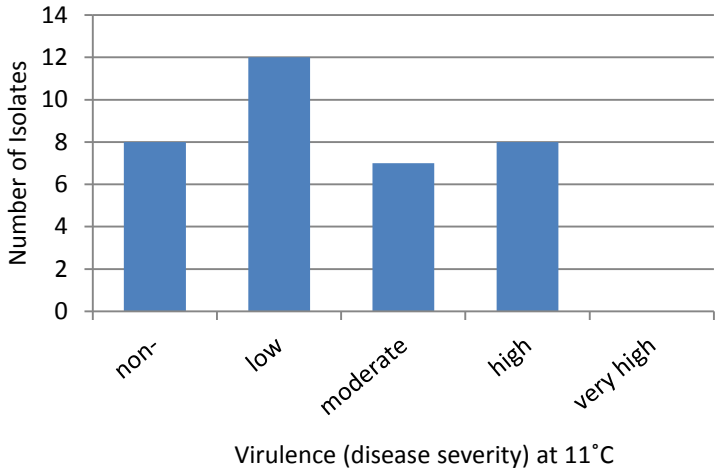

Figure 3.2 Disease severity of 35 *Rhizoctonia solani* AG2-2 isolates on sugar beet seedlings at 11˚C by virulence category. Plants were rated on a 0 to 5 scale where 0 = no disease and 5 = plant dead. Virulence categories are defined as 'non-' (DS = 0 to 1.20), 'low' (DS = 1.21 to 2.00), 'moderate' (DS = 2.01 to 3.00), 'high' (DS = 3.01 to 4.00) and 'very high' (DS = 4.01 to 5.00). The 'non-'category was not significantly different than the mock-inoculated control (Dunnett's, $\alpha = 0.05$).

Mean disease scores at 21˚C ranged from 1.10 to 5.00 (Table 3.2) with 34 isolates (97%) having disease severity significantly higher than the mock-inoculated controls (Dunnett's, $\alpha = 0.05$). Average disease severity at 21˚C was 3.46 with a median score of 3.77. The majority of the isolates (71%) were in the 'high' (DS = 3.0 to 4.0) or 'very high' (DS = 4.0 to 5.0) virulence categories (Fig. 3.3). Only four isolates (11%) had disease severity scores in the 'low' or 'non-virulent' categories at 21˚C of which one isolate (isolate 'F36') had scored in the 'high' virulence category at 11˚C (Table 3.2).

At 11˚C, isolates in phylogenetic group 2A were, on average, more aggressive than isolates in group 2B (Tukey, α = 0.05) while isolates in group 1 were intermediate between group 2A and group 2B (Fig. 3.4). All three phylogenetic groups had significantly higher average disease severity scores than the mock-inoculated control. Similar results were obtained at 21˚C (Fig. 3.4).

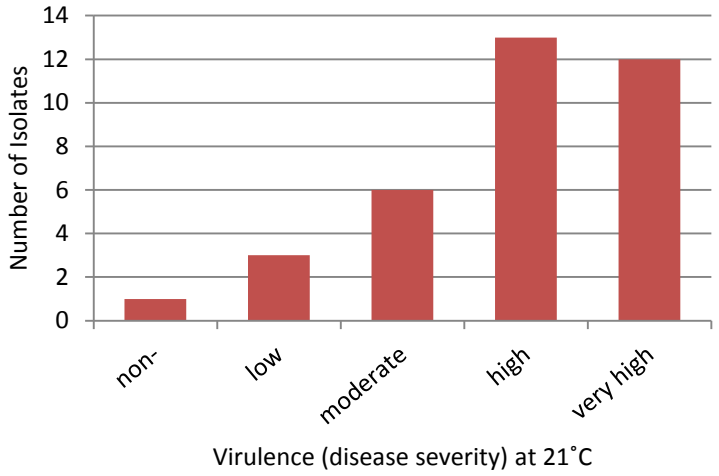

Figure 3.3 Disease severity of 35 *Rhizoctonia solani* AG2-2 isolates on sugar beet seedlings at 21˚C grouped by virulence category. Plants were rated for disease on a scale of 0 to 5 where 0 = no disease and 5 = plant dead. Virulence categories are defined as 'non-' (DS = 0 to 1.10), 'low' (DS = 1.11 to 2.00), 'moderate' (DS = 2.01 to 3.00), 'high' (DS = 3.01 to 4.00) and 'very high' (DS = 4.01 to 5.00). The 'non-'category was not significantly different than the mock-inoculated control (Dunnett's, α = 0.05).

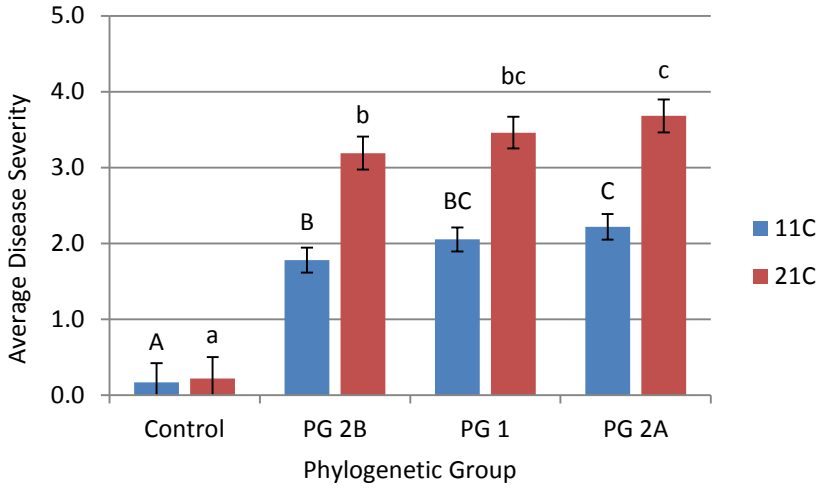

Figure 3.4 Average disease severity of 35 *Rhizoctonia solani* AG2-2 isolates on sugar beet seedlings at 11˚ and 21˚C by phylogenetic group. Plants were rated for disease severity on a scale of 0 to 5, where 0 = no disease and 5 = plants dead. Means with same letter are not significantly different (Tukey, α = 0.05), where upper case letters compare means at 11˚C and lower case letters compare means at 21˚C. Phylogenetic grouping is according to Martin et al. (2014). Error bars indicate standard error of the means.

Comparison of virulence at 11˚C within each phylogenetic group (Fig. 3.5) shows that group 1 and group 2A had about an equal proportion of isolates in each virulence category except for the 'very high' virulence category, in which there were no isolates. Group 2B had a large proportion of isolates (55%) in the 'low' virulence category and a smaller proportion of isolates in the 'high' virulence category (9%). Group 2A had the highest proportion of isolates in the 'high' virulence category (33%).

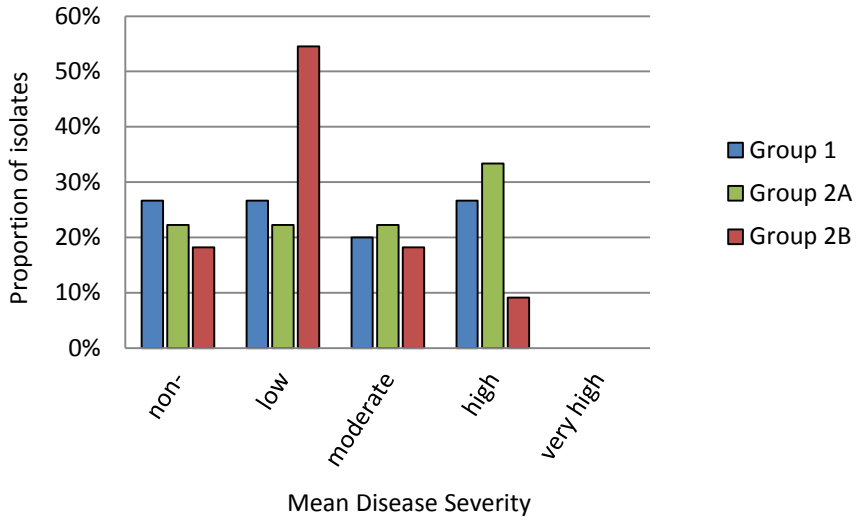

Figure 3.5 Proportion of 35 *Rhizoctonia solani* isolates in each virulence category at 11˚C arranged by phylogenetic group. Plants were rated for disease on a scale of 0 to 5 , where 0 = no disease and 5 = plant dead. Virulence categories are defined as: 'non-' (DS = 0 to 1.20), 'low' (DS = 1.21 to 2.00), 'moderate' (DS = 2.01 to 3.00), high (DS = 3.01 to 4.00) and 'very high' (DS = 4.01 to 5.00). The 'non-'category was not significantly different than the mock-inoculated control (Dunnett's, α = 0.05). Phylogenetic grouping (PG) is according to Martin et al. (2014).

Virulence at 11˚C was greatly reduced compared to 21˚C when the time of exposure to the pathogen is taken into account. Plants screened at 11˚C were exposed to the pathogen for three times longer than those screened at 21˚C which makes direct comparison of disease severity scores difficult. In this situation, we assumed Rhizoctonia damping-off would behave like a monocyclic disease and therefore disease progress was considered to be linear according to the following model (Arneson, 2001):

Equation 3.1 $$\int \frac{dx}{dt} = QR_y \quad \rightarrow \quad R_y = \frac{x}{Qt}$$

where R_y = rate of disease progress at temperature *y;* x = disease severity; t = time; and Q = initial amount of inoculum.

Reduction in virulence at 11˚C is then calculated as:

Equation 3.2 $$\frac{R_{21} - R_{11}}{R_{21}}$$

where R_{21} = rate of disease progress at 21˚C and R_{11} = rate of disease progress at 11˚C. Reduction in virulence ranged from 42% to 94% (Table 3.2) with an average reduction of 80%. There was no significant difference in the reduction in virulence at 11˚C by phylogenetic group (ANOVA, p = 0.787) or by traditional subgroups 'IIIB' or 'IV' (t-test, df = 29, p = 0.206).

Virulence of AG2-2 Isolates Related to Sub-groups IIIB and IV

There were 18 isolates that were previously designated as sub-group 'IIIB', 13 isolates previously designated as sub-group 'IV' and 4 isolates whose sub-group designation was either unknown or classified as intermediate. Comparison of means based on previous sub-group designations was not significant for either the 11˚C screen (t-test, df = 25, p = 0.474) or the 21˚C screen (t-test, df = 26, p = 0.939). At 11˚C, there were a greater number of AG2-2IIIB isolates with disease severity ratings higher than the median value (median = 1.78) compared to AG2-2IV isolates (11 vs. 4). However, this difference was not statistically significant (X^2 = 1.70, df = 1, p = 0.192). Isolates whose sub-group designation was unknown or intermediate were not included in this comparison.

Growth Rate of AG2-2 Isolates in vitro

Twenty-four isolates were tested for growth rate on media at 11˚C and 21˚C. Nine isolates were selected from group 1, six from group 2A and nine from group 2B (Table 3.3). Comparison of runs using control isolates 'R1' and 'F503' showed no significant difference between runs (ANOVA, p = 0.096 for 11˚C and p = 0.111 for 21˚C), so runs were analyzed together.

Table 3.3 Growth rate of 24 *Rhizoctonia solani* AG2-2 isolates at 11˚ and 21˚C. Isolates were grown on PDA for 4 days at 21˚C and 11 days at 11˚C and growth rate expressed in mm/day. Phylogenetic group (PG) is according to Martin et al. (2014). Reduction in growth rate indicates the percent reduction in growth rate at 11˚C compared to 21˚C as calculated by the formula (R_{21} - R_{11}) / R_{21} where R_y is the growth rate at temperature y.

Isolate	PG	Growth rate 11C	Growth rate 21C	Reduction in growth rate
24BR	1	0.024	0.396	94%
C116	1	0.058	0.357	84%
Rs393	1	0.087	0.400	78%
R09-2	1	0.091	0.420	78%
F36	1	0.097	0.418	77%
F30	1	0.108	0.407	74%
F503	1	0.115	0.406	72%
Slovakia	1	0.116	0.413	72%
R09-28	2A	0.050	0.314	84%
F508	2A	0.056	0.394	86%
Rzc16	2A	0.061	0.315	81%
R1	2A	0.077	0.351	78%
Rs866	2A	0.084	0.316	74%
R09-25	2A	0.092	0.355	74%
Italian	2A	0.107	0.377	72%
Rs588	2B	0.036	0.214	83%
Rzc94	2B	0.041	0.366	89%
Rs599	2B	0.073	0.386	81%
Rs106	2B	0.079	0.411	81%
Rs1146	2B	0.088	0.413	79%
Rs481	2B	0.089	0.368	76%
2C13	2B	0.122	0.373	67%
Rs200	2B	0.131	0.375	65%
Rs1090	2B	0.137	0.393	65%

Growth rates at 21˚C ranged from 0.214 to 0.420 mm/day (Table 3.3). Average growth rates of the three phylogenetic groups (Fig. 3.6) were significantly different from one another (Tukey, α = 0.05) at this temperature. Group 1 had the highest average growth rate (0.402 mm/day), followed by group 2B (0.370 mm/day) with group 2A having the slowest growth rate (0.347 mm/day).

Growth rates at 11˚C ranged from 0.024 to 0.137 mm/ day. Similar to growth rates at 21˚C, group 2A had the lowest average growth rate at 0.075 mm/day (Fig. 3.6), which was significantly less (Tukey, α = 0.05) than group 1 and group 2B (0.090 and 0.089 mm/day respectively). Growth rate at 11˚C was considerably reduced in all isolates compared to 21˚C but the amount of reduction varied from 65% to 94% (Table 3.3). There was no significant difference in the amount of reduction based on phylogenetic group (ANOVA, p = 0.762).

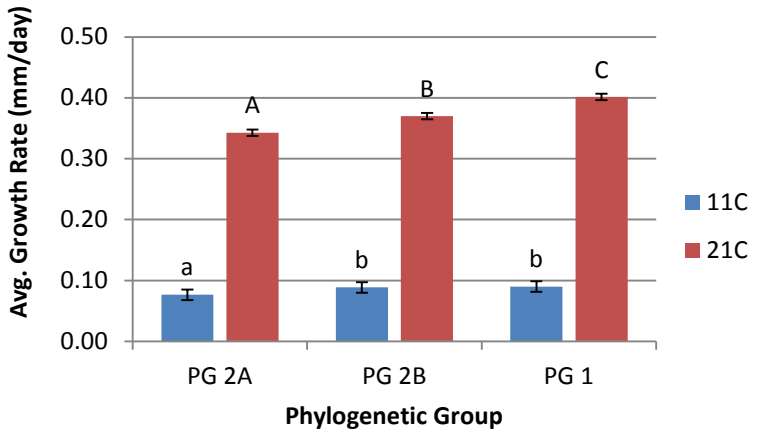

Figure 3.6 Average growth rate of 24 *Rhizoctonia solani* AG2-2 isolates by phylogenetic grouping at 11˚ and 21˚C. Isolates were grown on potato dextrose agar (PDA) for 4 days at 21˚C and 11 days at 11˚C and growth rate expressed in mm/day. Phylogenetic group (PG) is according to Martin et al. (2014). Means with the same letters are not significantly different (Tukey, α = 0.05), where upper case letters compare means at 21˚C and lower case letters compare means at 11˚C.. Error bars indicate standard error of the means.

Relationship between Growth Rate, Temperature and Virulence

A simple linear regression was used to predict growth rate at 11˚C based on growth rate at 21˚C. A significant linear relationship was found ($F_{1,22}$ = 5.91, p = 0.024) with an R^2 value of 0.212 (Fig. 3.7A). The coefficient for growth rate at 21˚C was 0.298 indicating the growth rate at 11˚C increases 0.298 mm/day for every 1 mm/day increase in the growth rate at 21˚C.

The relationship between virulence at 11˚C and virulence at 21˚C also had a significant linear component ($F_{1,33}$ = 5.13, p = 0.030) with an R^2 value of 0.135 (Fig. 3.7B). The coefficient for virulence was 0.374 indicating disease severity score at 11˚C increases by 0.374 for an increase in disease severity score of 1.0 at 21˚C.

There was no significant linear relationship between growth rate at 11˚C and virulence at 11˚C ($F_{1,22}$ = 1.31, p = 0.264) (Fig. 3.7C) or between growth rate at 21˚C and virulence at 21˚C ($F_{1,22}$ = 2.75, p = 0.112) (Fig. 3.7D).

