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Characterization and Optimization of Real-Time PCR in Detection of Novel Viral Sequences From Select Isolate of Rhizoctonia Solani

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CHARACTERIZATION AND OPTIMIZATION OF REAL-TIME PCR IN DETECTION OF NOVEL VIRAL SEQUENCES FROM SELECT ISOLATE OF *RHIZOCTONIA SOLANI*

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Title: Characterization and Optimization of Real-Time PCR in Detection of Novel Viral Sequences From Select Isolate of *Rhizoctonia solani*

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Rhizoctonia solani is a soil-borne pathogenic fungus with several distinct isolates that have been classified based on their anastomosis groups. Many isolates of this fungus contain double-stranded (ds) viral RNA that are cytoplasmic and viral in nature. In the present study, the dsRNA from isolates EGR4, Tom7 and a hyphal anastomosis reaction of EGR4/Tom7 was extracted and analyzed by gel-electrophoresis and micro-chip analysis. Interaction between the isolates showed effects on ribosomal RNA which may be due to the presence of the viral dsRNA. Real-time PCR was optimized with primers and probes, specific to the dsRNA sequence, via plasmid cultures that had previously been transformed using the extracted dsRNA from the isolate EGR4. The ideal primer/probe concentration was determined through comparing the lowest real-time PCR cycle threshold values supporting that a specific isolate can be selectively identified by means of an optimized real-time PCR protocol.

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ABBREVIATIONS

%	Percentage
BHQ	Black hole quencher
cDNA	Complementary DNA
C_t	Cycle threshold
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethyl alcohol
FAM	6-carboxyfluorescein
FP	Forward primer
g	Grams
G-C	Guanine-cytosine
GPS	Glycine-disodium phosphate saline
IPTG	Isopropzlthio-beta-D-galactoside
LB	Liquid broth
M	Molar (mol/Liter)
MEB	Malt extract broth
mg	Milligram
mL	Milliliter
mm	Millimeter
Na2EDTA	Sodium EDTA

NaOAc	Sodium acetate
NaOH	Sodium hydroxide
nM	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pH	Potentia hydrogenii
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Reverse primer
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Real-time PCR
SDS	Sodium dodecylsulfate
ssRNA	Single-stranded RNA
STE	Sodium chloride tris ethylenediamine tetraacetic acid
TAE	Tris-acetate EDTA
UV	Ultra-violet
V	Volts

CHAPTER ONE

INTRODUCTION

A heterogeneous assemblage of filamentous fungal taxa which share similar characteristics in their anamorphic states and do not produce asexual spores make up the genus *Rhizoctonia* (Garcia, *et al.*, 2006). De Candolle (1815) first established *Rhizoctonia* as a genus with some common basic characteristics such as production of sclerotia of uniform texture and association of mycelium with the roots of plants (Garcia, *et al.*, 2006). The fungi in this genus are generally pathogenic soil fungi, mostly associated with roots, and are distributed worldwide in both forest and agricultural soils (Garcia, *et al.*, 2006). Some species in the genus *Rhizoctonia* create a major agricultural problem, causing root rot diseases in many crops.

Rhizoctonia solani (teleomorph = *Thanatephorus cucumeris* Frank (Donk)) was first isolated from infected potatoes and characterized by Julius Kuehn in 1858 (Garcia, *et al.*, 2006; Ogoshi, 1987). *R. solani* is a multinucleated filamentous fungus which can survive for years in the soil. The fungus can germinate from either present mycelia or sclerotia in the soil (Ceresini, 2002). A wide range of agricultural and ornamental crops around the world, including rice, peanuts, strawberry, potato, tomato, grasses, cotton, wheat, tree seedlings, pine, and lettuce, are attacked by *R. solani (*Adams, 1988; Sneh, *et al.*, 1996; Garcia, *et al.*, 2006). *R. solani* does most of its damage primarily through "damping off" of seedlings, black lesions in root and seed, stem rot, and rot of plant parts in contact with soil (Garcia, *et al.*, 2006). The fungus has been reduced to a cosmopolitan plant parasite which is a major cause of death in crop plants.

The study of *Rhizoctonia solani* is complicated by the fact that it is a large species complex which consists of many genetically diverse species with different life histories

(Cubeta and Vilgalys, 1997; Carling, *et al.,* 2002). Classification of *R. solani* is based primarily on grouping the different fungal isolates into anastomosis groups. These groups are traditionally formed through hyphal anastomosis reactions. As of now, there are 13 different anastomosis groups (Cubeta and Vilgalys, 1997; Carling *et al.,* 1999; Carling, *et al.,* 2002). Many of the anastomosis groups are further divided into subgroups including anastomosis groups 1, 2, 3, 4, 6, 8, and 9. For example, anastomosis group 2 has seven subsets (Carling, Kuninaga, and Brainard, 2002). These subdivisions differ by one or more genetic, pathogenic or biochemical characteristics (Cubeta and Vilgalys, 1997). *R. solani* fungi from different anastomosis groups and different subdivisions have been found to show different levels of virulence (Woodhall *et al.,* 2008). These anastomosis groups and subdivisions are an important aspect in characterizing, understanding, and identifying *R. solani.*

Rhizoctonia solani was first subdivided through hyphal anastomosis in 1936 by Schultz (Adams and Butler, 1978). Hyphal interactions can help differentiate between genetically distinct individuals via somatic compatibility or incompatibility. When two isolates are paired, the resulting hyphal fusion will either lead to acceptance (self-pairings) or rejection (somatic incompatibility) (Cubeta and Vilgalys, 1997). If the pairing of isolates does not result in a hyphal fusion there is most likely a greater genetic difference between the two isolates (Cubeta and Vilgalys, 1997). Four different types of reactions occurring with isolate pairings have been classified (Table 1). C0 and C1 reactions occur when isolate between anastomosis groups are paired and result in little or no hyphal fusion (MacNish *et al.,* 1993; Cubeta and Vilgalys, 1997). C2 and C3 reactions occur between two isolates within the same anastomosis group. C2 is referred to as the killing reaction. When

two isolates in an anastomosis group are genetically diverse and are somatically incompatible a killing reaction occurs (MacNish *et al.*, 1993; Cubeta and Vilgalys, 1997). The C3 reaction indicates a "perfect" fusion between two isolates within the same anastomosis group which are very genetically similar, or is a fusion of the same isolate (MacNish *et al.*, 1993; Cubea and Vilgalys, 1997).

Table 1

Characterization of Hyphal Anastomosis Reactions of Rhizoctonia solani *(MacNish et al., 1993; Cubeta and Vilgalys, 1997; Chin, S. M., 2012).*

Category	Relatedness	Description of interaction	Nature of genetic relationship between isolates
C0	No interaction	Hyphae grow past each other, no recognition	Isolates have no genetic relationship and belong to different AG
C1	Hyphal contact only	Contact between hyphae; apparent connection of walls but no evidence of wall penetration or membrane-membrane contact; occasionally one or both anastomosing cells and adjacent cells die	Isolates have a distant genetic relationship and belong to either the same or different AG
C2	Killing reaction	Wall connection obvious; membranes contact uncertain; location of reaction site obvious; diameter of anastomosis point less than hyphal diameter; anastomosing and adjacent cells always die	Isolates represent genetically distinct individuals that belong to the same AG
C3	Perfect fusion	Walls fuse; membranes fuse; anastomosis point frequently not obvious; diameter of anastomosisi point equal or nearly equal to hyphal diameter; anastomosing cells and adjacent cells may die but generally do not	Isolates are genetically identical or closely related, individuals belong to the same AG and may represent clones

Several fungi have been shown to suffer from a degenerative disease. *R. solani* has been implicated as one of the fungi associated with a decline which affects the morphology and physiology of the fungus (Castanho and Butler, 1978; Castanho, *et al.*, 1978). Over 30 species of plant pathogenic fungi have been reported to contain mycoviruses (Castanho, *et al.*, 1978). Mycoviruses can be transmitted from infected fungi to healthy fungi via hyphal anastomosis (Castanho and Butler, 1978). Most of the fungi infected with these mycoviruses show no altered fungal phenotype, however, the decline of *R. solani* has been associated with a cytoplasmic double-stranded (ds) RNA mycovirus (Castanho, *et al.*, 1978). Infected fungal isolates are shown to contain these dsRNA mycoviral segments whereas healthy isolates are not often associated with the segments (Castanho, *et al.*, 1978).

A study done by Zanzig *et al.* (1984) questions the effects of the dsRNA on *Rhizoctonia solani*, suggesting that the presence of dsRNA had no association to the degree of virulence of the isolates. It has, however, been suggested that hypovirulent strains of the fungus may actually be devoid of any dsRNA, and loss of the dsRNA or parts of the dsRNA can lead a development of hypovirulence in virulent strains (Finkler *et al.*, 1985). In fact, transmission of virulence was found to occur with transmission of dsRNA from a virulent strain of *R. solani* to a native hypovirulent strain (Finkler *et al.*, 1985). These studies present conflicting evidence about the relationship of dsRNA and virulence. Further studies conducted on viral dsRNA and hypovirulent strains support that conversion of hypovirulence to virulence can occur with the presence of dsRNA and that there is a correlation with the level of virulence of *R. solani* and the presence of dsRNA (Nuss and Koltin, 1990).