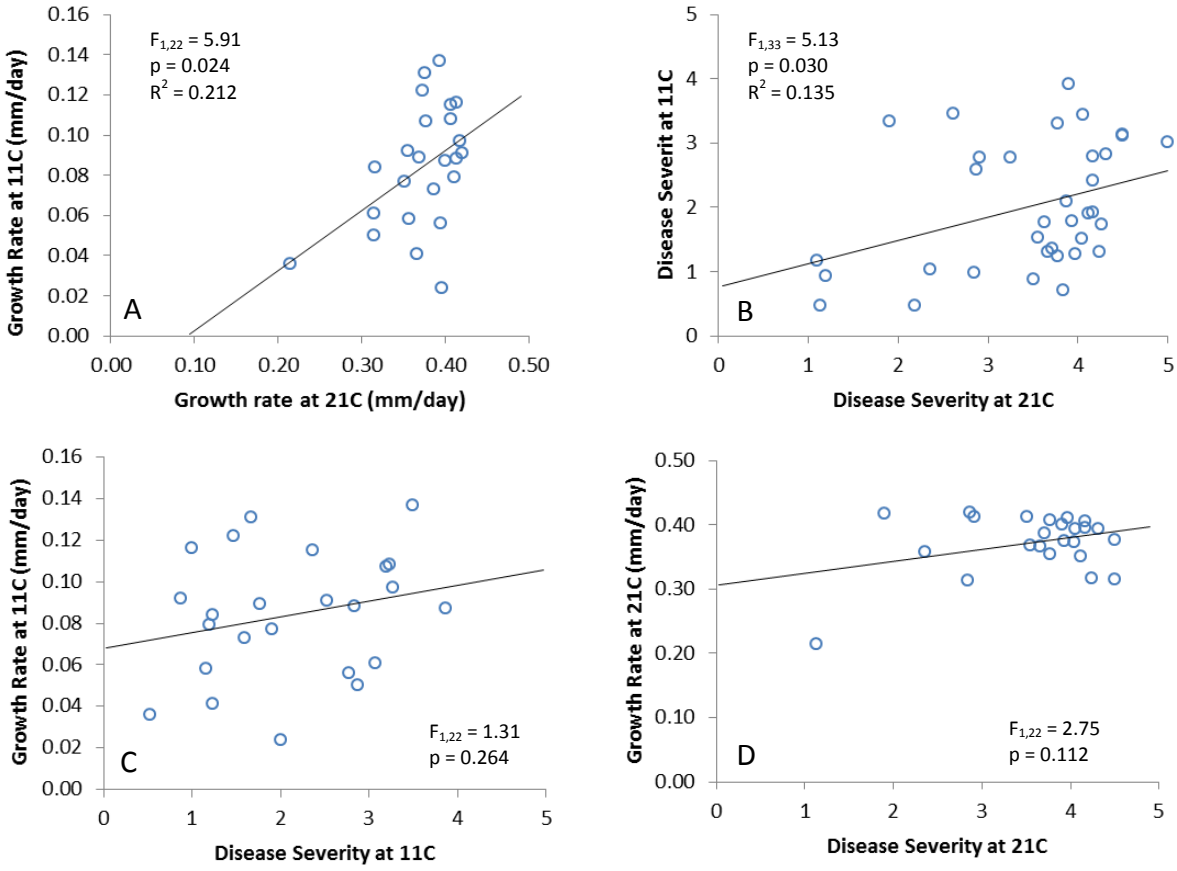

Figure 3.7 Scatterplots comparing growth rate and virulence of *Rhizoctonia solani* AG2-2 isolates at 11˚ and 21˚C. Disease severity was rated on a scale of 0 to 5, where 0 = no disease and 5 = plant dead. A) Comparison of the growth rate of 24 isolates at 11˚ and 21˚C. B) Comparison of disease severity of 35 isolates at 11˚ and 21˚C. C) Comparison of the growth rate and disease severity of 24 isolates at 11˚C. D) Comparison of growth rate and disease severity of 24 isolates at 21˚C.

Discussion

When grown at 11˚C, 43% of the isolates tested caused moderate or high levels of disease and 16% of the inoculated plants were dead by the end of the experiment. Virulence ratings ranged from 0.47 (non-virulent) to 3.92 (highly virulent) (Table 3.2), and distribution among the phylogenetic groups was fairly evenly distributed (Fig. 3.4). Group 2B had a lower mean disease severity score at 11˚C than group 2A (1.78 vs. 2.22) due to the high proportion of group 2B isolates that tested in the 'low' virulence category (Fig. 3.5). While the difference in virulence between group 2A and group 2B was statistically significant at 11˚C, the biological significance of that difference is questionable. In other words, the difference between the groups is unlikely to result in noticeable differences in a field level situation. Therefore, our data indicates that phylogenetic group is not diagnostic for virulence at low temperature as there was a wide range of virulence within the groups and the eight non-virulent isolates had representatives from each of the phylogenetic groups with a group 2A isolate having the lowest score. In addition, the three isolates with the highest disease severity scores at 11˚C were distributed among the three genetic groups.

The expression of disease symptoms can lag pathogen spread and infection resulting in substantial underestimation of disease progress (Leclerc et al., 2014). This problem can be exacerbated for pathosystems that primarily cause disease on underground plant parts, as these symptoms are not readily visible without destructive sampling. Detection in these systems generally relies on the development of symptoms on above ground parts (Leclerc et al., 2014; Rush et al., 1992). In our experiments, plants that were rated as a '3' or below exhibited little or no above ground symptoms and any damage to the hypocotyl was not visible without

careful examination of the tissue below the surface. At 11˚C, 45% of the plants were rated with a disease score of '2' or higher (Fig. 3.8), indicating substantial disease progression; yet, less than 25% had ratings that included the presence of above ground symptoms (DS = 4 or 5). We hypothesize that early season infection may allow the pathogen to initiate disease largely undetected but that the rate of disease progression would increase substantially once temperatures rise to a more favorable level.

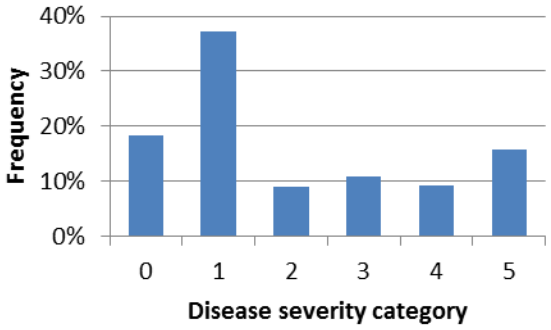

Figure 3.8 Proportion of plants in each disease severity category rated 21 days after inoculation with *Rhizoctonia solani*. A total of 1110 plants were inoculated using 35 strains of *R. solani* and were rated on a scale of 0 to 5; where 0 = no disease; 1 = lesion covering < 25% of the tissue; 2 = lesion covering 20 - 60% of the tissue; 3 = lesion covering 60 - 90% of the tissue and top still healthy; 4 = lesion covering > 90% and plant not dead; 5 = plant dead.

Virulence at 11˚C was reduced compared to virulence at 21˚C with 30 out of 35 isolates having a reduction in virulence of at least 70% (Table 3.1). This would most likely translate into reduced symptoms in the field and infected plants might not display any visible above-ground symptoms. It could be tempting to consider the lack of visible symptoms in the field to be an indication that there is no infection in the field and therefore, no risk. However, given the clear

evidence that at least some isolates of AG2-2 are moderately to highly virulent at low temperatures, it would be a mistake to ignore the potential for early season infection.

The virulence of the *R. solani* AG2-2 isolates in this study does not appear to be related to hyphal growth rate on artificial media (Fig. 3.7C & D). For instance, group 2A had an average growth rate that was significantly lower than that of group 2B isolates at both 11˚ and 21˚C but was significantly more virulent than group 2B at both temperatures (Fig. 3.4). This agrees with Leach (1947) who found that the incidence of damping-off did not correlate with either growth rate of the pathogen or the host. Instead, disease incidence was inversely related to the ratio between how rapidly the seedlings emerged (coefficient of velocity of emergence, CVE) and the growth rate of the pathogen (Leach, 1947). Disease was more severe when temperatures were relatively less favorable to the host than to the pathogen (Baker & Martinson, 1970; Leach, 1947).

Temperature alone has been shown to be insufficient as a predictor of disease severity (Dorrance et al., 2003; Leach, 1947; Kirk et al., 2008) and our results support this conclusion. While there was a significant linear component ($p = 0.030$) in the relationship between disease severity at 11˚C and disease severity at 21˚C, the R^2 value was only 0.135 (Fig. 3.7B), which indicates that less than 14% of the variability in virulence can be explained by differences in temperature. Some of the variability in our experiments might be explained by slight differences in moisture, inoculum density, and distance from inoculum source to host plant, which are factors known to influence disease severity (Bolton et al., 2010; Dorrance et al., 2003; Kirk, et al., 2008). However, the majority of the variability in disease severity should be attributed to differences in how the isolates responded to low temperatures.

Certain strains of *R. solani*, such as the strain of AG2 reported by O'Sullivan and Kavanagh (1991), have been reported to cause more severe disease at low temperatures than at higher temperatures (Baker & Martinson, 1970). While none of the isolates in the current study could be considered to be more aggressive at 11˚C than at 21˚C (once length of time of exposure to the pathogen is considered), at least one isolate was much less affected by lower temperatures than the others. Isolate 'F36' had a disease severity score of 1.90 at 21˚C (Table 3.2), which placed it in the 'low' virulence category for 21˚C (Fig. 3.3), but had a disease severity score of 3.33 at 11˚C (Table 3.2), which placed it in the 'high' virulence category for 11˚C (Fig. 3.2). When length of time of exposure to pathogen was taken into consideration (Eq. 3.1), isolate 'F36' had the lowest reduction in virulence due to low temperature of the isolates tested (Table 3.2). Low temperature does reduce the severity of disease but the amount of reduction depends on the response of the specific isolate.

Rhizoctonia solani cannot be thought of as a single entity but as a complex collection of related fungi that cause a range of diseases in a variety of crops (Baker, 1970; Cubeta & Vilgalys, 1997; Sneh et al., 1991). It also appears that *R. solani* AG2-2 should not be considered a single, homogeneous entity. The variability identified in this study agrees with previous studies that report *R. solani* AG2-2 to be a diverse group that has a wide range of virulence on sugar beet seedlings (Strausbaugh et al., 2011). Studies that examine the effects of *R. solani* AG2-2 on cropping systems need to take that variability into consideration. Unfortunately, the risk of early season disease development does not appear to be linked to a particular group within AG2-2 but rather is dependent on the specific isolate(s) present. This makes detection and identification of risk more difficult and it may be safer to consider all AG2-2 types capable of

causing early season damping-off. A set of microsatellite markers is currently being developed by our research group and we are investigating a possible connection between microsatellite genotype and low temperature virulence.

Conclusions

Early planting still remains an important part of sugar beet agronomics as it has been shown to provide increased yields (Scott, 1973) and does offer some measure of protection against *Rhizoctonia* induced damping-off (Leach, 1986). However, growers and agronomists should be cautious of the expectation that early season planting will provide 'no risk' of *Rhizoctonia solani* infection. Rather, the risk in any particular field will depend on the specific isolates present, the amount of inoculum, soil and moisture conditions, and how quickly the soil warms (Baker, 1970; Bolton et al., 2010; Dorrance et al., 2003). While this report cannot directly address the use of fungicides, our findings indicate that soil temperature should not be considered the determining factor for fungicide application timing. Instead, growers should consider including a protectant at planting that is effective against *Rhizoctonia solani,* especially in fields where there has been a history of disease.

APPENDIX

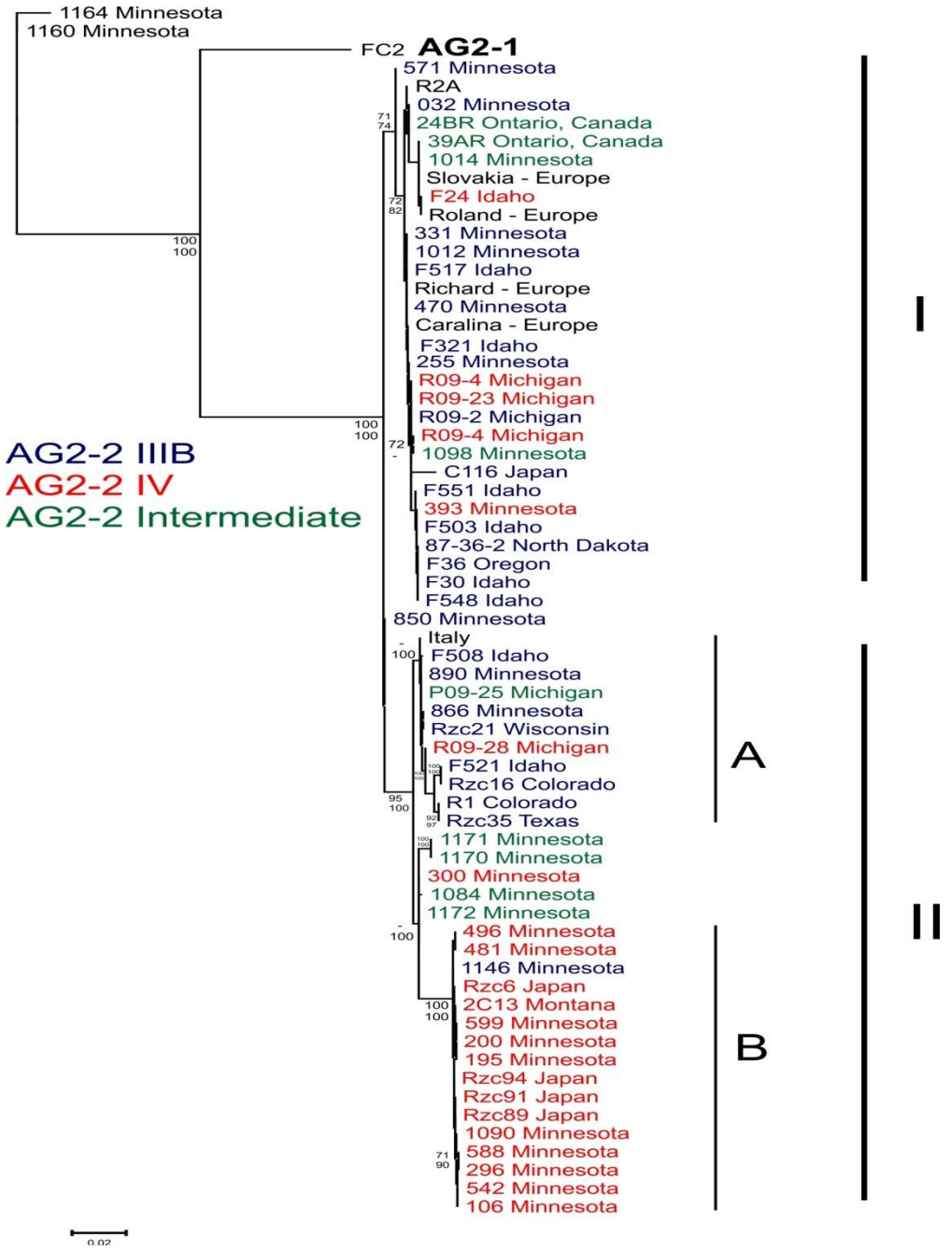

Figure A3.1 Multigene phylogeny of 63 *Rhizoctonia solani* AG2-2 isolates according to Martin et al. (2014). Genes sequenced included *rpb2*, tef1*, ITS,* and *LSU* as reported in Gonzalez et al. (2016) with minor modifications to improve reliability and specificity for AG2-2 (unpublished data). Isolates in blue were originally identified as AG2-2IIIB, those in red identified as AG2-2IV, and those in green were intermediates based on growth at 35˚C - where AG2-2IIIB grows well at 35˚C and AG2-2IV do not. An AG2-1 isolate was used for the outgroup. Phylogram used curtesy of Martin et al. (2014).

REFERENCES

REFERENCES

American Crystal Sugar Company (2016). Pest alert - *Rhizoctonia* fungicide application: Soil temp charts. Retrieved from: https://www.crystalsugar.com/sugarbeet-agronomy/pest-alert/ on 6/24/2017.

Anderson, N. A. (1982). The genetics and pathology of *Rhizoctonia solani*. Annual Review of Phytopathology, 20: 329-347.

Arneson, P.A. (2001). Plant disease epidemiology: Temporal aspects. The Plant Health Instructor. DOI: 10.1094/PHI-A-2001-0524-01. (Revised 2011).

Baker, K.F. (1970). Types of *Rhizoctonia* diseases and their occurrence. Pages 125-148 in *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr., University of California Press, Berkeley, CA.

Baker, R. and Martinson, C.A. (1970). Epidemiology of diseases caused by *Rhizoctonia solani*. Pages 172-188 in: *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J. R. University of California Press, Berkeley, CA.

Bolton, M.D., Panella, L, Campbell, L., Khan, M.F.R.(2010). Temperature, moisture and fungicide effects in managing Rhizoctonia root and crown rot of sugar beet. Phytopathology, 100: 689-697.

Carling, D.E., Baird, R.E., Gitaitis, R.D., Brainard, K.A. and Kuninaga, S. (2002a). Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. Phytopathology, 92: 893-899.

Carling, D., Kuninaga, S. and Brainard, K. (2002b). Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. Phytopathology, 92: 43-50.

Cubeta, M. A. and Vilgalys, R. (1997). Population biology of the *Rhizoctonia solani* complex. Phytopathology, 87: 480-484.

Dorrance, A.E., Kleinhenz, M.D., McClure, S.A. and Tuttle N.T. (2003). Temperature, moisture, and seed treatment effects on *Rhizoctonia solani* root rot of soybean. Plant Disease, 87: 533-538.

Gonzalez, D., Carling, D.E., Kuninaga, S., Vilglys, R. and Cubeta, M.A. (2001). Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs. Mycologia 93: 1138-1150.

Gonzalez, D., Rodriguez-Carres, M., Boekhout, T., Stalpers, J., Kuramae, E.E., Nakatani, A.K., Vigalys, R. and Cubeta, M.A. (2016). Phylogenetic relationships of *Rhizoctonia* fungi within the Cantharellales. Fungal Ecology, 120: 603-619

Harveson, R. (n.d.) Rhizoctonia root and crown rot. University of Nebraska-Lincoln Crop Watch. Retrieved from: http://cropwatch.unl.edu/plantdisease/sugarbeet/rhizoctonia-root-crown-rot on 6/24/17.