DsRNA has been found to be present in a large number of *Rhizoctonia solani* isolates. A study by Zanziger *et al.* (1984) showed that out of 50 field isolates of *R. solani*, forty-nine of them were found to contain dsRNA and several of the isolates contained up to eight dsRNA segments. Bharathan *et al.* (2005) found 36 of 36 isolates to contain dsRNA and a study by Kousik *et al.*, showed that in anastomosis groups 1 IA and IB, dsRNA was detected in 63% of isolates. The dsRNA seems to share a certain homology with isolates coming from similar geographic areas showing greater homology. Cross-hybridization of dsRNA from isolates in America showed 32-80% homology whereas dsRNA from Japanese isolates when compared to dsRNA from Maine showed homologies of 31% and 50% (Bharathan and Tavantzis, 1991).

Mycoviruses occur in most major groups of fungi. Unlike other viruses, mycoviruses spread via intracellular mechanisms. Hyphal anastomosis of an uninfected fungus with an infected fungus can result in mycoviral transmission (Nuss, 2011; Pearson *et al.*, 2009). With the fusing of the hyphae, viral particles are able to transmit from one fungus to the other. Another form of transmission which often occurs with mycoviruses is transmission from the fungal mycelium to the spores (Pearson *et al.*, 2009). Virus transmission through sexual spores is uncommon, but asexual spores are produced via hyphae modification (Pearson *et al.*, 2009). Because mycoviruses are so dependent on their host for transmission, mycoviral infections are persistent but usually lack severe symptoms which could result in cell death (Nuss, 2011). There are five families, (figure 1), in which these viruses have been classified: *Hypviridae, Totiviridae, Partiviridae, Chrysoviridae,* and *Reoviridae* (van de Sande *et al.*, 2010). These families are grouped by their type of genome and the number of genome segments.

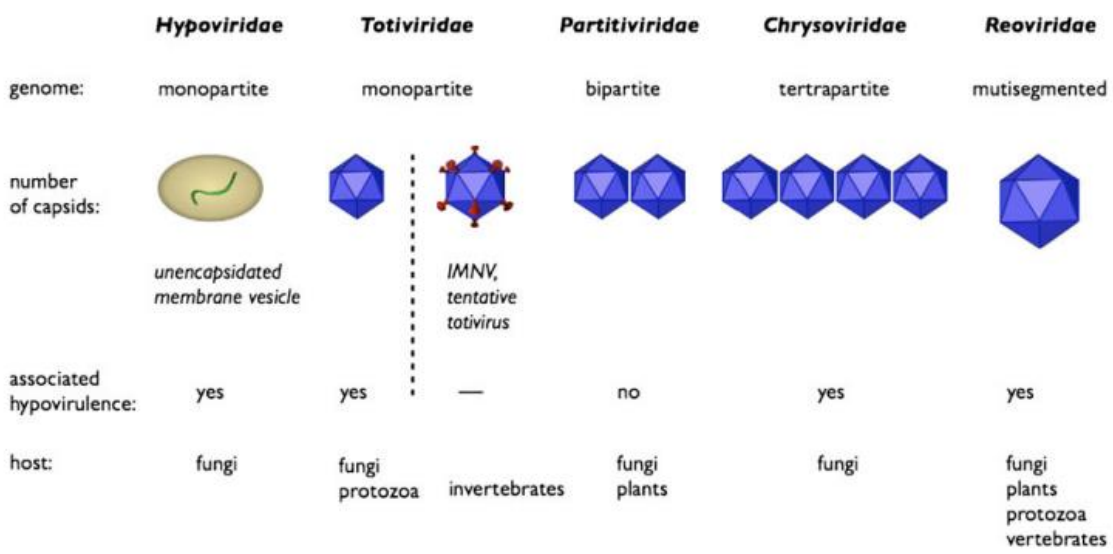

Figure 1. Mycoviral families with predominant genomic type, whether there is an associated hypovirulence, and host. Figure from van de Sande, *et al.* (2010).

The majority of mycoviruses contain double-stranded RNA (Bozarth, 1972; Nuss, 2011). There are some which contain single-stranded RNA, either with a reverse transcription step for replication or without, and a few which contain double-stranded DNA (van de Sande *et al.*, 2010). The dsRNA mycoviruses contain diverse lineages with some having multiple genomic segments while others just have one. There are at least ten monopartite, three bipartite, one tripartite and three quadripartite lineages of dsRNA mycoviruses (Liu *et al.*, 2012). DsRNA profiles of mycoviruses have a large amount of variability in their composition and the number of segments present, even in the same species.

While the mycoviruses remain primarily in the cytoplasm of the fungal host cells, they have been shown to be found in other places. Some dsRNA elements were found to be located within the mitochondria (Polashock and Hillman, 1994). The presence of the dsRNA in the fungal hosts involve a wide variety of possible locations. In fact, mycoviruses are suspected to involve an informational transfer among nuclear genes,

mitochondrial genes and the dsRNA genome (Wicker, *et al.*, 2007; Nakayashiki, 2011). DsRNA mycoviruses are complex in nature with little information known. Isolation of this viral dsRNA however has been long documented.

The isolation of dsRNA has been elucidated to permit a rapid and efficient yield from small amounts of mycelium (Morris and Dodds, 1979). DsRNA has been isolated from most of the *R. solani* anastomosis groups (Zanzinger *et al.*, 1984; Bharathan and Tavantzis, 1991; Kousik *et al.*, 1994). Often, RNA-dependent-RNA-polymerase is found to be associated with dsRNA viruses (Buck *et al.*, 1981; Ben-Tzvi *et al.*, 1984; Finkler *et al.*, 1985). RNA polymerase products can consist of single-stranded RNA which signifies transcription activity (Tavantzis and Bandy, 1988). Ethanol concentrations have an effect on the recovery of dsRNA versus ssRNA. DsRNA is efficiently recovered at 15% ethanol, with higher concentrations not improving recovery, whereas ssRNA recovery is favored above 20% ethanol (Morris and Dodds, 1979). DsRNA was found to be resistant to RNases under specific conditions. DsRNA was found to be resistant to RNases when placed in an high salt environment, while ssRNA did not retain the same resistance (Morris and Dodds, 1979). The high salt environment resistance to RNase may have something to do with the sequence of the nucleic acids. The dsRNA was found to contain 60-65% G-C base pairs, which suggests that the RNA can form hairpin loop structures, enabling it to form into dsRNA and to become more resistant to RNase in a high salt environment (Pays, 1976).

Polymerase chain reaction (PCR) is a technique used to selectively amplify a small sample of DNA, producing a greater amount for examination. PCR was first introduced by Kary Mullis in 1985 (Saiki *et al.*, 1985; VanGuilder *et al.*, 2008). PCR works by having two oligonucleotide primers that are on either side of the desired DNA sequence at the 3’

end and 5' end. Following a denaturation step, the primers anneal to the DNA sequence which they are complimentary to. DNA polymerase then extends from the primers, amplifying the DNA segment. This occurs over many cycles, which results in an exponential growth of DNA sample. A downside to this technique, however, was the sensitivity of the *Escherichia coli* DNA polymerase that was used to extend the DNA sequence from the annealed primers (Saike *et al.,* 1988). Due to this heat-instability and the denaturation step required in each cycle, fresh enzyme had to be added each time. This was very labor-intensive and also increased the risk of human error. This problem was solved, however, in 1988 when a thermostable DNA polymerase, which can survive at 95 degrees Celsius for an extended period of time, was purified from *Thermus aquaticus (Taq),* a thermophilic bacterium (Saiki *et al.,* 1988). With the introduction of this new enzyme, PCR has become a reliable and very useful technique in the amplification of DNA sequence.

Aside from amplification, PCR can also be used as a mode of detection of specific DNA sequences. This method incorporates the addition of a fluorescent component which can be used to detect the amount of DNA that has amplified. One of the first methods of simultaneous detection, via fluorescence, and amplification was with the usage of ethidium bromide (Higuchi *et al.,* 1992). Ethidium bromide was used due to the fact that its fluorescence increases when exposed to double-stranded DNA. This property of ethidium bromide allows for amplification of the DNA product to be monitored after each cycle via ultraviolet light. This method of simultaneous detection and amplification has some drawbacks however. Ethidium bromide will bind non-specifically to target DNA as well as primer-dimers, so there can be false amplification results. New technologies have overcome several of these limitations however and have included commercialized PCR

machines which are now available (Wittwer *et al.*, 1997). These commercial platforms allow samples to be rapidly heated through hot air, instead of via water baths, which greatly shortens the cycle duration and also incorporate SYBR Green I as a new DNA specific dye, which allows for fewer false positives (Wittwer *et al.*, 1989; Wittwer *et al.*, 1997).

Along with the new technology, a probe system has been introduced which allows the amount of amplification to be detected. This is achieved through the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase (Holland *et al.*, 1991). An oligonucleotide probe, which is non-extendable at the 3' end and labeled at the 5' end, anneals to the PCR product strand during amplification, providing a substrate for exonuclease activity (Holland *et al.*, 1991). The exonuclease activity of *T. aquaticus* DNA polymerase degrades the probe (Holland *et al.*, 1991). This application of the TaqMan probe avoids non-specific accumulation fluorescent signal. The TaqMan probe specifically works because it is labelled with two fluorescent dyes, a reporter fluorescent dye and a quencher dye. When the reporter dye is attached to the 5' end and the quencher dye is attached at the 3' end, the fluorescent dye transfers energy to the quencher dye, which is then released with cleavage from the exonuclease activity of the TaqMan DNA polymerase (Figure 2) (Livak *et al.*, 1995; Ranasinghe and Brown 2005; VanGuilder *et al.*, 2008).

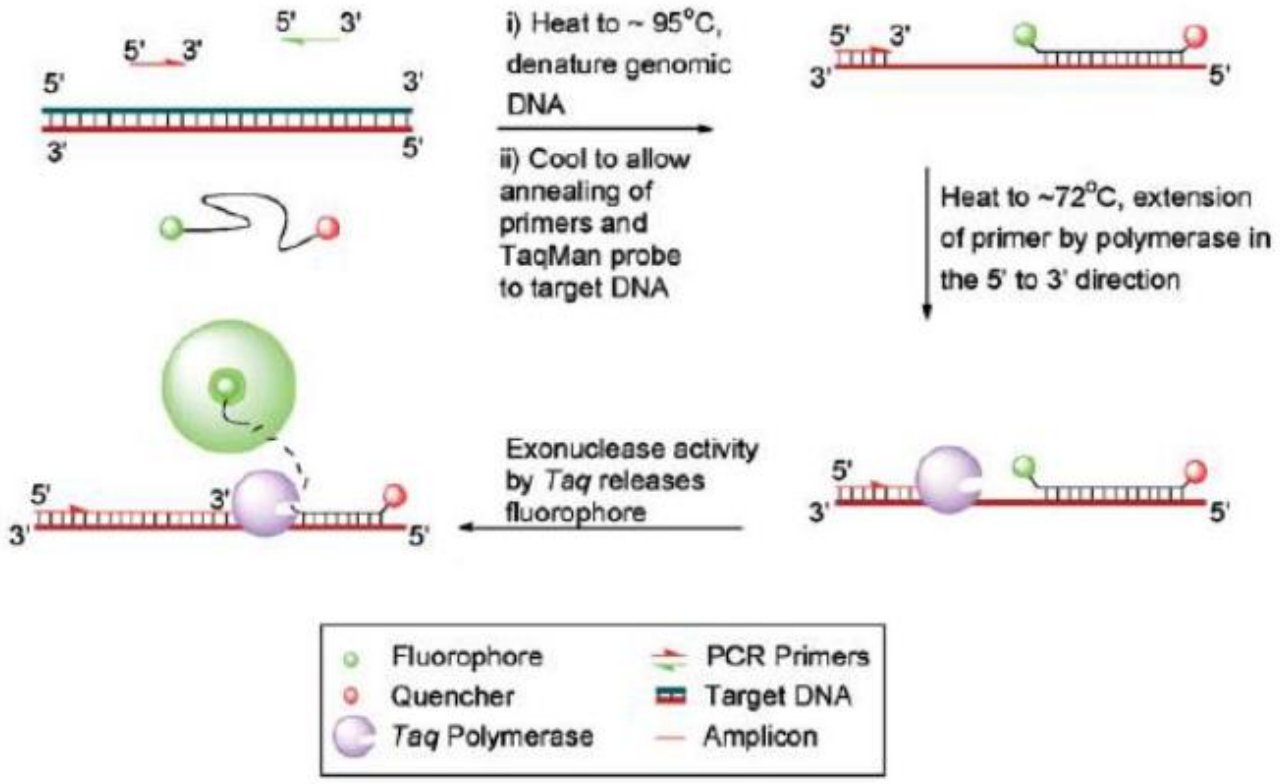

Figure 2. A model of TaqMan probe which shows fluorophore being cleaved. With each PCR cycle, target sequence is amplified. This allows more probe to bind to target sequence, more fluorophore to be cleaved, and therefore fluorescent signal to be released (Ranasinghe and Brown, 2005).

Real-time PCR (RT-PCR) has incorporated all these new technologies to achieve a method of PCR which allows for the PCR amplification product to be detected and observed while it is happening. RT-PCR uses a sequence detector which allows for the measurement of fluorescent spectra to be monitored in the wells of the thermal cycler continuously during PCR amplification (Heid *et al.*, 1996). This is advantageous for several reasons. Because the amplified products can be analyzed in real-time, there is no need to open the reaction tubes (Heid *et al.*, 1996). This lessens the risk of contamination. A second advantage is the time that RT-PCR saves because the sample does not need to be

handled after PCR amplification (Heid *et al.*, 1996) Overall RT-PCR has several advantages applications and is a useful tool in regards to the detection and identification of *Rhizoctonia solani* (Lees *et al.,* 2002).

The aims of this study are (1) to optimize RT-PCR in regards to primer and probe concentrations using a specific isolate of *Rhizoctonia solani* and (2) to further characterize *Rhizoctonia solani* through its interactions in an hyphal anastomosis killing reaction.

CHAPTER TWO

MATERIALS AND METHODS

Material

R. solani Culture

The isolates used in this experiment are: Tom7 and EGR4. EGR4 possesses detectable dsRNA. EGR4 belongs to anastomosis group 4 and originates from Egypt, with a host of *Solanum Tuberosum*. Tom7 is a reduced genome mutant isolate which does not contain the mycovirus.

Solid medium used to maintain/grow culture. Cultures were grown on Potato Dextrose Agar (PDA) which was prepared according to BD-Difco manufacturing instructions. For every 39g of PDA, 1 liter of autoclaved water was used. The solution was autoclaved for 30 minutes at 121 degrees Celsius and then poured into petri plates under a fume-hood. The petri plates were allowed to cool under the UV light, with the lids half off, in the fume-hood for approximately 30 minutes until no condensation was present. 125mL of the solution was used for 8 small petri plates (60mm) and 150mL of the solution was used for 8 large petri plates (100mm). Once the petri plates cooled down an approximate 4mm plug of an older fungal culture was transferred to the newly prepared petri plate. The plate was then closed and sealed with parafilm. Cultures were transferred every 2-3 weeks in order to ensure proper maintenance without contamination. When starting fresh cultures, ampicillin was added to the autoclaved solution before pouring the plates at approximately 1microliter per mL.

Liquid medium used to prepare/grow culture. Petri plate cultures were transferred to flasks using Malt Extract Broth (MEB) according to BD-Difco manufacturing instructions. For every 15 grams of MEB, one liter of autoclaved water was used. A solution was mixed up in a

large flask. This was poured into smaller flasks which were then sealed with a cotton swab and covered with aluminum foil. The flasks were autoclaved at 121 degrees Celsius for 30 minutes and then placed under UV light in the fume-hood for 30 minutes in order to cool. Once cooled, isolates were transferred from the petri plates to the flasks by cutting out 3 samples of the culture and dropping the pieces into the flask. The flasks were kept at room temperature and allowed to grow for at least three weeks.

Buffers

Buffers used for dsRNA extraction.

Chloroform/isoamyl alcohol:

Chloroform and isoamyl alcohol were mixed together in a 24:1 ratio.

GPS buffer (Glycine-Disodium Phosphate Saline):

In a liter of autoclaved water the following was dissolved: 15g of 0.2 molar glycine, 14.2g of 0.1 molar disodium dibasic phosphate, and 35g of 0.6 molar sodium chloride. This was adjusted to a pH of 9.5 with 5 molar NaOH and then autoclaved for 30 minutes at 121 degrees Celsius.

Saturated Phenol:

A 500mL phenol solution was brought up to 60 degrees Celsius. Then 0.1% of Hydroquinone was added.

10XSTE buffer (Sodium Chloride Tris Ethylenediamine Tetraacetic Acid):

In a liter of autoclaved water the following was dissolved: 58g of 1 molar sodium chloride, 60g of 0.5 molar tris, 3.72g of 0.01 molar Na2EDTA. This solution was adjusted to a pH of 7.0 with concentrated 12 molar HCl and autoclaved for 30 minutes at 121 degrees Celsius. For the dsRNA extraction procedure, the 10X STE was diluted to 1X STE.

1.18% STE EtOH:

To prepare 500mL of 1.18% STE EtOH solution, 59mL of 10X STE was first added to 441mL of autoclaved water. This solution was then added to 75mL of absolute EtOH until it reached 500mL.