Harveson, R. M. (2008). Rhizoctonia root and crown rot of sugar beet. Nebraska extension, G1841. University of Nebraska, Lincoln, NE.

Harveson, R. M. (2009). Seedling diseases. Pages 21-24 in Compendium of Beet Diseases and Pests. Edited by Harveson, R.M., Hanson, L.E. and Hein, G.L., APS Press, St. Paul, MN.

Herr, L. J. (1996). Sugar beet disease incited by *Rhizoctonia* spp. Pages 341-349 in *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Edited by: Sneh, B., Jabaji-Hare, S., Neate, S. and Dijst, G. Kluwer Academic Press, Dordrecht, Netherlands.

Kaminski, D.A. and Verma, P.R. (1985). Cultural characteristics, virulence, and *in vitro* temperature effect on mycelial growth of *Rhizoctonia* isolates from rapeseed. Canadian Journal of Plant Pathology, 7: 256-261.

Kirk, W.W., Wharton, P.S., Schafer, R.L., Tumbalam, P., Poindexter, S., Guza, C., Fogg, R., Schlatter, T., Stewart, J., Hubbell, L. and Ruppal, D. (2008). Optimizing fungicide timing for the control of Rhizoctonia root and crown rot of sugar beet using soil temperature and plant growth stages. Plant Disease 92: 1091-1098.

Kuninaga, S., Natsuaki, T., Takeuchi, T. and Yokosawa, R. (1997). Sequence variation or the rDNA ITS regions within and between anastomosis groups in *Rhizoctonia solani*. Current Genetics, 32: 237-243.

Leach, L.D. (1947). Growth rates of host and pathogen as factors determining the severity of preemergence damping-off. Journal of Agricultural Research, 75: 161-179.

Leach, L.D. (1986). Seedling diseases. Pages 4 - 8 in: Compendium of Beet Diseases and Insects. Edited by Whitney, E.D. and Duffus, J.E., APS Press, St. Paul, MN.

Leach, L.D. and Garber, R.H. (1970). Control of *Rhizoctonia*. Pages 189-198 in *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr., University of California Press, Berkeley, CA.

Leclerc, M., Doré, T., Gilligan, C.A., Lucas, P. and Filipe, J.A.N. (2014). Estimating the delay between host infection and disease (incubation period) and assessing its significance to the epidemiology of plant diseases. PLOS One, 9: 1-15.

Lewellen, R.T. (2004) Registration of rhizomania resistant, monogerm populations C869 and C869CMS sugarbeet. Crop Science, 44: 357-358.

Liu, Z.L. and Sinclair, J.B. (1992). Genetic diversity of *Rhizoctonia solani* anastomosis group 2. Phytopathology 82: 778-787.

Martin, F., Windels, C., Hanson, L. and Brantner, J. (2014). Analysis of population structure and pathogenicity of *Rhizoctonia solani* AG2-2 (ISG IIIB and IV) isolates from Michigan, Minnesota and North Dakota. Sugarbeet Research Reports. Beet Sugar Development Foundation, Denver, CO. Published on CD.

McGrath, J.M., Derrico, C.A., Morales, M., Copeland, L.O., and Christensen, D.R. (2000). Germination of sugar beet (*Beta vulgaris* L.) seed submerged in hydrogen peroxide and water as a means to discriminate cultivar and seedlot vigor. Seed Science and Technology 28: 607-620.

Neher, O.T. and Gallian, J.J. (2011). *Rhizoctonia* on sugar beet: Importance, identification and control in the Northwest. Pacific Northwest Extension. PNW629. University of Idaho, Moscow, ID.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annual Review of Phytopathology, 25:124-143.

Okura, M., Abawi, G.S., Smart, C.D. and Hodge, K.T. (2009). Diversity and aggressiveness of *Rhizoctonia solani* and *Rhizoctonia*-like fungi on vegetables in New York. Plant Disease, 93: 615-624.

O'Sullivan, E. and Kavanagh, J.A. (1991). Characteristics and pathogenicity of isolates of *Rhizoctonia* spp. associated with damping-off of sugar beet. Plant Pathology, 40: 128-135.

Rush, C.M., Mihail, J.D. and Singleton, L.L. (1992). Introduction. Pages 3-6 in Methods for Research on Soilborne Phytopathogenic Fungi. Edited by Singleton, L.L., Mihail, J.D. and Rush, C.M. APS Press, St. Paul MN.

Salazar, O., Julián, M.C., Hyakumachi, M., Rubio, V. (2000). Phylogenetic groupings of cultural types of *Rhizoctonia solani* AG 2-2 based on ribosomal ITS sequences. Mycologia, 92: 505-509.

Scott, R.K., English, S.D., Wood, D.W., and Unsworth, M.H. (1973). The yield of sugar beet in relation to weather and length of growing season. Journal of Agricultural Science, Cambridge 81: 339-347.

Sharon, M., Sneh, B., Kuninaga, S., Hyakumachi, M. and Naito, S. (2008). Classification of *Rhizoctonia* spp. using rDNA-ITS sequence analysis supports the genetic basis of the classical anastomosis grouping. Mycoscience, 49: 93-114.

Strausbaugh, C.A., Eujayl, I.A., Panella, L.W. and Hanson, L.E. (2011). Virulence, distribution and diversity of *Rhizoctonia solani* from sugar beet in Idaho and Oregon. Canadian Journal of Plant Pathology, 33: 210-226.

Sneh, B., Burpee, L. and Ogoshi, A. (1991). Identification of *Rhizoctonia* species. APS Press, St. Paul, MN, USA.

Veldre, V., Abarenkov, K., Bahram, M., Martos, F. Selosse, M., Tamm, H., Kõljalh, U. and Tedersoo, L. (2013). Evolution of nutritional modes of Ceratobasidiaceae (Cantharellales, Basidiomycota) as revealed from publicly available ITS sequences. Fungal Ecology 6: 256-268.

Windels, C. E. and Brantner, J. R. (2005). Early-season application of azoxystrobin to sugarbeet for control of *Rhizoctonia solani* AG4 and AG2-2. Journal of Sugar Beet Research, 42: 1-16.

Windels, C.E., Jacobsen, B. J. and Harveson, R.M. (2009). Rhizoctonia root and crown rot. Pages 33-36 in Compendium of Beet Diseases and Pests. Edited by Harveson, R.M., Hanson, L.E. and Hein, G.L. APS Press, St. Paul, MN.

Windels, C.E. and Nabben, D.J. (1989). Characterization and pathogenicity of anastomosis groups of *Rhizoctonia solani* isolated from *Beta vulgaris*. Phytopathology 79: 83-88.

CHAPTER 4:

IDENTIFICATION AND VALIDATION OF MICROSATELLITE MARKERS FOR USE IN *RHIZOCTONIA SOLANI* AG2-2 POPULATION ANALYSIS

Introduction to Microsatellites

Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STR), are comprised of nucleotide motifs of one to six base pairs that are tandemly repeated between five and fifty times (Oliveira et al., 2006). They are widespread in all eukaryotic genomes (Katti et al., 2001) and have been shown to be invaluable for use in many areas of biology that include genome mapping (Shimoda et al., 1999), parental analysis (Jones et al., 2010), population genetics (Biasi et al., 2016; Coupat-Goutaland et al., 2016; dos Santos Pereira et al., 2016) and resource conservation (Perez-Enriquez et al., 1998; Ernest et al., 2000). Microsatellites have become one of the most widely used and highly versatile genetic markers available for the study of plant pathogen populations (Benali et al., 2011).

The versatility of microsatellites can be attributed to the fact that they are ubiquitous, relatively abundant, co-dominant, and exhibit high levels of polymorphisms (Bhargava & Fuentes, 2009; Ellegren, 2004; Selkoe & Toonen, 2006). These characteristics give properly designed microsatellite marker sets sufficient statistical power and resolution for discriminating between closely related genotypes (Oliveira et al., 2006). Therefore, microsatellites can be powerful tools in addressing important problems in plant pathology such as identifying sources of primary inoculum, determining spatial and temporal patterns of genotypes, providing evidence for sexual or asexual recombination, tracking the dispersal of inoculum, and examining the evolution of virulence, host range and pesticide resistance (Milgroom & Peever, 2003).

Mutational Mechanisms

Although microsatellites have gained wide acceptance as an effective marker for use in population genetics, the mechanisms behind microsatellite mutations are still not well understood. Two major aspects of microsatellites need to be considered in a mutation mechanism model. One is that mutation rates for microsatellite regions are very high compared to the rates of mutation in coding regions (Bhargava & Fuentes, 2010; Fan & Chu, 2007). The other is that microsatellite alleles vary in the number of repeat units, indicating that mutations involve groups of nucleotides that are added or subtracted from the repeat array (Bell & Jurka, 1997). Several potential mechanisms have been proposed to explain the mutation process in microsatellite regions (Fan & Chu, 2007) and these are discussed in the following sections.

Unequal crossing over during meiosis is a well-known mechanism that can generate large scale mutational changes (Brown, 2002). This process has been proposed as a possible explanation for expansions and contractions in repeat arrays (Bhargava & Fuentes, 2009; Treco & Arnheim, 1986). However, since recombination involves the exchange of units between different chromosomes, it is unlikely to play a primary role in microsatellite expansion and contraction (Bhargava & Fuentes, 2009; Fan & Chu, 2007). It is still possible that this mechanism plays a role in some large scale changes and multistep mutations in microsatellites (Fan & Chu, 2007).

DNA replication slippage, also known as polymerase slippage or slipped strand mispairing, is widely accepted as the main mechanism for microsatellite mutation (Ellegren, 2004; Fan & Chu, 2007; Selkoe & Toonen, 2006). During DNA replication, the polymerase and the nascent strand may become temporarily disassociated from the template strand (Fig. 4.1B).

When the strands re-associate they can become mispaired because the repeat units can readily pair with the wrong repeat units on the template strand (Fig. 4.1C). If this happens, the DNA strand is forced to loop out at the mismatched sites. When DNA synthesis continues, the number of repeat units will be altered, increasing in number if the loop is on the nascent strand and decreasing in number if the loop is on the template strand (Fig. 4.1D). DNA replication slippage occurs at high rates *in vitro,* but *in vivo*, most loops are recognized and removed by the mismatch repair system (Schlötterer & Tautz, 1992). Therefore, the observed mutation rate depends on the rate of slippage and the efficiency of the repair system.

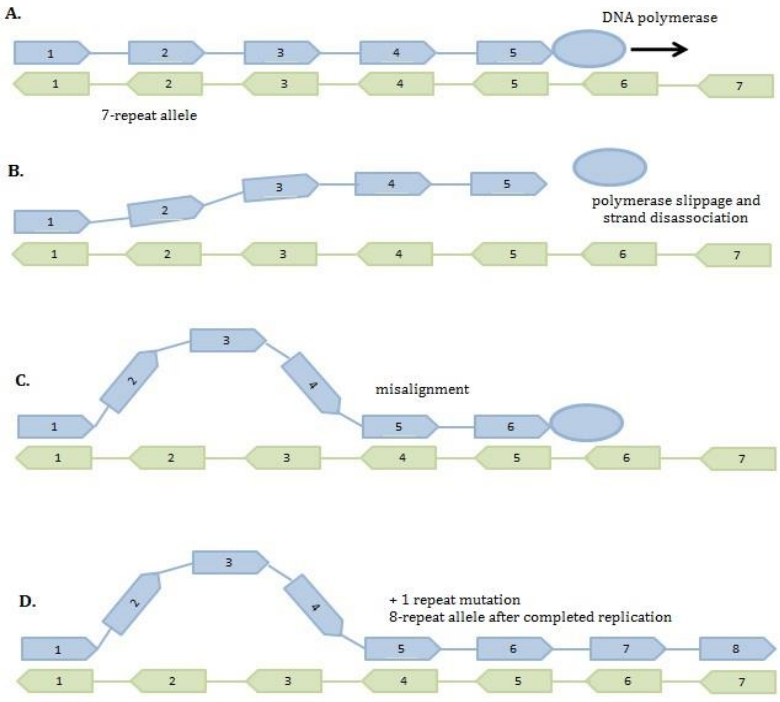

Figure 4.1 Schematic illustrating the stepwise mutational model. (A) DNA polymerase copying a section of a microsatellite locus. Nascent strand is shown in blue and template strand is shown in green. Each block represents identical repeat units of 3 to 6 base pairs. (B) Polymerase and nascent strand become disassociated and replication pauses. (C) When the nascent strand re-associates with the template strand, it can become misaligned since repeat units are identical. (D) When replication resumes, the nascent strand has one extra repeat unit compared to the original. If it had been the template strand that looped out, the newly replicated strand would be one repeat unit shorter. Figure adapted from Fan & Chu (2007).

Indel slippage was first proposed by Zhu et al. (2000) to explain patterns of microsatellite distributions that were not well supported by the mechanisms of base substitution and DNA replication slippage alone. This mechanism is based on the observation that indel-like processes tend to duplicate short flanking sequences, which creates a short microsatellite (Bhargava & Fuentes, 2009; Zhu et al., 2000). The indel slippage process is not length dependent, as is replication slippage, but is presumed to occur at a constant rate. Further support for this mechanism was provided by Dieringer and Schlötterer (2003) using computer simulations that demonstrated the need for a model that combined the mechanisms of base substitution, length dependent DNA replication slippage and a length-independent process in order to explain the observed pattern of microsatellite distribution.

It is important to recognize that the divergent patterns of microsatellite distribution among species and higher taxonomic groups implies that different rates of these processes work to shape microsatellite distributions (Bhargave & Fuentes, 2009; Dieringer & Schlötterer, 2003). In addition, the rates of all these processes are species dependent and also vary according to repeat unit size and type (Dieringer & Schlötterer, 2003; Ellegren, 2004; Fan & Chu, 2007; Katti et al., 2001). It is also likely that other factors that have yet to be described are involved. However, despite these other factors that may contribute to microsatellite distribution patterns, it is still widely accepted that replication slippage is the primary mechanism involved in microsatellite mutation (Ellegren, 2004; Fan & Chu, 2007; Selkoe & Toonen, 2006).

Mutation Models

In order to determine genetic distance between groups of individuals using microsatellite allele frequency data, a mutational model is needed. Several mutational models have been proposed to describe the evolutionary dynamics of microsatellites (Fan & Chu, 2007; Selkoe & Toonen, 2006). The original model that was applied to microsatellites was the stepwise mutational model (SMM; Ohta & Kimura, 1973). The SMM describes microsatellite mutation as occurring one repeat unit at a time with an equal probability of an increase or decrease (Fig. 4.2A). This model assumes independence of the rate and size, with no limit to allele size. The SMM is in agreement with the primary mutational mechanism of replication slippage. (Ellegren, 2004; Fan & Chu, 2007; Selkoe & Toonen, 2006).

Several observations have led to the conclusion that a simple SMM is inadequate to fully explain the distribution of microsatellite alleles. For example, microsatellites seem to show an upper limit on allele size (Naula & Weissing, 1996; Stefanini & Feldman, 2000). Additionally, mutational changes that involve more than one repeat unit are possible (Bhargava & Fuentes, 2009; Di Rienzo et al, 1994; Ellegren, 2004) and would still be consistent with the replication slippage mechanism. Therefore, more complex variations of the stepwise model have been proposed to account for these features. The two-phase mutation model (TPM; Di Rienzo et al, 1994) allows for mutations that change the array length by more than one repeat unit (Fig. 4.2B). There is still an equal probability of expansion or contraction, but steps larger than one repeat unit may occur at some frequency. Another modification of the standard SMM involves the tendency for there to be an upper limit on allele size. In this model, the mutation rates for long alleles are biased towards the contraction of allele size (Fig. 4.2C). The same type of

modification can be made for short alleles since short alleles tend towards expansion (Fig. 4.2D). Other more complex models have been proposed and tested against microsatellite distributions in genomic datasets (Dieringer & Schlotterer, 2017; Ellegren, 2004; Kruglyak et al., 1998; Renwick et al., 2001). Despite their fitting the data better than the SMM, these models still have difficulty with distance comparisons (Calabrese et al., 2001; Ellegren, 2004). Thus microsatellites are not the preferred marker for studying phylogenetic relationships (Estoup et al., 2002).