3 M NaOAc:

In 50mL of autoclaved water the following was dissolved: 20.4g of sodium acetate. Glacial acetic acid was added to adjust the pH to 5.2.

Buffer used for electrophoresis.

10X TAE buffer (Tris-Acetate EDTA):

In a liter of autoclaved water the following was dissolved: 48.4g of tris base, 7.4g of sodium EDTA, 16.4g of sodium acetate, and 17.0mLof glacial acetic acid. This was then autoclaved on the liquid-cycle at 121 degrees Celsius for 30 minutes.

Plasmids

LB/Ampicillin/X-gal plate:

In 500mL of autoclaved water 15.5g of Difco Nutrient Agar 1.5% was dissolved. This was mixed gently and autoclaved for 20 minutes. The autoclaved agar was then placed into a water bath in order to cool down to 55 degree Celsius. 2mL of 50mg/mL ampicillin was added into it along with 1mL of x-gal solution and 250microliters of IPTG. This solution was mixed thoroughly, avoiding any bubble formation, and then was poured into plates. 500mL can make 20 plates.

Nutrient Broth:

Nutrient broth was prepared according to BD-Difco manufacturing instructions. A liter of autoclaved sterilized water was used for every 15g of nutrient broth. The solution was autoclaved for 30 minutes. 2microliters per 1mL of 100mg/mL ampicillin is added.

Primers/Probe

Primers and Probes used were previously sequenced and ordered from Biosearctech (Chin, S. M., 2012). The EGR4 Probe had a sequence of AGTGCCGATCAGCCCTCCACCG with 5' end of FAM and a 3' end of BHQ-1.The EGR4 forward primer had a sequence of AGCGCTGACCTTGCTATCGAATC. The EGR4 reverse primer had a sequence of CCACCGGAAGAGGGAAATCC.

Methods

Extraction of DsRNA

The extraction of dsRNA was done using a procedure developed by Morris and Dodds (1979) with modifications as described by Bharathan and Tavantizis (1990). DsRNA was extracted from the fungal flask cultures after approximately four weeks of growth. 15-20g of mycelium was harvested by pouring the flask into a funnel filtered with two autoclaved sterile napkins. The mycelium was rinsed with autoclaved distilled water. The mycelium was then placed into a plastic bag and squeezed to remove any excess water. The bag with the mycelium was then placed into liquid nitrogen for fifteen minutes. Once frozen the mycelium was crushed using a pestle until it was broken up into small fine pieces. The bag was then cut at the top and the mycelium contents were poured into a homogenizing jar which contained: 30mL of GPS buffer, 30mL of 24:1 chloroform:isoamyl alcohol, 1mL of 10% SDS, and 10mL of saturated phenol. The mixture was homogenized, using a Polytron PT 6100, in increments of 1 minute on and 1 minute off until the mixture was liquid with no fragments seen. The homogenized mixture

was poured into 4 30mL glass corex tubes and was centrifuged, on an Avanti Centrifuge J-25 Beckman Coulter, at 8000rpm for 10 minutes at 4 degrees Celsius. The upper aqueous phase was carefully removed and added into a small 100mL beaker with a stirbar. This was adjusted to 15% absolute ethanol. 1g of CF-11 Whatman cellulose powder was added and the mixture was stirred slowly on ice for 90 minutes. The mixture was then poured into two corex glass tubes and centrifuged again at 8000rpm for 10 minutes at 4 degrees Celsius. The supernatant was discarded and the pellet resuspended in 1.18% STE EtOH. The cellulose suspension was then poured into a column and washed with approximately 200mL of 1.18% STE EtOH until the cellulose column turned white in color. The dsRNA was then eluted, in a sterile 38mL polyallomer tube, from the cellulose column with 10mL of 1X STE which was added a few drops at a time so as not to disturb the column. 40microliters of 3M sodium acetate was added. Cold absolute EtOH was added until the level reached approximately a half inch from the top of the tube. The tube was then labeled and stored in -20 degrees Celsius and allowed to precipitate overnight.

Gel Electrophoresis

To prepare for electrophoresis, the dsRNA that was allowed to precipitate overnight was pelleted through centrifugation at 12000rpm for 30 minutes at 4 degrees Celsius using an Optima L-90K Ultracentrifuge Beckman Coulter. The pellet was allowed to air dry for 45 minutes while on ice and then resuspended with 60microliters of DEPC treated water. While this was being done, a 1% agarose gel was prepared using 1g of agarose for every 100mL of 1X TAE buffer. The mixture was heated in order to thoroughly dissolve the agarose and then 20microliters of ethidium bromide stain was added to the agarose solution and gently mixed. This solution was then poured into a casting tray with a comb inserted and allowed to cool and solidify for 60 minutes. Once ready, the dsRNA samples, mixed with 6x loading buffer dye, were loaded onto

the gel along with lambda DNA HindIII/EcorI marker. The gel was run at 40V for approximately 4 hours. Pictures were taken using the Kodak Image Station 4000R Pro.

Determination of DsRNA Concentration

Concentration of dsRNA was determined on a Beckman DU-800 spectrophotometer. Absorbance was found at 260nm and 280nm. The spectrophotometer was first blanked with 100microliters of DEPC treated water pipetted into a cuvette. 1microliter of the sample was then added into a cuvette followed by 99microliters of DEPC treated water, mixing the two by drawing it back up into the pipette and then back into the cuvette a couple of times. Absorbance was found at 260nm and 280nm. Equation 1, as described by Barbas III *et al.* (2001), was then used to detect the purity of the sample using an OD260/OD280 ratio. Equation 2 was used to quantify the amount of nucleic acid found in each sample (Figure 3).

$$\frac{OD260_{nm}}{OD280_{nm}} = \text{dsRNA Purity}$$

Equation 1. Purity of nucleic acid indicated by an optical density ratio.

$$[\text{RNA}]\ \text{micrograms/mL} = OD_{260nm} * \text{Dilution Factor} * \text{Extinction Coefficient}$$

Equation 2. This equation measures the amount of dsRNA in a sample.

Figure 3. Equations used in determining dsRNA purity and amount of dsRNA in the sample.

Plasmid Plating and Colony Selection

Liquid colonies, which had been previously transformed (Chin, S. M., 2012) and stored in -80 degrees Celsius, were thawed by placing them in -20 degrees Celsius for approximately 5 hours and then moved to -4 degrees Celsius for approximately 24 hours. Serial dilutions were made at room temperature. 50 microliters of each thawed sample was plated on the X-gal, IPTG, ampicillin plates using two dilutions to ensure that single colonies were seen. The plates were placed in an incubator at 37 degrees Celsius. Growth was seen approximately 24 hours later and colonies were picked at approximately 48 hours. Approximately 4 colonies from each plate were picked using a pipette tip. The pipette tips were dropped into separate 10mL tubes, containing approximately 4mL of nutrient broth, and the tubes were shaken. The tubes were placed in an incubator at 37 degrees Celsius at 110rpm. The tubes were allowed to grow for 48 hours before the plasmids were purified.

Plasmid Purification

The plasmids were purified using a Fermentas GeneJet Plasmid Miniprep Kit. 1.9mL of each growth culture was taken and placed into separate sterilized 2mL tubes and centrifuged at 8000rpm for two minutes at room temperature. The tubes were centrifuged for a second time at 8000rpm for two minutes at room temperature. The supernatant was then poured into a waste beaker and the pellets resuspended by vortexing in 250microliters of the resuspension solution. In each tube, 250microliters of lysis solution was added and the tubes mixed by inverting the tube 4-6 times. 350microliters of the neutralization solution was then added and the tubes and mixed immediately by inversion of the tube 4-6 times. The tubes were centrifuged for 5 minutes at 12000rpm at room temperature. The supernatant from each tube was transferred to a separate

supplied column. The columns were centrifuged for 1 minute at 12000rpm at room temperature. The flow-through was discarded. 500microliters of wash solution was added and centrifuged at 12000rpm at room temperature for 1 minute and the flow-through discarded. This wash step was done a second time. The empty column was then centrifuged at 12000rpm for another minute to remove residual wash solution. The column was transferred to a new centrifuge tube and 50microliters of the elution buffer was added. The column was allowed to incubate at room temperature for 2 minutes and then centrifuged for 2 minutes at 12000rpm to collect the flow-through. Plasmid DNA was stored at -20 degrees Celsius.

Plasmid Digestion

The plasmids were digested by placing 2microliters of each DNA sample into a separately labeled tube. 15microliters of DEPC treated water, 2microliters of 10x Fast Digest Buffer, and 1microliter of EcoR1 were also added with the samples. The tubes were incubated in a water bath at 37 degrees Celsius for 5 minutes.

Real-time PCR: Optimization of Primer and Probe

Real-time PCR, using the SensiMix II Probe Kit, was optimized using different concentrations of primers and probes with the plasmid cDNA. Forward and reverse EGR4 specific primers were first diluted to concentrations of 200nM, 400nM, and 600nM. The EGR4 specific probe was diluted to 50nM, 100nM and 150nM concentrations. Table 2 shows the amounts of Sensi 2x, reverse primers (RP), forward primers (FP), probe, DEPC-treated water and cDNA template added into each sample tube for each specific primer/probe concentration.

Table 2

Primer/Probe Concentrations in nM and Amounts Added to Sample Tubes for RT-PCR Optimization.

	Volume (uL)						
Primer:Probe	Sensi 2X	RP	FP	Probe	Water	Template	Total
200:50	12.5	0.500	0.500	0.250	8.750	2.50	25
200:100	12.5	0.500	0.500	0.500	8.500	2.50	25
200:150	12.5	0.500	0.500	0.750	8.250	2.50	25
400:50	12.5	1.000	1.000	0.250	7.750	2.50	25
400:100	12.5	1.000	1.000	0.500	7.500	2.50	25
400:150	12.5	1.000	1.000	0.750	7.250	2.50	25
600:50	12.5	1.500	1.500	0.250	6.750	2.50	25
600:100	12.5	1.500	1.500	0.500	6.500	2.50	25
600:150	12.5	1.500	1.500	0.750	6.250	2.50	25
NTC	12.5	1.500	1.500	0.750	8.750	0.00	25

The tubes were placed into an Eppendorf Realplex2 Mastercycler. The program was set to TaqMan probe with FAM color calibration, using Eppendorf White Plate/Tubes 10microliters as a background. An initial first cycle of 10 minutes at 95 degrees Celsius was run. This was followed by 30 cycles consisting of first 10 seconds at 95 degrees Celsius and then 60 seconds at 60 degrees Celsius (Figure 4).

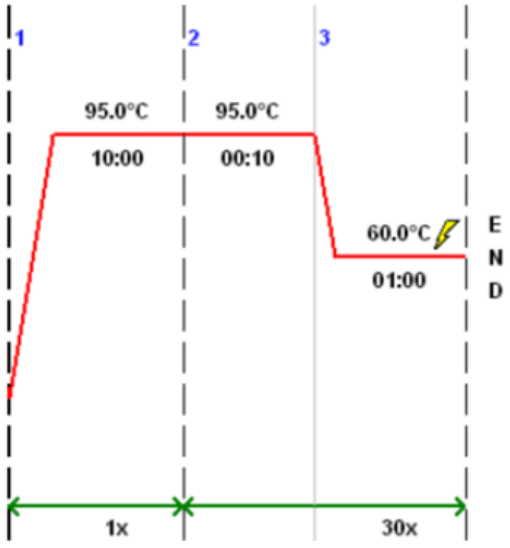

Figure 4. The RT-PCR program.

Plating/Flasking Hyphal Anastomosis Barrage Lines

PDA was prepared and poured into petri plates. The plates were allowed to cool under UV light for approximately 30 minutes. Plugs taken from two separate *Rhizoctonia solani* isolates were placed on one petri plate following a template placed underneath the plate. This ensured that the plugs were placed equidistant to each other and to the edges of the plate. Cultures were allowed to grow until a barrage line formed at approximately 4 days. Three pieces of the barrage line were taken and transferred to a flask containing prepared MEB. The flasks were allowed to grow for 3-4 weeks.

Micro-Chip Analysis of Barrage Lines

Micro-chip analysis was performed on the dsRNA that was extracted from the flasks containing the barrage line cultures as well as on dsRNA from single isolates. The dsRNA was pelleted through centrifugation at 12000rpm for 30 minutes at 4 degrees Celsius using an Optima L-90K Ultracentrifuge Beckman Coulter. The dsRNA concentration was determined and the sample was diluted with DEPC treated water to between 0.5-50ng/microliters.

An Agilent DNA 7500 kit was used with the Agilent 2100 Bioanalyzer for the micro-chip analysis. The Gel-Dye Mix was prepared following manufacturing instructions. The DNA dye concentrate and DNA gel matrix were first allowed to equilibrate to room temperature for 30 minutes. The DNA dye concentrate was vortexed and 25microliters of the dye concentrate was added to a DNA gel matrix vial. The solution was vortexed and spun down and then transferred to a spin filter. This was centrifuged at 13000 rpm for ten minutes. The solution was stored at 4 degrees Celsius until used, being careful not to expose the solution to light.

The samples were loaded onto an Agilent DNA chip. The prepared gel-dye mix was allowed to equilibrate to room temperature and a new chip was placed onto the priming station.

9microliters of the gel-dye mix was first pipetted into a gel-dye marked well by pushing down on the plunger of the syringe attached to the priming station. The plunger was held down by a clip for 30 seconds. Once the clip was released, after five seconds, the plunger was pulled slowly back to its original position. 9microliters of gel-dye mix was pipetted into a second gel-dye marked well. 5microliters of marker was pipetted into all the wells. 1microliter of DNA ladder was then pipetted into the specific ladder well. 1microliter of each sample was pipetted into separate sample wells while 1microliter of de-ionized water was pipetted into the un-used sample wells. The fully-loaded chip was vortexed for a minute at 2400 rpm. The chip was then run in the bioanalyzer.

CHAPTER THREE

RESULTS

Extraction of DsRNA

The dsRNA extracted from the EGR4 isolate of *R. solani* was detected on a 1% agarose gel electrophoresis stained with ethidium bromide (Figure 5). The gel showed the characteristic viral dsRNA band of around 3.3kb that is associated with this particular isolate. This falls within the medium (M) range of 1.0kb to 4.5kb (Bharathan and Tavantzis, 1991).

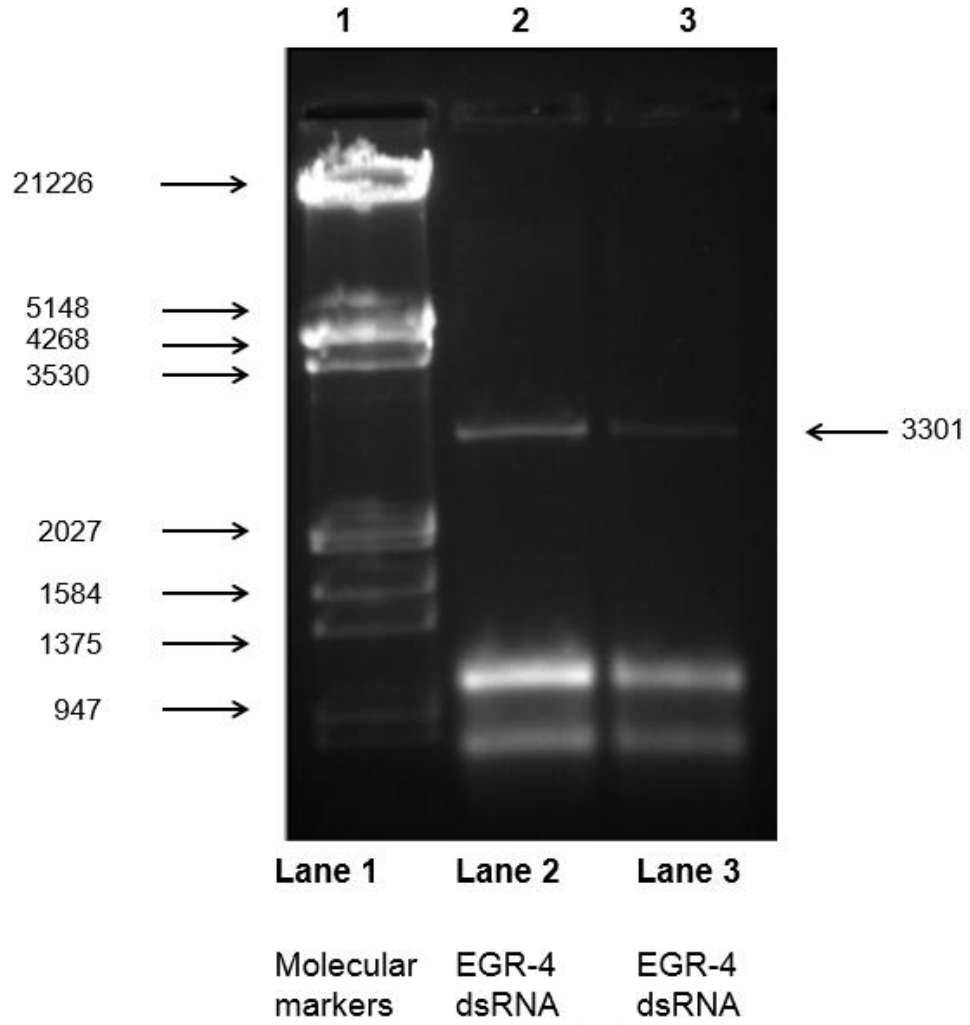

Figure 5. Lanes 2 and 3 show the characteristic viral dsRNA bands extracted from an EGR4 fungal culture and run on a 1% agarose electrophoresis gel.