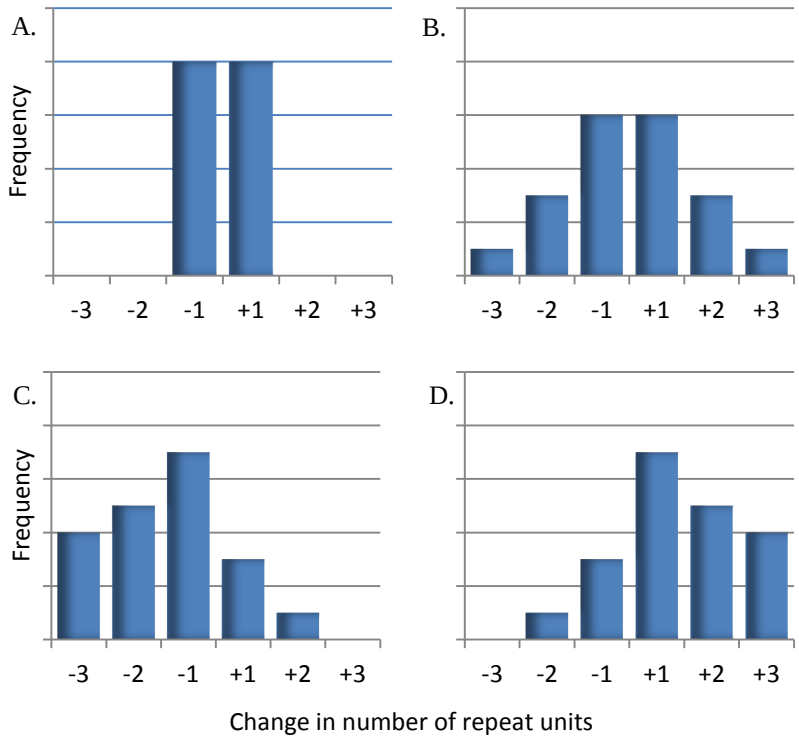

Figure 4.2 Multistep mutational models for microsatellite loci. (A) In the strict stepwise mutational model, there is an equal probability for the addition or subtraction of 1 repeat unit. (B) The two-phase model allows for mutations of more than 1 step to occur at some frequency but with expansion or contraction occurring with equal probability. Variations of the two-phase model can take into account that (C) long microsatellite alleles have a tendency to contract in size and (D) short alleles have a tendency to expand.

Microsatellite Applications

Microsatellites have become a popular marker for population studies in recent years due to properties such as being relatively abundant, codominant, highly polymorphic and selectively neutral (Fan & Chu, 2007). Because microsatellites are DNA based and small in size, they are capable of being utilized even in degraded and challenging samples such as mountain lion feces (Butler, 2007; Ernest et al., 2000) and human forensic practices (Su et al., 2016). Another valuable characteristic of microsatellites is their substantial resolving power. Because of the relative instability of the molecular structure of microsatellites, they have a high mutation rate (Ellegren, 2004; Fan & Chu, 2007; Selkoe & Toonen, 2006). This allows researchers to discriminate amongst closely related genotypes and can provide answers to fine-scale ecological questions (Selkoe & Toonen, 2006).

A common application for microsatellites is the determination of population structure (Moges et al., 2016). Properly designed microsatellite panels are discriminating enough to detect differentiation even in very closely related populations (Balloux & Lugon-Moulin, 2002). For example, Coupat-Goutland et al. (2016) used microsatellites to examine genetic variation in *Naegleria fowleri*, an amoeboic human pathogen, and stated that the six microsatellite markers they used provided a level of discrimination better than any marker to that point. Wang and Chilvers (2016) were able to identify genetic diversity among a population of *Fusarium virguliforme* that previously were thought to be genetically identical using standard phylogenetic markers such as ribosomal RNA internal transcribed spacer (ITS) and transcription elongation factor 1 alpha (EF-1α). Thus microsatellites can be an effective tool for studying very closely related populations.

Other researchers have used the resolution that microsatellites can provide to examine gene flow among geographically distant populations (Moges et al., 2016; dos Santos Pereira, 2017). A microsatellite panel with a sufficient number of markers should be sensitive enough to detect even very low levels of gene flow (Selkoe & Toonen, 2006). Another area of ecological interest is to connect pathogen populations to a particular host (Biasi et al., 2016). Again, this may require a marker that can differentiate between very closely related genotypes. These and other population genetics problems are important to understanding the evolutionary processes in response to pressures exerted by management practices and changing distribution patterns (Milgroom & Peever, 2003).

Drawbacks to Microsatellite Markers

Despite the utility of microsatellites, they do have some challenges and drawbacks that can complicate data analysis and limit their utility (DeWoody et al., 2006; Selkoe & Toonen, 2006). However, their ability to address important ecological questions greatly outweighs their drawbacks and makes the effort to develop a suitable marker set worthwhile. With careful selection of loci during the validation process and awareness of potential issues during the analysis process, the complications can be minimized or even avoided altogether (Selkoe & Toonen, 2006). It is, therefore, important to understand the limitations and potential problems associated with microsatellites in order to reduce challenges in data analysis and minimize the possibility of reaching flawed conclusions.

Narrow Taxonomic Range

One of the major issues in developing a set of microsatellite markers is identifying primer sequences that can amplify the selected loci over the entire range of taxa studied

(Selkoe & Toonen, 2006). Unlike single gene markers where primers are usually located in highly conserved regions, microsatellites are more abundant in non-coding regions which tend to be highly variable (Sharopova, 2008; Katti et al., 2001). Because primers need to be located in the regions that flank the repetitive sequences, prior knowledge of the sequences in these flanking regions is necessary. Algorithms have been designed that can search genomic sequences for repetitive regions (Leclercq et al., 2007) and so suitable repeat sequences and associated flanking regions can be identified from whole genome sequence data. However, genetically distinct individuals within the same taxa may have variation within these flanking regions and primers designed for one individual may not work for other individuals. This would necessitate identifying and testing significantly more loci than required with the expectation that many of them will not work across all individuals within the considered taxa. Alternately, multiple individuals representing the range of genetic diversity in the taxa could be sequenced and evaluated for suitable loci and only those loci with consistency in the target region would be selected and tested against a larger set of individuals. This would potentially reduce the number of loci that need to be screened but would require a larger investment up front in sequencing and assembling multiple genomes (Biasi et al., 2015). For these reasons, microsatellite markers rarely work across broad taxonomic groups (Selkoe & Toonen, 2006).

Hidden Allelic Diversity

Microsatellites are typically scored by means of size-based identification which reduces the time and expense of genotyping compared to sequencing each allele in each individual (Flores-Renteria & Krohn, 2013). However, not all genetic variation can be detected using this method. Alleles can be the same length but have a different evolutionary history and sequence

variations that are not detectable by size-based identification alone (Estoup et al., 2002). This phenomenon, referred to as 'size homoplasy', can be quite common and may result in an underestimation of allelic diversity (Taylor et al., 1999).

Scoring microsatellite alleles by length relies on the assumption that all unique alleles differ in length and that length is a factor of the number of repeat units (Selkoe & Toonen, 2006). However, alleles can vary in ways other than the number of repeat units. These occurrences of size homoplasy can either be 'detectable' or 'undetectable' (Flores-Renteria & Krohn, Selkoe & Toonen, 2006). Detectable homoplasy is when two fragments are identical in length but are not identical in sequence. For example, a point mutation might represent genetic diversity that would not be revealed by size detection. Similarly, insertions or deletions in the flanking regions may create an allele of a different size without changing the number of repeat units. Detectable homoplasy can be discovered by sequencing alleles.

Undetectable homoplasy occurs when two alleles are identical in sequence but have different genealogical histories (Selkoe & Toonen, 2006). The step-wise mutational process can both add and subtract repeat units and can result in convergence in size (Garza & Freimer, 1996). For instance, consider two copies of a locus that are identical by descent. If copy A undergoes a loss of one repeat unit and then a gain of two units while copy B a gain of two repeat units and then a loss of one unit, they would again be identical in length. Thus these alleles would appear identical, even by sequencing, but would be separated by four mutational steps. (Fig. 4.3).

In general, homoplasy for size has a minimal effect on population studies involving groups with shallow evolutionary histories (Adams et al., 2004; Selkoe & Toonen, 2006). The

degree of homoplasy increases with the rate of mutation and time of divergence (Adams et al., 2004). In other words, the chance of homoplasy increases with genetic distance. Because of this and other issues discussed in this section, microsatellites are generally unsuitable for more distantly related taxa (Estoup et al., 1995). However, the appropriate level of relatedness can differ by organism based on the varying rate of mutation.

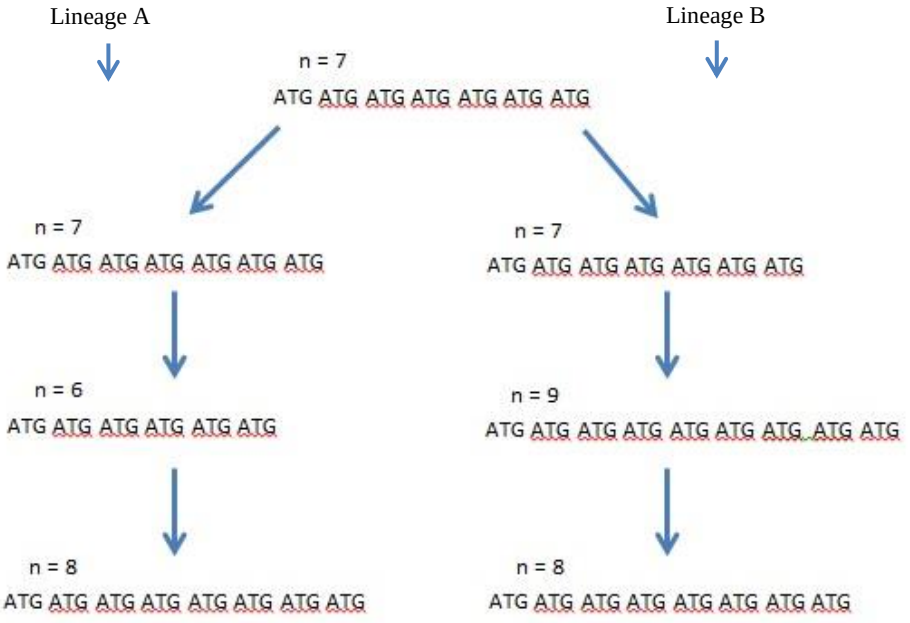

Figure 4.3 Schematic illustrating convergent evolution of microsatellites. Lineage A and lineage B start out identical by descent. Lineage A experiences a mutation event and loses 1 repeat unit but later gains 2 repeat units through another mutational event. In contrast, the mutational history of lineage B involves first a gain of 2 repeat units and then a loss of 1. Both alleles end up with a length of 8 repeat units and identical nucleotide sequences. This type of homoplasy would be considered 'undetectable' since length based detection and sequencing would indicate these alleles were identical but they would actually be separated by 4 mutations. Figure adapted from Garza & Freimer (1996).

Null Alleles

A null allele is any allele at a microsatellite locus that fails to amplify to detectable levels during polymerase chain reaction (PCR) (Dakin & Avise, 2004). Null alleles pose an important and persistent challenge for population geneticists. Because they fail to produce a visible product, null alleles are particularly difficult to detect.

There are at least three potential causes for the occurrence of null alleles: (1) Mutations in the priming regions, (2) inconsistent or poor DNA template quality and (3) large allele dropout due to the competitive nature of PCR (Dakin & Avise, 2004). Mutations in the priming regions can cause primers to bind inefficiently or not at all, particularly when they are near the 3' terminus where extension begins (Kwok et al., 1990). Redesigning the primers with degenerate bases at the sites of mutation can for allow amplification of alleles that would not amplify with more stringent primers (Pemberton et al., 1995; Selkoe & Toonen, 2006). However, this approach requires knowledge of the specific mutations present which is not often plausible. Alternatively, adjusting PCR conditions can often improve the amplification success of recalcitrant loci (Selkoe & Toonen, 2006).

Problems with template quality can be troublesome to detect because all loci may not be affected equally (Dakin & Avise, 2004; DeWoody et al., 2006). Therefore, it is advisable to start with the highest quality DNA template possible to provide the best chance of avoiding difficulties (Selkoe & Toonen, 2006). Re-extracting DNA from the sample in question is advisable for those loci that failed to amplify any product.

Large allele dropout is a consequence of the competitive nature of PCR which can cause small alleles to amplify more efficiently than larger one (Wattier et al., 1998). In cases where

there are large size differences between alleles in an individual, only the smaller allele might be detected from a heterozygous individual. This is sometimes referred to as 'partial null' because detection can often be made possible by loading more sample or altering primer concentration (Dakin & Avise, 2004).

In addition to these primary causes of null alleles, there are several population genetic phenomena that can give the false impression that null alleles are present in a population. Certain biological factors, such as inbreeding, can cause a deficit in heterozygotes that might be interpreted as an indication that null alleles are present (Chakraborty et al, 1992). Proper multilocus analysis should be able to distinguish this type of problem since population genetic factors should register more or less consistently across all loci. Another possible source of erroneous evidence for null alleles is sex linkage (Dakin & Avise, 2004). In diploid organisms, sex chromosomes carry only one allele at a locus and can result in the identification of a locus having a heterozygous deficit. Gender-specific analysis may be needed to identify sex linked loci and eliminate them as a potential source of error.

Mitigating Scoring Errors

Scoring errors can have a substantial impact on downstream analysis and it is important to take precautions to mitigate or minimize the effects of potential scoring errors. The three types of scoring errors that are of primary concern are stuttering, large allele dropout and null alleles. These errors tend to create consistent scoring bias and can affect data interpretation (DeWoody et al., 2006).

Re-amplification and rescoring of samples provides the opportunity to identify and quantify scoring errors (Dakin & Avise, 2004; DeWoody et al., 2006). This approach is

particularly important during the development of the marker set and an estimate of the error rate for each locus should be determined and reported. It may be necessary to abandon loci with excessive error rates. Once a marker set has been developed it is recommended that, given high quality DNA template, a random 10% of samples are reanalyzed at all loci (DeWoody et al., 2006). Loci with questionable peak patterns or samples with low quality DNA template may require additional resampling.

Detecting scoring errors in microsatellite datasets typically relies on testing for heterozygote deficiencies using software such as Microchecker (DeWoody et al., 2006; van Oosterhout et al., 2006). Microchecker tests the microsatellite data for Hardy-Weinberg equilibrium and uses the presence of excess homozygotes to estimate the incidence of large allele dropout and null alleles. Loci with problems in these areas should be re-evaluated or eliminated.

Several programs have been developed for the scoring of microsatellite data. It is recommended to use automatic allele calling to provide consistency and efficiency (DeWoody et al., 2006). Microsatellite scoring systems typically rely on binning, which creates a range of values which, when a peak falls within that range, it is assigned the allele size of that bin. This allows some flexibility in peak sizes since the peak values will not always match allele sizes exactly. In addition to automatic allele calling, each sample should be visually inspected to identify any novel alleles, potential mistypes or other problematic patterns. This combination of automatic scoring and visual inspection provides a suitable balance between efficiency and accuracy.

Objectives

Rhizoctonia solani AG2-2 is a genetically diverse plant pathogen that causes disease on a number of economically important crops (Ogoshi, 1987; Sneh et al., 1991). In the current study, the intent was to develop a set of microsatellite markers to use for the analysis of *R. solani* AG2-2 populations. We have identified a set of potential markers through *in silico* analysis and tested them for suitability for use *in vitro*. Thirteen potential markers were fluorescently labeled and multiplexed for automatic allele sizing. Markers were evaluated for scoring errors, polymorphism information content, and genotypic diversity using 23 isolates of *R. solani* AG2-2 to verify suitability of selected loci for use in population studies

Methods

In-Silico Identification and Evaluation of Potential Loci

Potential microsatellite loci were identified by Frank Martin (USDA-ARS, Salinas, CA.) using an *in-silico* approach. Briefly, one isolate was selected from each of the three genetic groups within *R. solani* AG2-2 that were described by Martin et al. (2014). Rs850 was arbitrarily selected from group 1, Rs866 from group 2A and Rs588 from group 2B. These three isolates were sequenced on a HiSeq4000 (Illumina Inc., San Diego, CA, USA) and raw sequences assembled using CLC Genomic Workbench (Qiagen, Redwood City, CA, USA). Since isolate Rs850 would be used as the initial sequence for marker selection, additional effort was used on this assembly. The assembly was filtered to discard sequences with less than 15x coverage. The remaining contigs were exported to SeqMan NGen (DNASTAR, Inc., Madison, WI, USA) and

assembled *de novo*. Contigs were imported back into CLC Genomic Workbench and a second *de novo* assembly was performed.

Initial design of markers was done with isolate Rs850 using BatchPrimer3 (You et al., 2008). Search parameters were set to not search for dinucleotide repeats and to limit fragment size to between 100 and 250 bp. One locus was chosen for each contig of isolate Rs850 and evaluated for suitability. The assemblies of the other two isolates were checked to determine if there were differences in the number of repeats, no indels in the flanking regions and primer design was appropriate for all isolates. If there was a problem with one of the isolates, the locus was discarded and the next marker on the contig was evaluated until a suitable marker was identified.

DNA Extraction

Twenty three *Rhizoctoni solani* AG2-2 isolates (Table 4.1) representative of the three genetic clades as determined by Martin et al. (2014) were used to evaluate the *in silico* selected markers. Isolates were grown on malt extract broth (MEB; Sigma-Aldrich, St. Louis, MO USA) without shaking for 5 to 7 days. The mycelial mat was harvested using forceps, placed in a sterile 50 ml centrifuge tube, and rinsed with sterile distilled water. Tissue was lyophilized in a freeze drier (VirTis Genesis; SP Scientific; Warminster, PA) and ground in a modified paint shaker using 6 mm ceramic grinding beads (Zircoa, Inc.; Solon, OH).

Table 4.1 Twenty-three isolates of *Rhizoctonia solani* AG2-2 used in the current study. Phylogenetic group is according Martin et al. (2014). Multilocus genotype was determined from 13 microsatellite loci using Genotype version 1.2 (Meirmans & Van Teinderen, 2004).