Plasmid Purification, Digestion, and Spectrophotometry

Plasmids transformed using the viral dsRNA were grown, purified and digested with the digestion enzyme EcoR1. The resultant bands were detected on a 1% agarose gel electrophoresis stained with ethidium bromide showing that the transformation was successful with the presence of cDNA (Figure 6). The plasmid product in lane 1 on the electrophoresis gel suggested the best transformation due to its higher base pair amount. The purified plasmid products were run through the spectrophotometer to determine cDNA purity (Figure 7). Based on the absorbance at wavelengths 260nm and 280nm a 260/280 ratio was determined for each purified plasmid (Table 3). A ratio above 1.5 is ideal. Due to both its base pair amount and its purity, the plasmid in lane 1 was chosen for real-time PCR.

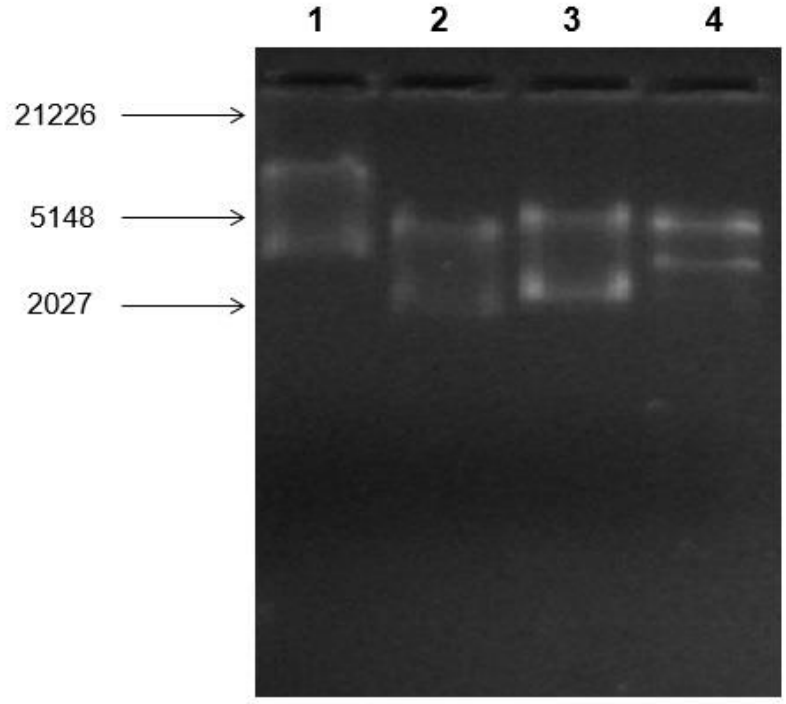

Figure 6. Electrophoresis gel of plasmid products. Lanes 1, 2, 3, and 4 show the purified plasmids of four separate colonies after they have undergone a Fast Digest. The bands show a successful transformation and presence of cDNA.

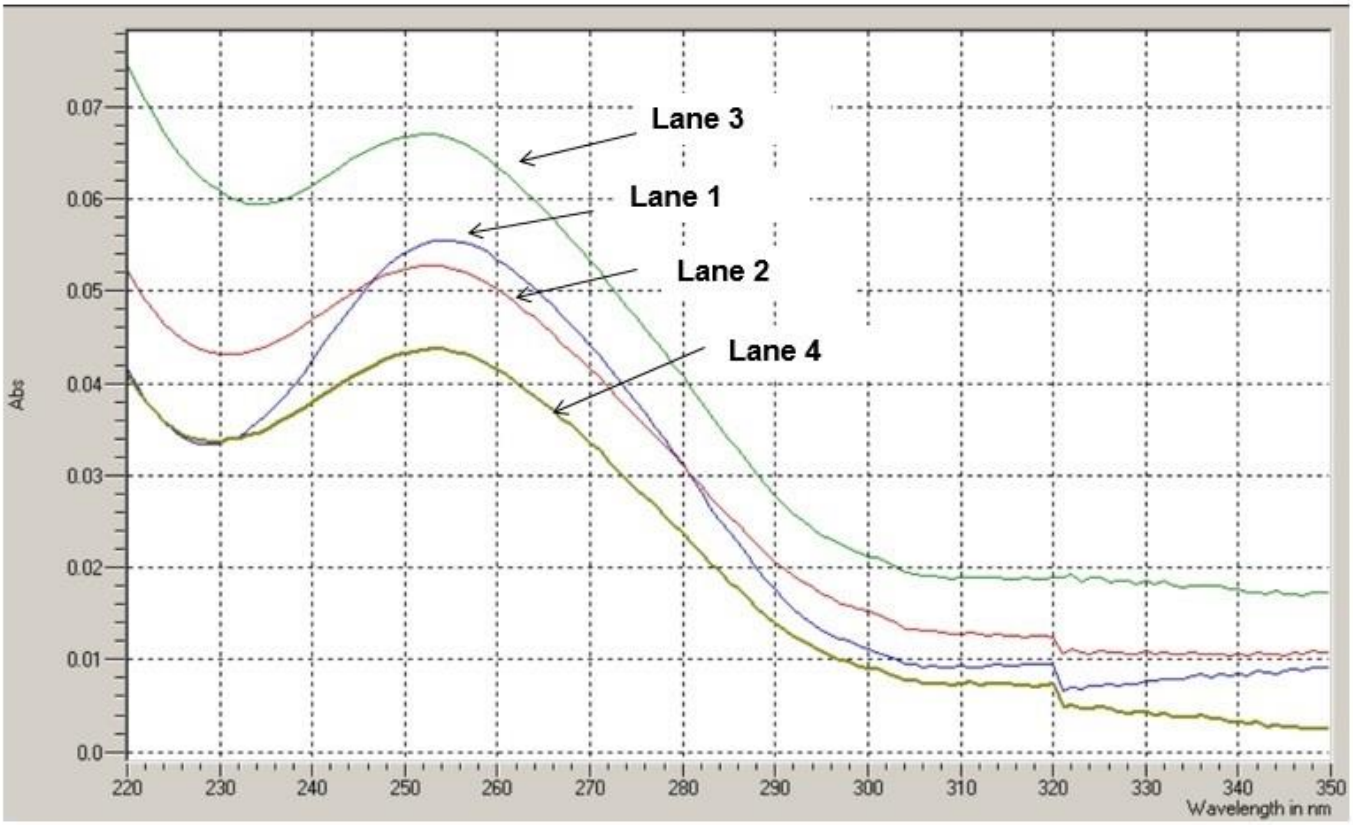

Figure 7. Spectrophotometric graph of the four purified plasmids' absorbance at a wavelength of 220nm to 350nm.

Table 3

260 and 280 Absorbance Values as Determined via Spectrophotometric Analysis for Each Purified Plasmid.

Lanes	260	280	260/280
Lane 1	0.0533	0.0313	1.703
Lane 2	0.0502	0.0311	1.614
Lane 3	0.0635	0.0408	1.556
Lane 4 (Blue colonies)	0.0415	0.0238	1.744

Real-Time PCR

Real-time PCR was performed with the cDNA that was previously purified. Varying concentrations of primer and probe were used in order to determine the best combination for detection of EGR4. A combination of 200nM/150nM primer/probe concentration yielded the smallest threshold value at 3.21 (Figure 8). A primer to probe concentration of 400nM/150nM was the second best combination. This suggests that a lower concentration of primer and a greater concentration of probe is associated with greater amplification. This also showed how important the right combination of primer and probe concentrations is. Some combinations resulted in absolutely no detectable amplification. The highest concentration of primer at 600nM showed almost no amplification regardless of probe concentration. Other combinations resulted in amplification at a greater cycle number.

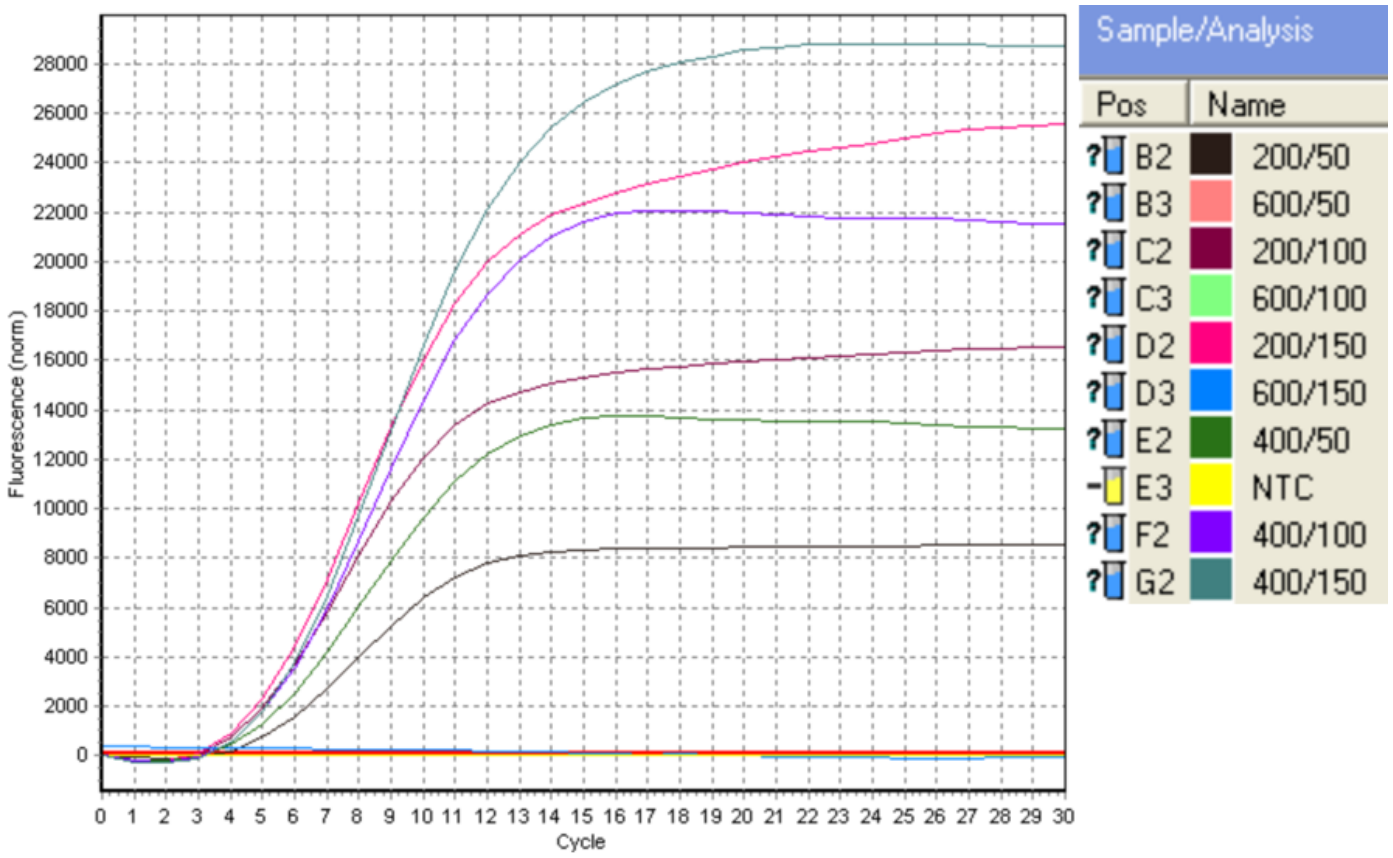

Figure 8. Amplification and C_t -FAM values for each primer/probe concentration combinations.

Barrage Line Analysis

Two *R. solani* isolates, Tom7 and EGR4, were placed equidistance from each on a petri dish and upon meeting in the middle, formed a barrage line (Figure 9). Double-stranded RNA that was extracted from the flasked cultures of the barrage lines and flasks containing the two isolates. This dsRNA was run on a 1% agarose gel electrophoresis (Figure 10). The same samples underwent microchip analysis. The resultant electropherograms (Figures 11, 12, and 13) and microchip-electrophoresis (Figure 14) showed the effects of a barrage line kill reaction in anastomosing isolates on the ribosomal RNA (rRNA) bands. While Tom7 had clear peaks on the

electropherogram the EGR4/Tom7 sample resulted in almost no rRNA peaks, similar to what was seen with EGR4.

Figure 9. Petri plate containing the isolates EGR4 and Tom7. In between the two isolates, where they meet, a barrage line from a killing reaction formed.

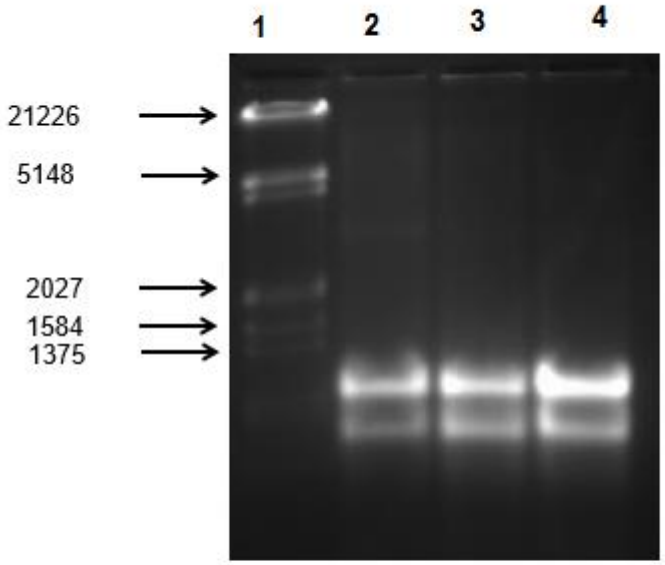

Figure 10. Gel electrophoresis of dsRNA extraction samples. Lane 1 contains lambda DNA markers. Lane 2 contains EGR4 dsRNA sample. Lane 3 contains EGR4/Tom7 barrage line dsRNA sample. Lane 4 contains Tom7 dsRNA sample.

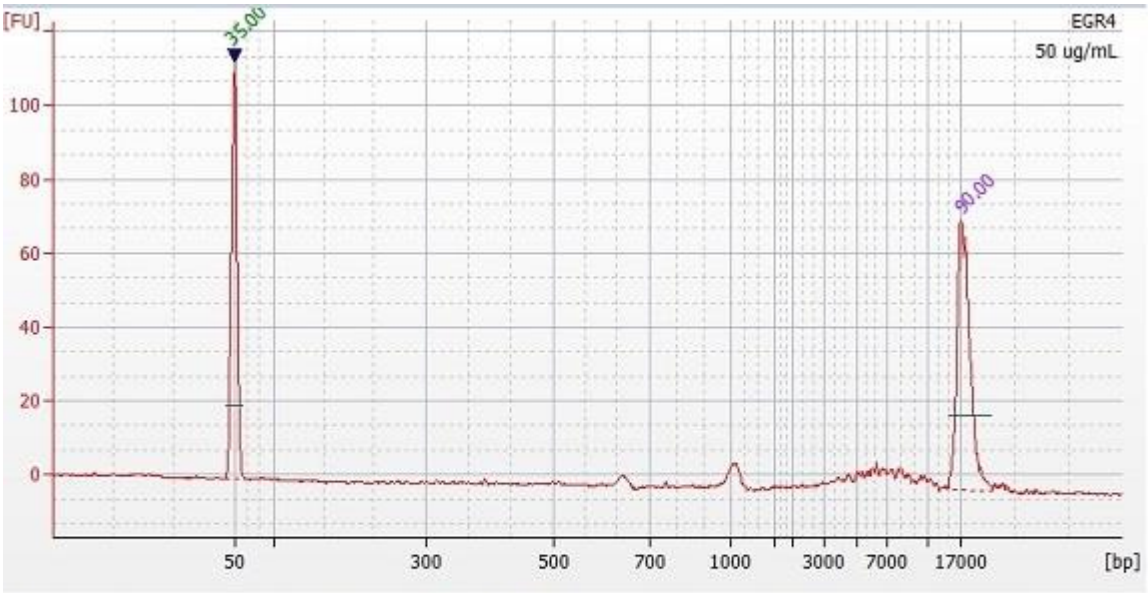

Figure 11. The electropherogram of the dsRNA extraction sample taken from EGR4.

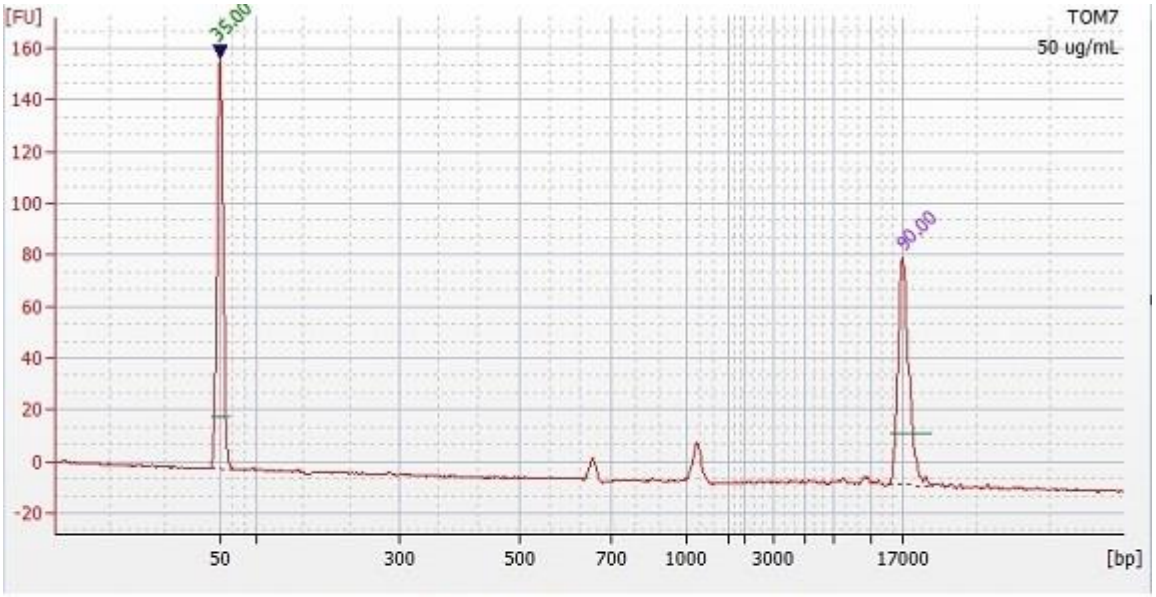

Figure 12. The electropherogram of the dsRNA extraction sample taken from Tom7.