Isolate name	Phylogenetic group	Origin	Multi-locus Genotype
Rs850 *	Undet.	Minnesota, USA	1
Rs866 *	2A	Minnesota, USA	2
Rs588 *	2B	Minnesota, USA	3
R09-23	1	Michigan. USA	4
Rs1146	2B	Minnesota, USA	4
F36	1	Oregon, USA	4
R09-2	1	Michigan, USA	5
2C13	Undet.	Montana, USA	5
Rs1012	1	Minnesota, USA	6
F517	1	Idaho, USA	7
Cavalie	1	Europe	8
Roland	1	Europe	9
39AR	1	Canada	10
24BR	1	Canada	11
R-9	2A	Colorado, USA	12
F521	2A	Idaho, USA	13
R-1	2A	Colorado, USA	14
W-22	2A	Wisconson, USA	15
F508	2A	Idaho, USA	16
Italian	2A	Europe	17
RH193	2B	Japan	18
Rs481	2B	Minnesota, USA	19
R164S	2B	Japan	20

* Initial isolates sequenced for preliminary marker identification

Total DNA was extracted using the OmniPrep for Fungus kit (G-Biosciences; St. Louis, MO) according to manufacturer's instructions with the following modifications. In a 2ml microcentrifuge tube, 20 to 25mg of ground, lyophilized tissue was added to 550µl of genomic lysis buffer and 5µl Proteinase K. The mixture was incubated at 65˚C for 1 hour and then extracted with 500µl chloroform. Samples were treated with 5µl RNase A for 30 min. at room temperature and extracted a second time with 500µl chloroform. Fifty microliters of DNA stripping solution was added and samples were incubated at 65˚C for 10 minutes. After samples

cooled to room temperature, 150µl of precipitation solution was added and samples incubated on ice for 10 to 15 min. Precipitate was pelletized by centrifugation (12,000 x g for 5 min.) and the supernatant transferred to a clean tube. DNA was precipitated with 500µl isopropanol and pelletized by centrifugation (12,000 x g for 5 min.). The supernatant was poured off and the DNA pellet dissolved in 450µl ddH_2O. At this point, the solution contained DNA and an unknown contaminant (possibly a charged polysaccharide, unpublished data) that was removed by precipitating with 100µl of 100% ethanol and incubation on ice for 15 min. The contaminant was collected by centrifugation at 2,500 x g for 2 min. The low speed was used to minimize the amount of DNA drawn out of solution in this step (unpublished data). The supernatant was poured into a clean tube and the DNA precipitated using 45µl 3M sodium acetate and an additional 900µl of 100% ethanol. The pellet was washed twice with 70% ethanol, air dried and dissolved in 50µl Tris-EDTA buffer (doi:10.1101/pdb.rec11661Cold Spring Harb Protoc 2009). DNA quality was evaluated using a spectrophotometer (Nanodrop ND-8000; Thermo Fisher; Waltham, MA) and DNA concentration measured using a fluorometer (Qubit 4; Thermo Fisher; Waltham, MA). DNA concentration was standardized to 15ng/µl using ddH_2O.

PCR Amplification and Marker Evaluation

In order to reduce error from stuttering, Phusion II High-Fidelity polymerase (Thermo Fisher, Waltham, MA USA) was used for all PCR amplifications (Fazekas et al., 2010). Initially primer pairs were grouped by annealing temperature (T_a) as calculated by Thermo Fisher's Tm calculator (https://www.thermofisher.com). Thirty three primer pairs were categorized into four T_a groups of 56˚, 59˚, 61˚ and 67˚C containing five, twelve, fifteen and one pair(s) respectively. To determine optimum amplification conditions, DNA from isolates Rs850, Rs866

and Rs588 was amplified with the primer pairs that had a T_a of 59˚ or 61˚C using four $MgCl_2$ concentrations. Reactions were performed in a total volume of 20µl containing 15ng of DNA template, 1 x Phusion II HF buffer, 0.2mM dNTPs, 0.5mM of each primer and 1 unit of Phusion II HF polymerase with total $MgCl_2$ concentrations of 1.5mM, 2.0mM, 2.5mM or 3.0mM. Amplification conditions consisted of 1 cycle of 98˚C for 2 min. followed by 25 cycles of 98˚C for 15 s, 59˚ or 61˚C for 20 s, 72˚C for 12 s and a final extension cycle of 72˚C for 5 min. PCR products were separated on 4% agarose gels including ethidium bromide (.5µg / ml) in 1 x Tris-acetate buffer (Thermo Fisher; Waltham, MA) and visualized with UV light.

No noticeable differences in amplification quality were observed between the various $MgCl_2$ concentrations; therefore, a total concentration of 2.5mM $MgCl_2$ was used for all subsequent reactions. A single annealing temperature of 58˚C was used in subsequent reactions regardless of whether the primer pair was in the 59˚ or 61˚C category. Twenty four primer pairs were initially tested on 9 isolates using reaction conditions noted above and were evaluated for amplification across all isolates, band intensity, noticeable size differences between isolates, and suitability for multiplexing. Sixteen loci that amplified for all nine isolates and had observable polymorphisms were selected and fluorescently labeled for automatic fragment sizing (Table 4.2). Forward primers were labeled with either 6-FAM or HEX fluorescent dyes (Integrated DNA Technologies, Coralville, IA USA) for use in duplex analysis (Table 4.2). Reverse primers were evaluated with and without a 5’ GTTT- PIG tail (Brownstein et al., 1996) to evaluate incidence of stutter peaks. Amplification of fluorescently labeled primers was conducted as described above.

Table 4.2 Microsatellite loci evaluated in the current study for use on *Rhizoctonia solani* AG2-2. The 5' end of the forward primers were labeled with either HEX or 6-FAM fluorophores for automatic sizing. Loci with the same letter in parenthesis were run in duplex reactions. Amplicon length indicates the range of fragment lengths across all 13 loci. Primer concentration was adjusted to the values in the 'conc. μM' column in order to yield similar fluorescent peak levels in multiplexed reactions.

Locus	Repeat motif	Amplicon length	Dye	Primer sequences 5' → 3'		conc μM
				Forward	Reverse	
5402 (a)	TCG	138-156	HEX	CCATACGCTCATACTTGAGAC	CGTAGACGAAAGTGGAMRTAG	.30
7420 (a)	CGA	170-176	6-FAM	TATCARGCAAACTTRACCAAT	AGACCACTCTACGAACCTTGY	.20
759 (b)	CAG	131-170	6-FAM	CAACAGCACGCCMTYATG	CAGAGGGYAATTGTTGTTGAA	.35
2893 (c)	GGTGTT	119-143	HEX	CAGCTGGYGTAGTAGAAGTGG	GAATCRACRCCRGCAGTAGA	.45
8224 (c)	CAAA	186-190	6-FAM	CCAAGACTCCGCTCATTG	CTATCTATCACTCGTTCCGC	.20
6150 (d)	TTTC	130-158	HEX	TGATATCACCACATTCTTTSA	CRATTGACGGTCTACTGTTGY	.25
5583 (d)	AGA	183-198	6-FAM	CGTCGAGGATCTCAAATATGT	TTGCTAATGGTTCCTTTACTG	.10
6145 (e)	CAG	146-158	HEX	ATGCAGATGGTTTTGTACG	CTAGAGATCGATGCTGTGTCT	.30
4660 (f)	CGA	132-159	HEX	GTRATGGTGAGAGTGAGAGAA	CTCSTCGTCTGAAGAGTCATA	.45
8703 (f)	GTT	201-216	6-FAM	TGRGGTGGKGGATGTATTG	TCTCGGTCRAGTTACAATGG	.20
5487 (g)	ACG	132-141	HEX	ATACCGAGAGTGTCTTTACSC	AAAACGACTGGGGAGGAA	.30
5877 (g)	GTG	226-232	6-FAM	TACTTTGTACTCCCCGACG	TTTGTCGTAACTTGGCTACA	.35
2547 (h)	AACA	214-222	6-FAM	AATCRCTCGAATCGGTAATT	ATCGGGAATCATACTACCGG	.10

Fluorescently labeled PCR products were evaluated for isolates Rs850, Rs588 and Rs866 using a genetic analyzer (Applied Biosystems 3130; Applied Biosystems; Foster City, CA). Samples were cleaned on gel filtration columns (Sephadex G-50 superfine; GE Healthcare Life Sciences; Pittsburg, PA) and diluted 1:40, 1:60 or 1:80 with sterile distilled water before submission. Analysis was performed by the Michigan State University Genomics Core (East Lansing, MI USA) according to the manufacturer's instructions with GeneScan 400HD-ROX (Applied Biosystems, Foster City, CA) used as the size standard. Several runs were conducted and primer concentrations adjusted to provide similar levels of fluorescent signal for all loci (Table 4.2). Loci were evaluated for fragment length patterns inconsistent with repeat unit length, stutter peaks, failure to amplify, and overlap in allele sizes of the duplexed loci. Three

loci had allele patterns inconsistent with repeat unit and were eliminated from further consideration.

The remaining 13 markers were tested on a total of 23 isolates (including the nine original isolates referenced above) in two runs of 12 isolates with isolate Rs850 used as a positive control in each run. Final reactions were performed in 20µl volumes with 15ng of DNA template, 1 x Phusion II HF buffer, 0.2mM dNTPs, 2.5mM total $MgCl_2$ and 1 unit of Phusion II HF polymerase. Primer concentrations were as shown in Table 4.2. PIG-tailed reverse primers were unnecessary for reducing stuttering and unmodified reverse primers were used. Final PCR conditions consisted of 1 cycle of 98˚C for 2 min. followed by 27 cycles of 98˚C for 20 s, 57˚C for 20 s, 72˚C for 12 s and a final extension cycle of 72˚C for 5 min. PCR products were diluted 1:50 before submission to the MSU Genomics Core for analysis.

Data Analysis

Chromatograms were analyzed using Geneious 9.0.2 microsatellite plugin 1.4.4 (Biomatters, Inc.; Newark, NJ). Peaks were called using the Third Order Least Squares sizing algorithm. The first 12 isolates screened were used to predict bin sizes. Additional bins were added when needed as further samples were processed. Allelic data was analyzed for scoring errors using Micro-Checker (Van Oosterhout et al., 2004). Scoring errors evaluated included homozygote excess, errors due to stuttering, large allele dropout and possible null alleles. Allelic diversity was evaluated using MSAnalyzer 4.05 (Dieringer & Schlotterer, 2003).

Simpson's Diversity Index (D; Simpson, 1949) was calculated using the formula:

Equation 4.1 $$D = \sum_{i}^{R} {p_i}^2$$

where *R* is the total number of alleles in the dataset and p_i is the proportional abundance of the *i-th* allele.

Genepop 4.5.1 (Rousset, 2008) was used for genotypic and genic differentiation tests. The default settings were used for the Markov chain parameters in both analyses. Multi-locus genotypes and pairwise distances were determined with GenoType 1.2 (Meirmans & Van Tienderen, 2004) using the stepwise mutation model with missing data counted as one mutational step. Relationships of the isolates were inferred from pairwise distances using the neighbor-joining method (Saitou & Nei, 1987). Phylogenetic analyses were conducted in Mega 6.0 (Tamura, et al., 2013).

Results

In-Silico Identification of Potential Loci

Whole genome sequencing of isolate Rs850 yielded 236 million reads that were assembled into 13,926 contigs over 1kb in length with a N50 value of 15.9. De novo assembly and clean up in SeqMan NGen (DNAStar; Madison, WI) and CLC Workbench (Qiagen; Redwood City, CA) improved assembly quality by reducing the number of contigs to 13,792 with a N50 value of 16.6 kb. Isolates Rs866 and Rs588 yielded 23,128 and 18,179 contigs respectively (Table 4.3).

Thirty three potential maker loci were identified using the BatchPrimer3 software (You et al., 2008) to analyze the genomes of isolates Rs850, Rs688 and Rs588. The most abundant microsatellites were trinucleotide (18), followed by tetra- (8), hexa- (6) and penta- (1). The number of repeat units for each locus for isolate Rs850 varied from 5 to 11 and the predicted fragment length varied from 104 to 244 bp. Primers were between 18 and 22 bp in length.

Table 4.3 Results of NextGen sequencing and assembly for three isolates of *Rhizoctonia solani* AG2-2. Isolates were sequenced on a HiSeq4000 and assembled using CLC Workbench. Additional effort was used on isolate Rs850 and contigs were filtered and *de novo* assembled using SeqMan NGen before an additional *de novo* assembly using CLC Workbench. Data is shown for isolates Rs850 before the additional assembly (raw) and after (final).

Isolate	# reads	# contigs	N50 (bp)	Avg. length	% identity
Rs850 (raw)	236 million	13,926	15.9 kb	7 kb	96%
Rs850 (final)	-	13,792	16.6 kb	-	96%
Rs866	245.5 million	23,128	6.1 kb	4.1 kb	96%
Rs588	265 million	18,179	4.2 kb	3.1 kb	96%

PCR Amplification and Marker Evaluation

Of the 33 potential markers initially identified *in silico*, six were omitted from further consideration because including them would have resulted in more than a 5˚C difference in annealing temperature when run in a single PCR reaction. Another eight loci were eliminated because they failed to amplify or had weak amplification for one or more of the three initial isolates tested. Three loci had fragment sizes for isolate Rs850 that were inconsistent with predicted product size and were not tested further. Three additional markers were eliminated after automatic sizing analysis because they had allele patterns inconsistent with repeat unit. The remaining 13 markers and the primer concentrations used in final PCR reactions are shown in Table 4.2.

Stutter was only a minor issue in a couple of loci with stutter peaks generally 1 bp shorter than the actual allele with a peak intensity of less than 10% of the main peak. By slightly increasing the peak threshold for allele calling, false allele calls were minimized. PIG-tailed reverse primers showed no improvement over standard primers in reducing stutter peaks and were not used in the final analysis.

Data Analysis

Genotyping of the 23 *R. solani* AG2-2 isolates using the selected 13 microsatellite markers confirmed a high level of diversity with a total of 20 multi-locus genotypes identified (Table 4.1). The number of genotypes at each marker ranged from 3 to 11 and 10 of the 13 loci (77%) had five or more unique genotypes (Table 4.4). Polymorphism information content (PIC; Anderson et al., 1993) ranged from 0.332 to 0.794 with an average value of 0.618. Ten (77%) loci had a PIC value greater than 0.50 which indicates those loci were highly informative (Anderson et al., 1993). A total of 61 alleles were detected, of which 16 (27%) were rare, having a frequency of less than 5%. The number of alleles per locus varied from 3 to 10 with an average of 4.7 alleles per locus. The total number of alleles for each isolate varied from 13 to 24 with an average of 19 alleles per isolate (Table 4.4).

Significant deviations from Hardy-Weinberg equilibrium were found in four loci (8224, 5487, 2893 and 5877) indicating a deficiency of heterozygotes and the potential presence of null alleles. The calculated null allele frequency for each locus is listed in Table 4.5. There was no evidence of large allele dropout in any loci (Table 4.5).

Table 4.4 Microsatellite alleles detected in 23 *Rhizoctonia solani* AG2-2 isolates. Group designation is according to Martin et al. (2014). Allelic diversity was determined using MSAnalyzer 4.0.5.

		Allele Size by Locus													
	Isolate	**759**	**8224**	**2893**	**8703**	**4660**	**7420**	**5402**	**5877**	**5487**	**6145**	**5583**	**6150**	**2547**	**N_a**
Group 1	Rs850	131/167	186/190	119/137	207/213	144/150	170/173	144/147	226	135	149/152	183/198	134	218	22
	Roland	131/140	186/190	131	207/213	144/150	170	144	229	135/138	149	183/189	138	214/218	20
	24BR	131/140	186/190	119/125	207/210	144/156	170	144	229	135/138	149/158	183/189	134/138	218/222	23
	39AR	131/140	186/190	119/131	207/213	144/150	170	144	226/229	135/138	149/158	183/189	134/138	214/218	24
	Rs1012	131/140	190	125	204/216	147/150	170	144/156	226	132	149	186	130/134	218/222	19
	F517	131/140	190	125/131	204/210	150/159	170	144/156	226	132/135	146/149	183/186	130/142	218/222	23
	Cavalie	131	190	125	204/216	150/159	170	144/156	-	132	146/149	186	130/134	214	17
	R09-23	131/137	190	131/143	204/216	147/150	170	144/156	226	132	149	183/186	130/138	218/222	21
	Rs1146[(a)]	131/137	190	131/143	204/216	147/150	170	144/156	226	132	149	183/186	130/138	218/222	21
	F36	131/137	190	131/143	204/216	147/150	170	144/156	226	132	149	183/186	130/138	218/222	21
	R09-2	131/137	190	131/143	204/216	147/150	170	144/156	226/232	132	149	183/186	130/138	218/222	22
	2C1	131/167	190	131/143	204/216	147/150	170	144/156	226/232	132	149	183/186	130/138	218/222	22
Group 2A	R-1	146/152	186	119	201/207	144	170/176	138/144	-	135	152/155	186	138/142	222	18
	R-9	152	186	119	201/207	144	176	141/144	226	135	152/155	186/198	138/142	222	18
	F521	152	186	119	201/207	144	176	141/144	226	135	152/155	186	138/142	222	17
	W-22	149/158	186	119	204/207	144	170	141/144	226	138/141	152/155	198	134/142	222	19
	F508	152/158	186	119	201/207	144	170/173	141/144	226	135	152/158	186/198	134/142	222	20
	Rs866	152/170	186	119	201/207	144	170/173	141/144	226	135/138	149/158	186/198	142	222	20
	Italian	152/161	186	119	201/207	144	170/176	141/144	226	135/138	149/158	186/198	142/158	222	21
Group 2B	Rs588	140	186	119	207	144	170	144	229	132	152	189	138	218	13
	R164S	140	186	119	207	144	170	144	229	135	152	189	138	218	13
	RH193	140	186	119	207	132	170	144	229	138	152	189	138	218	13
	Rs481	140	186	119	207	132	170	144	229	135	152/158	189	138	218	14
Statistics	N_a	10	2	5	6	6	3	5	3	4	5	4	5	3	
	N_G	11	3	8	7	6	4	5	4	6	7	8	10	5	
	R_a	1.81	1.50	1.65	1.76	1.70	1.34	1.54	1.50	1.65	1.71	1.73	1.73	1.59	
	H_E	.812	.502	.651	.761	.702	.339	.538	.501	.654	.706	.730	.728	.587	
	H_O	.696	.174	.391	.826	.522	.217	.696	.130	.348	.609	.609	.696	.435	
	PIC	.794	.491	.637	.745	.687	.332	.526	.489	.640	.691	.715	.712	.575	

Table 4.4 (cont'd).N_a indicates the number of alleles detected in each isolate or at each locus. N_G = number of genotypes at each locus, R_a = allelic richness, H_E = expected heterozygosity, H_O = observed heterozygotes, PIC = polymorphism information content (Anderson et al., 1993). Footnote (a) isolate Rs1146 was identified as belonging to group 2B according to Martin et al. (2014). Virulence data (Chapter 2, this thesis) and microsatellite data indicate it likely belongs to group 1. Re-examination of original sequence data is necessary.

Two loci (8224 and 5877) also showed evidence of scoring errors due to stuttering as indicated by the significant shortage of heterozygote genotypes with alleles of one repeat unit difference (Table 4.5). Chromatograms of these loci were examined visually for stuttering patterns. Locus 5877 had peaks that were only one base pair apart but the longer allele was consistently called as it had the larger peak height (Fig. 4.4). The more problematic issue for locus 5877 was that isolates R-1 and Cavalie failed to amplify any alleles. Locus 8224 had a more problematic pattern of peaks. Again, peaks within the same isolate differed in length by only one base pair, but which peak was called was inconsistent between isolates (Fig. 4.4). Bin sizes had to be set asymmetrically (-1 to +2) in order to accommodate the variation in peaks. However, this type of pattern is not indicative of true stutter, in which peaks typically differ by one repeat unit, but is more suggestive of the presence of indels. Cloning and sequencing of representative samples for affected loci will be required to confirm condition.

Pairwise distances between the 23 isolates in this study are shown in Table 4.6. The neighbor-joining tree generated from this data (Fig. 4.5) was generally consistent with the multi-gene phylogeny of Martin et al. (2014) (Fig. A4.1) with one exception (Fig. 4.5). Isolate Rs1146 was expected to cluster with group 2B isolates according to Martin et al. (2014), but was found to have a microsatellite genotype identical to two group 1 isolates (R09-23 and F36) (Table 4.4).

Although all four isolates in group 2B had unique multi-locus microsatellite genotypes, they were highly homogeneous. Except for a single isolate at one locus, all isolates were completely homozygous (Table 4.4). Only three out of thirteen loci (23%) had more than one allele with an average of 1.31 alleles per locus while group 1 and group 2A had an average of 3.62 and 2.62 alleles per locus respectively (Table 4.4). Simpson's Diversity Index (D; Simpson, 1949) measures the probability that two alleles selected at random from the sample will be identical and the value increased from 0.459 for group 1 to 0.892 for group 2B (Table 4.7). This means that, on average, two alleles selected from a locus in group 2B are roughly twice as likely to be identical as two alleles selected from the same locus in group 1.

Genotypic and genic differentiation of groups 2A and 2B indicate that the populations are highly similar with five and six loci respectively not significantly differentiated (Table 4.8 and 4.9). However, across all loci both tests returned significant differentiation between all three populations.

Table 4.5 Scoring errors for the 13 microsatellite loci evaluated in the current study. Microsatellite data was collected on 23 isolates of *Rhizoctonia solani* AG2-2 and analyzed for scoring errors using Microchecker v. 2.2.3 (van Oosterhaut et al., 2004). Confidence interval was set to 95% with 1000x simulations.

Locus	Expected Homozygotes	Observered Homozygotes	Homozygote excess	Scoring error due to stuttering	Large allele dropout	Null alleles	Null allele frequency
7420	15.369	18	no	no	no	no	0.086
5402	10.891	7	no	no	no	no	0
759	4.739	7	no	no	no	no	0.055
8224	11.695	19	yes	yes	no	yes	0.213
5487	8.282	15	yes	no	no	yes	0.178
4660	7.195	11	no	no	no	no	0.098
2547	9.782	13	no	no	no	no	0.089
2893	8.347	14	yes	no	no	yes	0.150
6145	7.108	9	no	no	no	no	0.049
6150	6.630	7	no	no	no	no	0.009
5583	6.565	9	no	no	no	no	0.062
8703	5.869	4	no	no	no	no	0
5877	10.738	18	yes	yes	no	yes	0.379

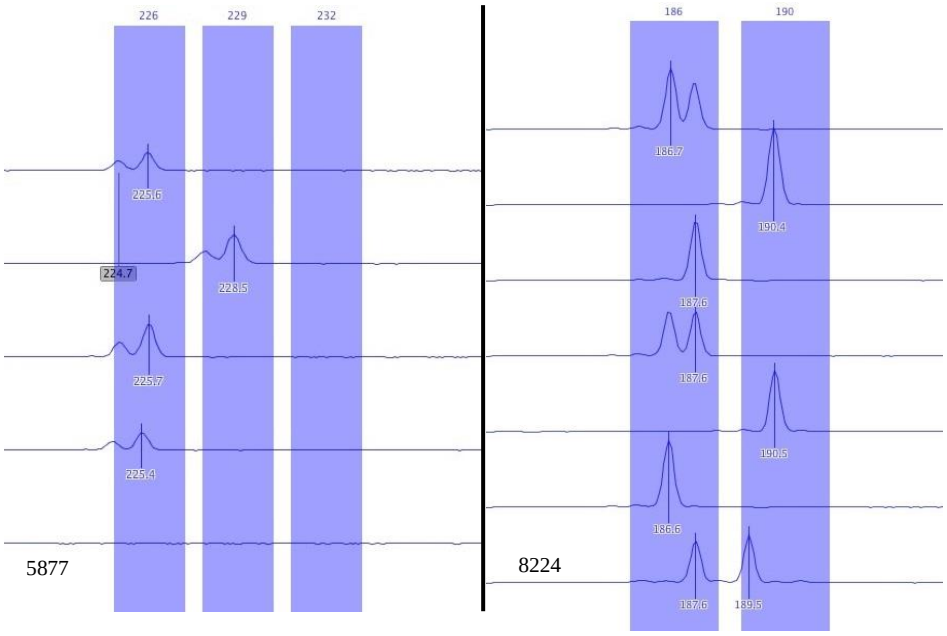

Figure 4.4 Chromatograms of locus 5877 and 8224 showing stutter-like patterns. Forward primers were labeled with either HEX or 6-FAM fluorescent dyes (both loci above were labeled with the 6-FAM dye) and amplified with PCR. Resulting microsatellite fragments were automatically sized on an Applied Biosystems 3130 genetic analyzer and chromatograms were analyzed using Genieous 9.0.2 microsatellite plugin 1.4.4. Shaded bands show the size and range of the bins used for automatic allele calling. Stutter-like peaks shown above are 1 base pair shorter (or longer) than the major peak and are not characteristic of true stutter which is typically 1 repeat unit shorter. Locus 5877 has a repeat unit of 3 nucleotides and locus 8224 has a repeat unit of 4 nucleotides. Bin range for locus 8224 was set asymmetrically (-1 to +2 bp.) to accommodate the variation in peak sizes.

Table 4.6 Pairwise distances of 23 *Rhizoctonia solani* AG2-2 isolates based on 13 microsatellite loci. Pairwise distances were determined with Genotype 1.2 using the stepwise mutational model with missing data counted as 1 step.

	F508	F36	R09-23	24BR	F517	Rs1012	39AR	Cavalie	R09-2	Roland	Rs866	R-9	R-1	W-22	F521	Italian	Rs588	Rs1146	R164S	RH193	Rs481	Rs850	2C1
F508	-																						
F36	170	-																					
R09-23	170	0	-																				
24BR	108	98	98	-																			
F517	151	49	49	75	-																		
Rs1012	144	34	34	78	41	-																	
39AR	116	88	88	26	83	80	-																
Cavalie	182	66	66	100	49	38	100	-															
R09-2	176	6	6	98	55	40	88	70	-														
Roland	144	74	74	48	81	78	28	96	74	-													
Rs866	26	190	190	122	171	164	130	202	196	150	-												
R-9	31	165	165	109	146	139	117	177	171	131	49	-											
R-1	39	155	155	101	136	129	111	163	159	123	57	20	-										
W-22	33	179	179	117	160	153	125	191	185	147	53	58	60	-									
F521	34	162	162	112	143	136	120	174	168	134	52	3	17	61	-								
Italian	36	200	200	132	181	174	140	212	206	160	28	53	61	63	56	-							
Rs588	79	131	131	57	120	105	63	131	131	77	97	72	64	88	75	107	-						
Rs1146	170	0	0	98	49	34	88	66	6	74	190	165	155	179	162	200	131	-					
R164S	82	128	128	60	117	102	66	128	128	68	100	69	61	85	72	110	9	128	-				
RH193	112	158	158	84	147	132	90	158	158	92	124	99	91	103	102	134	39	158	30	-			
Rs481	100	158	158	78	147	132	84	158	158	98	118	93	85	109	96	128	27	158	30	12	-		
Rs850	104	106	106	86	113	102	68	132	112	84	106	129	125	119	132	128	117	106	108	138	138	-	
2C1	176	6	6	98	55	40	88	70	0	74	196	171	159	185	168	206	131	6	128	158	158	112	-

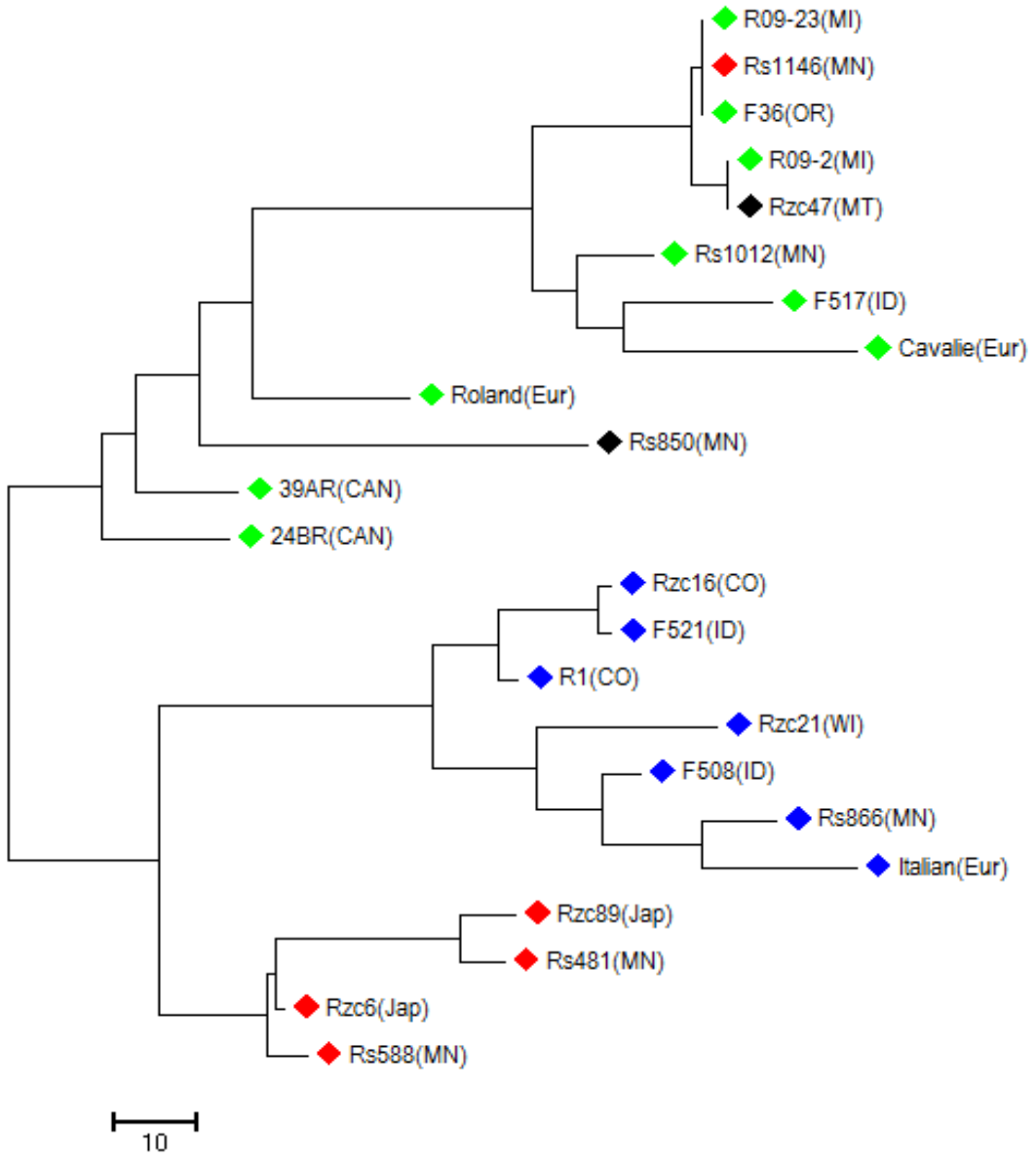

Figure 4.5 Neighbor-joining tree of 23 *Rhizoctonia solani* AG2-2 isolates based on 13 microsatellite loci. Pairwise distances were determined with Genotype 1.2 using the stepwise mutation model with missing data counted as 1 mutational step. Relationship of the isolates was inferred from pairwise distances using the neighbor-joining method (Saitou & Nei, 1987). Analysis was performed using Mega 6.0. Region of origin is indicated in parentheses after the isolate name. Color of diamond signifies phylogenetic group according to Martin et al. (2014); green = group 1, blue = group 2A, red = group 2B and black = undetermined. Scale bar indicates number of mutational steps.Locale abbreviations are as follows: MI = Michgan, MN = Minnesota, OR = Oregon, MT = Montana, ID = Idaho, Eur = Europe, CAN = Canada, CO = Colorado, WI = Wisconson, Jap = Japan.

Table 4.7 Simpson diversity index by group for 23 *Rhizoctonia solani* AG2-2 isolates. Simpson diversity index was calculated according to Simpson (1949) (see text for formula). Group designation is according to Martin et al. (2014). $\overline{X}$ is the average diversity across all 13 loci. Shaded values are 1.00 and indicate the all alleles at that locus are identical for that group.

	Total No. Alleles	Alleles per locus	Simpson Diversity Index by Locus													
			7420	**5402**	**759**	**8224**	**2893**	**5583**	**6150**	**6145**	**8703**	**4660**	**5877**	**5487**	**2547**	$\overline{X}$
Overall	61	4.69	0.668	0.474	0.206	0.509	0.363	0.285	0.288	0.309	0.255	0.313	0.511	0.360	0.425	0.376
Group 1	47	3.62	0.920	0.503	0.382	0.722	0.264	0.365	0.316	0.642	0.247	0.309	0.525	0.469	0.389	0.459
Group 2A	34	2.62	0.388	0.439	0.367	1.000	1.000	0.459	0.398	0.276	0.439	1.000	1.000	0.561	1.000	0.620
Group 2B	17	1.31	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.625	1.000	0.500	1.000	0.469	1.000	0.892

Table 4.8 Genotypic differentiation (exact G test) for each population pair of *Rhizoctonia solani* AG2-2. Populations were separated by phylogenetic group according to Martin et al. (2014). Pairwise differentiation was determined using Genepop 4.5.1. Shaded values indicate non-significant relationships ($p > 0.05$). A designation of ' - ' indicates no variability in allele size between populations at that locus.

	p-value by Locus													
Population pair	**759**	**8224**	**2893**	**8703**	**4660**	**7420**	**5402**	**5877**	**5487**	**6145**	**5583**	**6150**	**2547**	**Across All Loci**
Grp 1 & Grp 2A	<.0001	<.0001	<.0001	.0002	.0002	.0008	<.0001	.1504	.0062	.0004	.0019	<.0001	<.0001	<.0001
Grp 1 & Grp 2B	<.0001	<.0001	.0005	.0007	.0022	1.000	.0381	.0110	.1538	.0006	.0006	.0002	.0114	<.0001
Grp 2A & Grp 2B	.0028	-	-	.0154	.1088	.0496	.0145	.0049	.8938	.1341	.0029	.0003	.0030	<.0001

Table 4.9 Genic differentiation (exact G test) for each population pair of *Rhizoctonia solani* AG2-2. Populations were separated by phylogenetic group according to Martin et al. (2014). Pairwise differentiation was determined using Genepop 4.5.1. Shaded values indicate non-significant relationships (p > 0.05). A designation of ' - ' indicates no variability in allele size between populations at that locus.

	p-value by Locus													
Population pair	**759**	**8224**	**2893**	**8703**	**4660**	**7420**	**5402**	**5877**	**5487**	**6145**	**5583**	**6150**	**2547**	**Across All Loci**
Grp 1 & Grp 2A	<.0001	<.0001	<.0001	<.0001	<.0001	.0001	<.0001	.0522	.0002	<.0001	0024	.0004	<.0001	<.0001
Grp 1 & Grp 2B	.0003	<.0001	<.0001	.0004	.0002	1.000	.0927	.0002	.0625	<.0001	<.0001	.0077	.0005	<.0001
Grp 2A & Grp 2B	<.0001	-	-	.0508	.0097	.0233	.0518	<.0001	.8446	.1573	<.0001	.0013	<.0001	<.0001

Discussion

The objective of the current study was to develop and validate a set of microsatellite markers for the fungal pathogen *Rhizoctonia solani* AG2-2. The initial *in-silico* selection of potential markers made validation relatively straightforward as all of the tested markers amplified in the three isolates that had been used for *in-silico* selection. More traditional methods for identifying potential microsatellite loci, such as restriction enzyme fragmentation and ligation into a plasmid vector, can be time-consuming and inefficient (Glenn & Schable, 2005; Squirrell et al., 2003; Zane et al., 2002). Generating microsatellite-enriched libraries can improve success rates and efficiency (Edwards et al., 1996; Zane et al., 2002), but can introduce selection bias as repeat motifs must be pre-defined (Leese et al., 2008; Zane et al., 2002). Thus, the initial *in-silico* identification of potential markers provided an efficient method for identifying microsatellite loci.

One criticism that has been leveled against the methodology presented in the current study is that since the cost of sequencing is relatively inexpensive, it would have been more efficient to just sequence all of the isolates as opposed to only a subset. While sequencing all 23 isolates would have provided more data on these individuals, the larger goal of this project is to use these markers on much larger populations. Microsatellites are still much less expensive per individual than whole genome sequencing and would be a more cost effective approach to larger sample sizes (Table 4.10). The other issue in favor of microsatellites is the simplicity of analysis. Although there are some peculiarities of microsatellites that can make interpretation tricky (Selkoe & Toonen, 2006), analysis is fairly straightforward and can typically be completed in a few hours. NextGen sequencing requires assembly and filtering of large amounts of data

which can require a high-performance computer system and a considerable amount of time. Often the services of a bioinformatician are employed to analyze NextGen sequence data which may incur additional expenses. Sequencing more isolates to use for initial selection of microsatellite loci may have reduced the number of rejected markers, but would not have eliminated the need for testing potential markers *in-situ*. In short, NextGen sequencing does have the potential to enhance population studies but the cost and effort required have not yet made microsatellites obsolete. Furthermore, the methodology used in this study is comparable to that used in other recent studies involving the identification and validation of microsatellite markers (Biasi et al., 2015; Moges et al., 2016; Vaghefi et al., 2017a; Wang & Chilvers, 2016).

Table 4.10 Cost comparison for Illumina sequencing and microsatellite analysis. Estimates for Illumina sequencing cost is based on prices advertised by Michigan State University Genomics Core. Microsatellite cost estimates are based on expenses incurred in the course of the current study.

Illumina sequencing			**Microsatellite analysis**	
Coverage	80x	50x		
Number of samples	20	25	Number of samples	12
Library Prep	$1850	$2300	PCR Materials	$150
HiSeq 4000 (paired end)	$2400	$2400	Labeled primers	$12
			ABI 3730 genetic analyzer	$150
Cost per sample	$215	$190	Cost per sample	$26

The presence of stutter peaks can greatly complicate the interpretation of microsatellite data and in extreme cases can result in improper or ambiguous results (DeWoody et al, 2006; Leclair et al., 2004). In order to minimize the occurrence of stuttering, we employed two approaches. The first was to utilize a high-fidelity proofreading polymerase. Stutter peaks are

thought to occur through a mechanism similar to that which causes the length polymorphisms in microsatellites, namely disassociation of the polymerase and subsequent slippage of the strands (Fan & Chu, 2007). The Phusion II polymerase is a *Pyrococcus*-like enzyme that is highly accurate and rapid (Fazekas et al., 2010; Lundberg et al., 1991; Uemori et al., 1997). There was virtually no observed stuttering in the amplicons generated with this polymerase. One explanation for the reliability of the Phusion type polymerases in amplifying microsatellite regions is the increased contact surface provided by the addition of a non-specific dsDNA binding protein (Fazekas et al., 2010). This additional affinity is thought to minimize polymerase stalling and disassociation that can allow mispairing and strand slippage (Fazekas et al., 2010).

The second technique we employed to help eliminate stuttering problems was to include a "GTTT" PIG-tail on the 5' end of all non-fluorescently labeled primers (reverse primers) (Brownstein et al., 1996). This additional tail is intended to decrease misalignments of the template and the generation of secondary structures that contribute to polymerase slippage. In the present study, there was no improvement with the PIG-tailed products compared to non-PIG-tailed products. This was presumably due to the use of the Phusion II polymerase (Fazekas et al., 2010), since there was no noticeable stuttering even in the non-PIG-tail products. However, no comparison was made with other types of polymerases to conclusively connect the lack of stuttering in our study with the use of the Phusion II polymerase. Nonetheless, the final analysis was completed without the PIG-tailed primers.

An important aspect of the microsatellite panel developed in the current study is the suitability for duplex PCR reactions, which reduces the time and cost associated with setup (Biasi et al., 2015; Li et al., 2012). Since two marker sets are included in a single PCR reaction

and are run as a single sample through the bioanalyzer, the cost of this step is reduced to about half of what it would be for running them individually. The goal was to have each sample occupy a single column on a 96-well plate, allowing up to 16 markers per sample and 12 samples per 96-well plate. The intent was to make setup and loading of the samples as efficient as possible by enabling the use of multi-channel pipettes. Markers were sorted by fragment length and shorter fragments were paired with longer fragments to minimize spectral overlap. The more difficult attribute to adjust for was that the 6-FAM dye produces a more intense fluorescent signal than does the HEX dye. What this means for analysis is that when the dilution is sufficient so that the HEX signal is appropriate, the signal for the 6-FAM is overly intense. To balance the signals from both dyes, the concentration of primers was varied to produce similar peak levels from each dye (Table 4.3).

Twenty unique multi-locus genotypes were identified among the 23 isolates screened, indicating a high level of diversity. This level of diversity was not surprising as the isolates used in this study were specifically chosen from diverse regions (Table 4.1). What was unexpected was that several isolates from diverse regions were more closely related to one another than they were to isolates from similar regions (Fig. 4.5). For example, none of the European isolates cluster together. Both Japanese isolates (Rzc6 & Rzc89) have a microsatellite pattern more similar to isolates from Minnesota than to each other. Isolates from three states, Michigan (R09-23), Minnesota (Rs1146) and Oregon (F36) have identical microsatellite genotypes even though they are from diverse regions of the country. Likewise, an isolate from Michigan (R09-2) and an isolate from Montana (Rzc47) are identical according to their microsatellite genotype.

Although a sample size of 23 isolates is insufficient to confidently draw conclusions regarding geographic distribution, the general observation is that genotype had little relation to locale of isolation. Instead, the distribution is suggestive of individuals being translocated from one region to another, possibly through human activity. *R. solani* has been reported to be carried in and on some types of seeds (Baker, 1947; Baker & Martinson, 1970, Neergaard, 1958; Crosier, 1968) and this is a potential source of transport between regions. However, there is little or no evidence that *R. solani* is carried on sugar beet seeds. In addition, the AG of reported seed-borne *R. solani* is either uncertain or unspecified. Therefore, *R. solani* AG2-2 being transported across regions via contaminated seed is questionable and there is likely another explanation for the observed distribution. This leaves the pattern of distribution or the source of migration uncertain. Recently, Vaghefi et al. (2017b) found that another common pathogen of sugar beet, *Cercospora beticola*, also shared genotypes across distant states and even between the US and Europe. They also identified contaminated seed as a potential source of genotype flow, but cited mixed reports as to the presence of *C. beticola* on seed. How genotype flow occurs in *C. beticola* also remains uncertain.

Polymorphism information content (PIC) is a measure of the usefulness of a genetic marker for detecting polymorphisms within a population (Anderson et al., 1993). The value ranges from zero to one and is dependent on the number of detectable alleles and the distribution of their frequencies. A value of zero is the least informative and means there is only one allele at that locus. The closer the value is to one, the more informative that locus will be. The total number of alleles at a locus determines the maximum PIC for that locus, while allele frequency determines the value between zero and the maximum (Table 4.11). Evenly

distributed alleles return the highest PIC values, while varied allele frequencies reduce the PIC value.

Table 4.11 Hypothetical polymorphism information content (PIC) values for loci with a differing number of alleles and frequencies. Maximum PIC value occurs when all allele frequencies are equal. PIC value with 1 allele at 50% illustrates how the PIC value is affected when the frequency of 1 allele is 50% of the other alleles, which are evenly distributed. PIC value with 1 rare allele is the value when 1 allele has a frequency of 0.05 and the other alleles are equally distributed.

	Number of Alleles					
PIC value	**1**	**2**	**3**	**4**	**5**	**10**
Maximum value	.000	.500	.667	.750	.800	.900
with 1 allele at 50%	N/A	.444	.640	.735	.790	.898
with 1 Rare Allele	N/A	.095	.546	.697	.772	.897

In the current study, PIC values ranged from 0.332 to 0.794 with an average value of 0.618. Ten loci had PIC values above 0.500, which indicates those loci were considered to be highly informative (Botstein et al., 1980). The remaining three loci had PIC values between 0.250 and 0.500 and so were considered to be reasonably informative. Locus 7420 had a total of three alleles with one allele having a frequency greater than 0.80 and one allele having a frequency of 0.065. This combination made locus 7420 the least informative marker of the set with a PIC value of 0.332. Locus 5877 had the lowest observed heterozygosity (0.130) of the loci in this study. In addition, two isolates failed to amplify for any allele at this locus. Of the three alleles detected, one was rare (frequency < 0.05) and the other two alleles were unevenly distributed (frequency of 0.643 and 0.310). PIC value was slightly below 0.50 (0.489) and considered reasonably informative. For these reasons, locus 7420 and 5877 may not be suitable markers for the purposes of this microsatellite panel.

Locus 8224 had only two alleles but they were fairly evenly distributed (0.565 / 0.435). This pattern resulted in a marginal PIC value (0.491). Heterozygosity at this locus was low (0.174) with only four individuals out of twenty three identified as heterozygous. Due to the low numbers of heterozygotes, this locus was identified as having the possibility of null alleles with the second highest predicted null allele frequency of all the loci screened (Table 4.5). In addition, locus 8224 was identified as having the potential for scoring errors due to stuttering. However, observations from the chromatograms are not indicative of stuttering but are more consistent with indels that have altered allele length by one base pair (Fig. 4.4). Screening of additional isolates and sequencing problematic alleles will be required to determine the suitability of this marker.

The neighbor-joining tree based on the microsatellite data from the current study (Fig. 4.5) largely agree with the multigene phylogeny of Martin et al. (2014) (Fig. 4.6). Microsatellites are not considered to be an effective marker type for reconstructing phylogenetic relationships (Estoup et al., 2002). However, the general agreement between the microsatellite tree and the multigene phylogeny indicates the selected microsatellite loci likely have a similar evolutionary history. The major disagreement between the microsatellite tree and the multigene phylogeny was isolate Rs1146. The analysis of Martin et al. (2014) placed isolate RS1146 in group 2B, but our microsatellite data includes the isolate with others in group 1. Evidence from virulence tests show that isolate Rs1146 is more like other isolates from group 1 than isolates from group 2B as it is highly aggressive on dry beans and sugar beet and isolates from group 2B are generally weaker (Chapter 2, this thesis). Resequencing the markers used for the multigene phylogeny

and re-running the microsatellite panel for isolate Rs1146 will be needed to examine this discrepancy.

Something that is unclear from the phylogeny of Martin et al. (2014) (Fig. 4.6) is whether the clades labeled 2A and 2B should be considered two separate clades or a single clade with two sub-clades. Data from pathogenicity studies indicate that group 2A is significantly more aggressive than group 2B on sugar beet and dry beans when inoculated at planting (Chapter 2 & 3, this thesis). Previously reported subgroups, AG2-2IIIB and AG2-2IV, have been associated with differences in virulence, with AG2-2IIIB identified as being more virulent on sugar beet (Engelkes & Windels, 1996; Panella, 2005; Strausbaugh et al., 2011). These distinctions can have a significant effect on management and resistance breeding efforts (Engelkes & Windels, 1996; Strausbaugh et al., 2013). Thus it may be prudent to consider phylogenetic groups 2A and 2B to be distinct, separate groups rather than subgroups.

In addition, microsatellite data from the current study indicate that group 2B is highly homozygous with only one locus in a single isolate being heterozygous. Average Simpson Diversity Index by group shows a decline in diversity within the groups with group 1 having the highest diversity and group 2B the lowest diversity (Table 4.7). Ten out of thirteen loci (77%) were fixed in group 2B while only five loci (38%) were fixed in group 2A.

Population differentiation tests indicate significant differences in allele distribution between groups 2A and 2B. Genic differentiation evaluates the distribution of alleles in the samples and uses a null hypothesis of H_0: 'alleles are drawn from the same distribution in all populations.' Pairwise comparison of groups 2A and 2B (Table 4.9) showed seven of thirteen loci (54%) and the comparison across all loci to have significant p-values ($p < 0.05$). Genotypic

differentiation, which considers the distribution of genotypes, had similar results (Table 4.8) with eight of thirteen loci (62%) and the comparison across all loci having significant p-values ($p < 0.05$). The conclusion is that the distribution of alleles and genotypes in groups 2A and 2B come from different distributions and appear to represent distinct populations.

Conclusions

Rhizoctonia solani AG2-2 is a highly diverse group of fungi. In a representative group of 23 isolates, 20 unique genotypes were identified. Ten microsatellite markers evaluated in the current study have 5 or more unique genotypes at the given loci and have a PIC value greater than 0.50, indicating they are highly informative. Groupings based on microsatellite distances largely agree with the multigene phylogeny of Martin et al. (2104), which supports the three proposed genetic groups. Genotypic differentiation supports the position that groups 2A and 2B should be considered separate, independent clades. Thus, this set of microsatellite markers were effective at discriminating genotypes of *Rhizoctonia solani* AG2-2 isolates and have shown some utility for use in population genetics work by their ability to discriminate the three clades identified by Martin et al. (2014).

APPENDIX

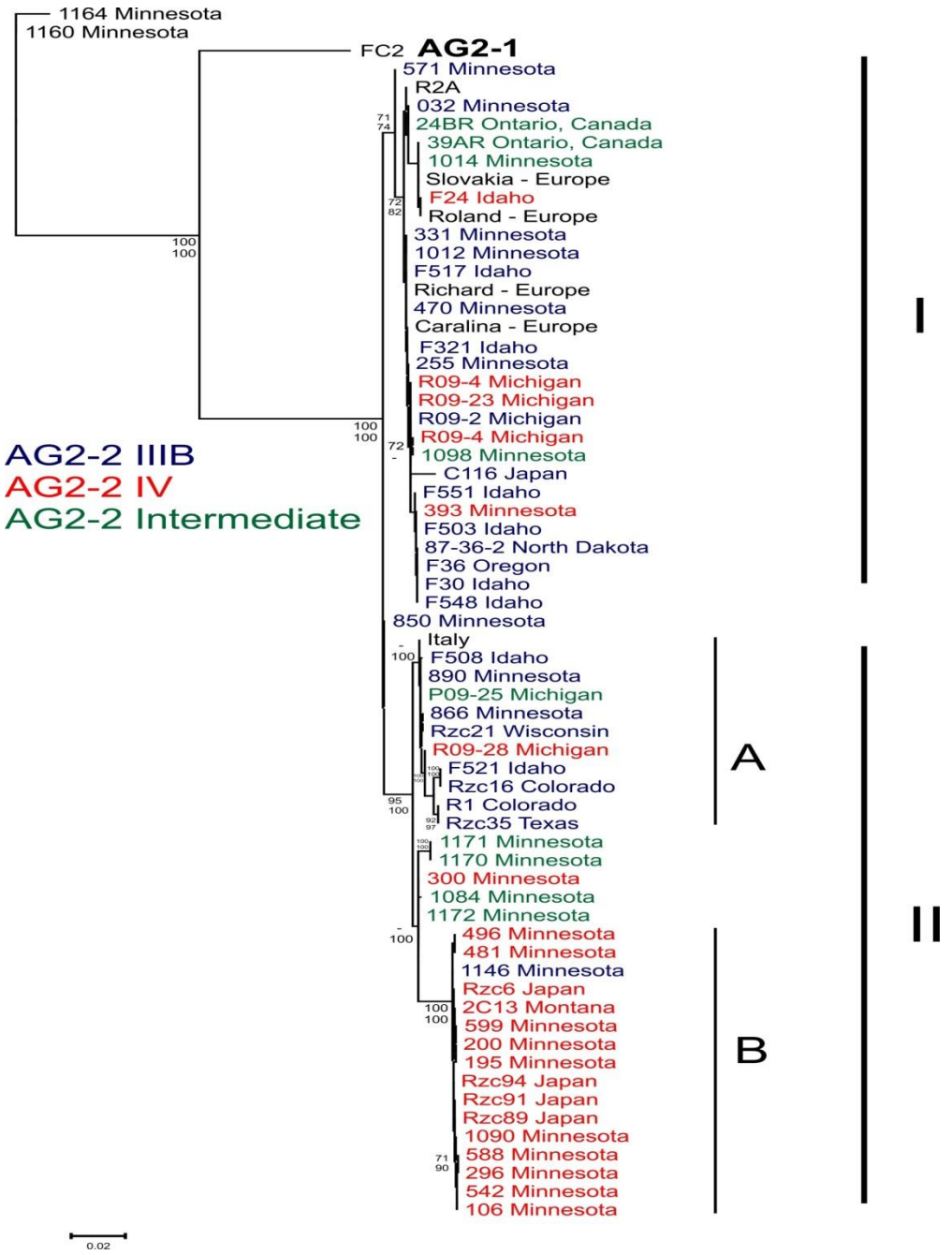

Figure A4.1 Multigene phylogeny of 63 *Rhizoctonia solani* AG2-2 isolates according to Martin et al. (2014). Genes sequenced included *rpb2*, tef1*, ITS,* and *LSU* as reported in Gonzalez et al. (2016) with minor modifications to improve reliability and specificity for AG2-2 (unpublished data). Isolates in blue were originally identified as AG2-2IIIB, those in red identified as AG2-2IV, and those in green were intermediates based on growth at 35˚C - where AG2-2IIIB grows well at 35˚C and AG2-2IV do not. An AG2-1 isolate was used for the outgroup. Phylogram used curtesy of Martin et al. (2014).

REFERENCES

REFERENCES

Adams, R.I., Brown, K.M. and Hamilton, M.B. (2004). The impact of microsatellite electromorph size homoplasy on multilocus population structure estimates in a tropical tree (*Corythophora alta*) and an anadromous fish (*Morone saxatilis*). Molecular Ecology, 13: 2579-2588.

Anderson, J.A., Churchill, G.A., Autrique, J.E., Tanksley, S.D. and Sorrells, M.E. (1993). Optimizing parental selection for genetic linkage maps. Genome, 36: 181-186.

Baker, K.F. (1947). Seed transmission of *Rhizoctonia solani* in relation to control of seedling damping off. Phytopathology, 37: 912-924.

Baker, R. and Martinson, C.A. (1970). Epidemiology of diseases caused by *Rhizoctonia solani*. Pages 172-188 in *Rhizoctonia solani*: Biology and Pathology. Edited by Parmeter, J.R. Jr. University of California Press, Berkeley, CA.

Banke, S. and McDonald, B.A. (2005), Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola*. Molecular Ecology, 14: 1881-1896.

Bell, G.I. and Jurka, J. (1997). The length distribution of perfect dimer repetitive DNA is consistent with its evolution by an unbiased single-step mutation process. Journal of Molecular Evolution, 44: 414-421.

Benali, S., Mohamed, B., Eddine, H.J. and Neema, C. (2011). Advances of molecular markers application in plant pathology research. European Journal of Scientific Research, 50: 110-123.

Bhargava, A. and Fuentes, F.F. (2010). Mutational dynamics of microsatellites. Molecular Biotechnology, 44: 250-266.

Biasi, A., Martin, F. and Schena, L. (2015). Identification and validation of polymorphic microsatellite loci for the analysis of *Phytophthora nicotianae* populations. Journal of Microbiological Methods, 110: 61-67.

Biasi, A., Martin, F.N., Cacciola, S.O., di San Lio, G.M., Grünwald, N.J. and Schena, L. (2016). Genetic analysis of *Phytophthora nicotianae* populations from different hosts using microsatellite markers. Phytopathology, 106: 1006-1014.

Bolloux, F. and Lugon-Moulin, N. (2002). The estimation of population differentiation with microsatellite markers. Molecular Ecology, 11: 155-165.

Botstein, D., White, R.L., Skolnick, M. and Davis, R.W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics, 32: 314-331.

Brown, T.A. (2002). Chapter 14: Mutation, repair and recombination. In Genomes (2nd ed.) Oxford: Wiley-Liss, Oxford, UK.

Brownstein, M.J., Carpten, J.D and Smith, J.R. (1996). Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. BioTechniques, 20: 1004-1010.

Butler, J.M. (2007). Short tandem repeat typing technologies used in human identity testing. BioTechniques, Supplement to Vol. 43: *Sii-Sv*.

Calabrese, P.P., Durrett, R.T. and Aquadro, C.F. (2001). Dynamics of microsatellite divergence under stepwise mutation and proportional slippage/point mutation models. Genetics, 159: 839-852.

Chakraborty, R., de Andrade, M., Daiger, S. and Budowle, B. (1992). Apparent heterozygote deficiencies observed in DNA typing data and their implications in forensic applications. Annuals of Human Genetics, 56: 45-57.

Coupat-Goutaland, B., Régoudis, E., Besseyrias, M., Mularoni, A., Binet, M., Herbelin, P. and Pélandakis, M. (2016). Population structure in *Naegleria fowleri* as revealed by microsatellite markers. PLOS One, 11: e0152434. http://doi:10.1371/journal.pone.0152434

Crosier, W.F. (1968). *Rhizoctonia solani* in seeds of small grains and other plants. Proceedings of the Association of Official Seed Analysts, 58: 111-117.

Dakin, E.E., and Avise, J.C. (2004). Null alleles in parentage analysis. Heredity, 93: 5004-509.

DeWoody, J., Nason, J.D. and Hipkins, V.D. (2006). Mitigating scoring errors in microsatellite data from wild populations. Molecular Ecology Notes, 6: 951-957.

Dieringer, D. and Schlötterer, C. (2003). Two distinct modes of microsatellite mutation processes: evidence from the complete genomic sequences of nine species. Genome Research, 13: 2242-2251.

Di Rienzo, A., Peterson, A.C., Garza, J.C., Valdes, A.M., Slatkin, M. and Freimer, N.B. (1994). Mutational proceses of simple-sequence repeat loci in human populations. Proceedings of the National Academy of the Sciences, 91: 3166-3170.

Edward, K.J., Barker, J.H.A., Daly, A., Jones, C. and Karp, A. (1996). Microsatellite libraries enriched for several microsatellite sequences in plants. BioTechniques, 20:758-760.

Ellegren, H. (2004). Microsatellites: simple sequences with complex evolution. Nature Reviews Genetics, 5: 435-445.

Engelkes, C.A. and Windels, C.E. (1996). Susceptibility of sugar beet and beans to *Rhizoctonia solani* AG2-2IIIB and AG2-2IV. Plant Disease, 80: 1413-1417.

Ernest, H.B. and Penedo, C. (2000). Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA. Molecular Ecology, 9: 433-441.

Estoup, A., Jarne, P.and Cornuet, J. (2002). Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Molecular Ecology, 11: 1591-1604.

Estoup, A., Tailliez, C., Cornuet, J.M. and Solignac, M. (1995). Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apis mellifera* and *Bombus terrestris* (Apidae). Molecular Biology and Evolution, 12: 1074-1084.

Fan, H. and Chu, J. (2007). A brief review of short tandem repeat mutation. Genomics, Proteomics and Bioinformatics, 5: 7-14.

Fazekas, A.J., Steeves, R. and Newmaster, S.G. (2010). Improving sequence quality from PCR products containing long mononucleotide repeats. BioTechniques, 48: 277-285.

Flores-Renteria, L. and Krohn, A. (2013). Scoring microsatellite loci. Pages 319-336 in Microsatellites: Methods in Molecular Biology (Methods and Protocols) vol. 1006, Edited by Kantartzi, S.K. Humana Press, Totowa, NJ. doi: 10.1007/978-1-62703-389-3_21

Garza, J.C. and Freimer, N.B. (1996). Homoplasy for size at microsatellite loci in humans and chimpanzees. Genome Research, 6: 211-217.

Glenn, T.C. and Schable, N.A. (2005). Isolating microsatellite DNA loci. Methods in Enzymology, 395: 202-222.

Gonzalez, D., Rodriguez-Carres, M., Boekhout, T., Stalpers, J., Kuramae, E.E., Nakatani, A.K. ... Cubeta, M.A. (2016). Phylogenetic relationships of *Rhizoctonia* fungi within *Cantharellales*. Fungal Biology, 120: 603-619.

Katti, M.V., Ranjekar, P.K. and Gupta, V.S. (2001). Differential distribution of simple sequence repeats in eukaryotic genome sequences. Molecular Biology and Evolution, 18: 1161-1167.

Kruglyak, S., Durrett, R.T., Schug, M.D. and Aquadro, C.F. (1998). Equilibrium distributions of microsatellite repeat length resulting from a balance between slippage events and point mutations. Genetics, 95: 10774-10778.

Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C. and Sninsky, J.J. (1990). Effect of primer - template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acid Research, 18: 999-1005.

Leclair, B., Frégeau, C.J., Bowen, K.L. nd Fourney, R.M. (2004). Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. Journal of Forensic Science, 49: 1-13.

Leclercq, S., Rivals, E. and Jarne, P. (2007). Detecting microsatellites within genomes: significant variation among algorithms. BMC Bioinformatics, 8: 125.

Leese, F., Mayer, C. and Held, C. (2008). Isolation of microsatellites from unknown genomes using known genomes as enrichment templates. Limnology and Oceanography: Methods, 6: 412-426.

Li, Y., Cooke, D.E.L., Jacobsen, E. and van der Lee, T. (2012). Efficient multiplexing simple sequence repeat genotyping of the oomycete plant pathogen *Phytophthora infestans*. Journal of Microbiological Methods, 92: 316-322.

Lim, S., Notley-McRobb, L., Lim, M. and Carter, D.A. (2004). A comparison of the nature and abundance of microsatellites in 14 fungal genomes. Fungal Genetics and Biology, 41: 1025-1036.

Lundberg, K.S., Shoemaker, D.D., Adams, M.W.W., Short, J.M., Sorge, J.A. and Mathur, E.J. (1991). High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. Gene, 108:1-6.

Martin, F., Windels, C., Hanson, L. and Brantner, J. (2014). Analysis of population structure and pathogenicity of *Rhizoctonia solani* AG2-2 (ISG IIIB and IV) isolates from Michigan, Minnesota and North Dakota. Sugarbeet Research Reports. Beet Sugar Development Foundation, Denver, CO.

Milgroom, M.G. and Peever, T.L. (2003) Population biology of plant pathogens: The synthesis of plant disease epidemiology and population genetics. Plant Disease, 87: 608-617.

Meirmans, P.G. and Van Teinderen, P.H. (2004). Genotype and Genodive: two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes, 4: 792-794.

Moges, A.D., Admassu, B. Belew, D., Yesuf, M., Njguna, J., Kyalo, M. and Ghimire, S.R. (2016). Development of microsatellite markers and analysis of genetic diversity and population structure of *Colletotrichum gloeosporioides* from Ethiopia. PLOS One, 11 (3): e0151257. doi.org/10.1371/journal.pone.0151257

Nauta, M.J. and Weissing, F.J. (1996). Constraints on allele size at microsatellite loci: implications for genetic differentiation. Genetics 143: 1021-1032.

Neergaard, P. (1958). Infection of Danish seeds by *Rhizoctonia solani* Kuehn. Plant Disease Reporter, 42: 1276-1278.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annual Review of Phytopathology, 25: 124-143.

Oliveira, E.J., Pádua, J.G., Zucchi, M.I., Vencovsky, R. and Vieira, M.L.C. (2006). Origin, evolution and genome distribution of microsatellites. Genetics and Molecular Biology, 29: 294-307.

Ohta, T. and Kimura, M. (1973). A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genetics Research, 22: 201-204.

Panella, L.W. (2005). Pathogenicity of different anastomosis groups and subgroups of *Rhizoctonia solani* on sugar beet. Proc. American Society of Sugarbeet Technologists. 33rd Meeting (Agriculture) Palm Springs, CA. March 2-5, 2005. Annual Meeting abstracts p. 166. 2005. (Abstr.)

Pemberton, J.M., Slate, J., Bancroft, D.R. and Barrett, J.A. (1995). Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. Molecular Ecology, 4: 249-252.

Perez-Enriquez, R., Takagi, M. and Taniguchi, N. (1999). Genetic variability and pedigree tracing of a hatchery-reared stock of red sea bream (*Pagrus major*) used for stock enhancement, based on microsatellite markers. Aquaculture, 173: 413-423.

Renwick, A., Davison, L., Spratt, H., King, J.P. and Kimmel, M. (2001). DNA dinucleotide evolution in humans: fitting theory to facts. Genetics, 159: 737-747.

Rousset, F. (2008). Genepop'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Molecular Ecology Resources, 8: 103-106.

Saitou N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

dos Santos Pereira, D.A., Ceresini, P.C., Castroagudín, V.L., Ramos-Molina, L.M., Chavarro-Mesa, E., Negrisoli, M.M. ... Takada, H.M. (2017). Population genetic structure of *Rhizoctonia oryzae-sativae* fom rice in Latin America and its adaptive potential to emerge as a pathogen on *Urochloa* pastures. Phytopathology, 107: 121-131.

Selkoe, K.A. and Toonen, R.J. (2006). Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters, 9: 615-629.

Schlötterer, C. and Tautz, D. (1992). Slippage synthesis of simple sequence DNA. Nucleic Acids Research, 20: 211-215.

Sharopova, N. (2008). Plant simple sequence repeats: distribution, variation and effects on gene expression. Genome, 51: 79-90.

Shimoda, N., Knapik, E.W., Ziniti, J., Sim, C., Yamada, E., Kaplan, S., ... Fishman, M.C. (1999). Zebrafish genetic map with 2000 microsatellite markers. Genomics, 58: 219-232.

Simpson, E.H. (1949). Measurement of diversity. Nature, 163: 688.

Sneh, B., Burpee, L. and Ogoshi, A. (1991). Identification of *Rhizoctonia* species. APS Press, St. Paul, MN.

Stefanini, F.M. and Feldman, M.W. (2000). Bayesian estimation of range for microsatellite loci. Genetics Research, 75: 167-177.

Strausbaugh, C.A., Eujayl, I.A. and Panella, L.W. (2013). Interaction of sugar beet host resistance and *Rhizoctonia solani* AG2-2IIIB strains. Plant Disease, 97: 1175-1180.

Strausbaugh, C.A., Eujayl, I.A., Panella, L.W. and Hanson, L.E. (2011). Virulence, distribution and diversity of *Rhizoctonia solani* from sugar beet in Idaho and Oregon. Canadian Journal of Plant Pathology, 33: 210-226.

Stukenbrock, E.H., Banke, S. and McDonald, B.A. (2006). Global migration patterns in the fungal wheat pathogen *Phaeosphaeria nodorum*. Molecular Ecology, 15: 2895-2904.

Su, Q., Jin, B., Luo, H., Li, Y., Wu, J. Yan, J., ... Zhang, L. (2016). Population study and mutation analysis for 28 short tandem repeat loci in southwest Chinese Han population. Journal of Forensic and Legal Medicine, 44: 10-13.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). Mega6: molecular evolutionary genetic analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

Taylor, J.S., Sanny, J.S.P. and Breden, F. (1999). Microsatellite allele size homoplasy in the guppy (*Poecilia reticulata*). Journal of Molecular Evolution, 48: 245-247.

Treco, D and Arnheim, N. (1986). The evolutionarily conserved repetitive sequence d(TG • AC)$_n$ promotes reciprocal exchange and generates unusual recombinant tetrads during yeast meiosis. Molecular and Cellular Biology, 6: 3934-3947.

Uemori, T., Sato, Y., Kato, I., Doi, H. and Ishino, Y. (1997). A novel DNA polymerase in the hyperthermophilic archaeon, *Pyrococcus furiosus*: gene cloning, expression and characterization. Genes to Cells, 2: 499-512.

Vaghefi, N., Kikkert, J.R., Bolton, M.D., Hanson, L.E., Secor, G.A. and Pethybridge, S.J. (2017a). *De novo* genome assembly of *Cercospora beticola* for microsatellite marker development and validation. Fungal Ecology, 26: 125-134.

Vaghefi, N., Kikkert, J.R., Bolton, M.D., Hanson, L.E., Secor, G.A., Nelson, S.C. and Pethybridge, S.J. (2017b). Global genotype flow in *Cercospora beticola* populations confirmed through genotyping-by-sequencing. PLOS One, 12: e0186488. https://doi.org/10.1371/journal.pone.0186488

van Oosterhout, C., Hutchinson, W.F, Wills, D.P.M. and Shipley, P. (2004). Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes, 4: 535-538.

Wang, J. and Chilvers, M.I. (2016). Development and characterization of microsatellite markers for *Fusarium virguliforme* and their utility within clade 2 of the *Fusarium solani* species complex. Fungal Ecology, 20: 7-14.

Wattier, R., Engel, C.R., Saumitou-Laprade, P. and Valero, M. (1998). Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental evidence at the dinucleotide locus GvqCT in *Gracilaria gracilis* (Rhodophyta). Molecular Ecology, 7: 1569-1573.

You, F.M., Huo, N., Gu, Y.Q., Luo, M., Ma, Y., Hane, D., ... Anderson, O.D. (2008). BatchPrimer3: a high throughput web application for PCR and sequencing primer design. BMC Bioinformatics, 9: 253.

Zane, L., Bargelloni, L. and Patarnello, T. (2002). Strategies for microsatellite isolation: a review. Molecular Ecology, 11: 1-16.