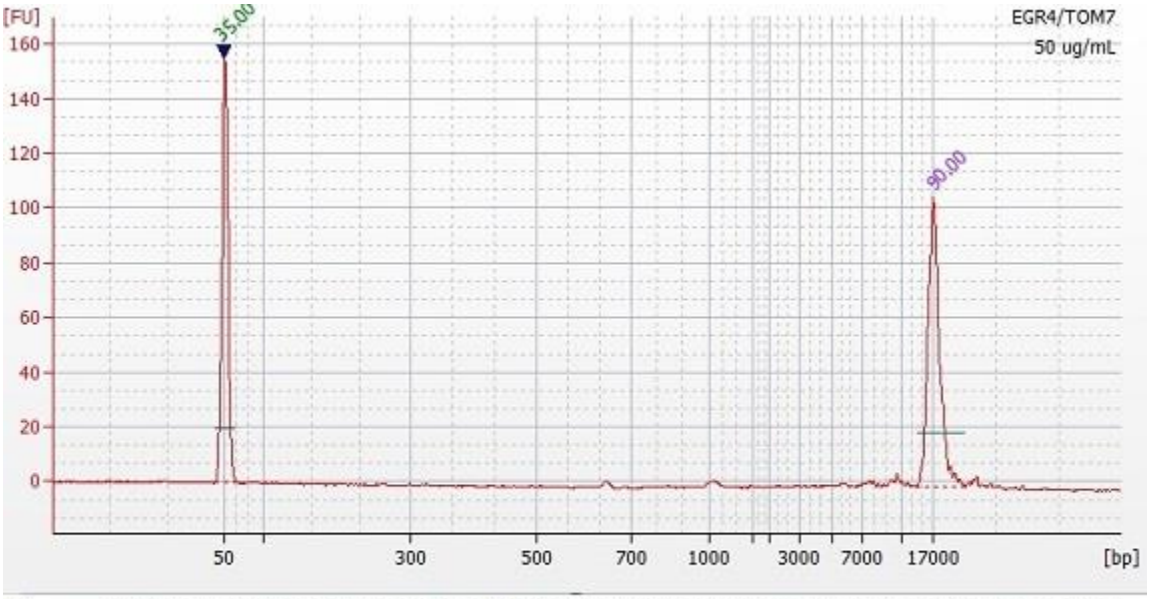

Figure 13. The electropherogram of the dsRNA extraction sample taken from the barrage line of EGR4/Tom7.

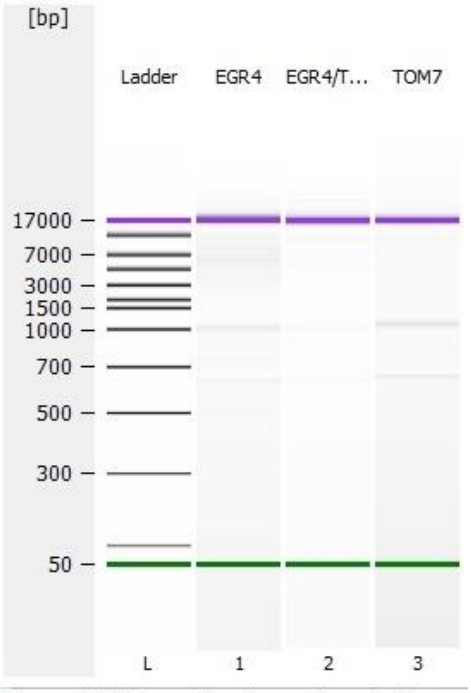

Figure 14. Micro-chip electrophoresis. L contained the ladder. Lane 1 contained sample from EGR4. Lane 2 contained sample from EGR4/Tom7. Lane 3 contained sample from Tom7.

CHAPTER FOUR

DISCUSSION

The main goal of my study was to develop an optimized RT-PCR protocol with specific regards to primer and probe concentrations. I used the *R. solani* isolate EGR4 in order to achieve this goal. A secondary goal of my study was to further characterize EGR4 specifically the effects of its virus in interactions with the non-virus-containing isolate, Tom7. Overall I was able to develop an optimized RT-PCR protocol that successfully amplified EGR4 within a few cycles. I was also able to elucidate further the interaction between a virus-containing isolate and a non-virus-containing isolate of *R. solani* and what happens during a barrage line reaction between those two isolates.

During this study I encountered several problems which needed to be addressed. At first, the dsRNA extraction that I performed would not yield the expected viral bands. After much trial and error I was able to discover that several solutions were bad. The GPS buffer had somehow become acidic, less than a pH of 1, instead of at a pH of 9.5 and the saturated phenol had degraded, turning dark brown in color. Also, a fifteen minute stirring step was added to the extraction protocol. Once the fungal solution had been homogenized, in order to better mix the solution, it was stirred for fifteen minutes with a glass stir rod. Fixing the GPS buffer and Phenol solution and incorporating this extra step fixed the dsRNA extraction problem and yielded a decent amount of dsRNA with few impurities.

Another problem during the study was getting the isolates to grow properly. Since the plasmids used in this study were previously transformed (Chin, S.M., 2012), they were kept stored at -80 degrees Celsius. A method for thawing out these plasmid isolates had not been previously described. My first attempt at thawing the isolates within a day was too quick and the

isolates did not grow on the nutrient agar. I then tried thawing the isolates out over a period of days and at three different increasing temperatures. This did work and growth was observed. The extra thawing steps resulted in both growth of the isolates, but also viable plasmids from the isolates.

The optimization of real-time PCR (RT-PCR) was done using different primer and probe concentrations. The most effective primer and probe concentrations were successfully determined for the identification of the *R. solani* isolate EGR4 cDNA. The lowest threshold cycle value (C_t-FAM), at 3, was found for a primer concentration of 200nM and a probe concentration of 150nM. This suggests that a lower concentration of primer and greater concentration of probe causes greater amplification. In fact, the largest concentration of primer that was tested, 600nM, showed no amplification. I believe that this is due to the formation of primer dimers with a lesser amount of available primers to assist in amplification. While the lowest concentration of probe did result in amplification, the amplification had higher C_t-FAM values and plateaued at a lower amplification amount. I feel that this is most likely due to not enough probe to release a fluorescent signal with the amplification.

In this study only the primer and probe concentrations were optimized with EGR4. I used a simple SensiMix II protocol program was run on the RT-PCR. I wanted to ensure that the primer/probe combinations were the primary focus of the study. Although future optimization studies should be done on different temperatures for the different PCR steps as well as on $MgCl_2$ concentrations. Both of these two things play important integral roles along with the primer and probe. Optimizing all of these components would be an excellent next step for future studies in this research. One main limitation of the RT-PCR of this study however is the lack of repetition. Repeated trials should be done to ensure that the results are replicable and reliable.

Along with the RT-PCR analysis, I performed a microchip analysis. The analysis was performed on dsRNA extraction samples taken from EGR4, Tom7, and the barrage line of those two isolates. The results showed modest ribosomal RNA (rRNA) in the barrage line sample as well as the EGR4 sample. The rRNA peaks were visibly larger in the Tom7 sample however. Since EGR4 contains the mycovirus and Tom7 does not, this lack of rRNA in the barrage line sample could be due to the virus. When the two samples meet on the plate, they anastomose and material gets passed between the two isolates. This suggests the possibility that the virus acts on the fungus in such a way as to reduce the rRNA. No viral dsRNA bands were observed on microchip analysis however for EGR4. I suspect that this is due to the fact that the dsRNA was not extracted in the peak virus timespan (within 3-4 weeks of inoculating the flasks). I am certain that this did not have an effect on the barrage line results however. I performed the extraction of the barrage line samples within a month of inoculating the flasks. Also, the isolates were freshly transferred to the petri plates to form the barrage lines.

I suspect that the reduction of rRNA seen in the barrage line is in fact related to the virus within the fungus. Reduction of rRNA should have an effect on overall protein expression. These results support the idea that the virus has an effect on the fungus somehow. I am uncertain however how the fungus would be effected by the virus. Whether the virus results in a hypo- or hyper- virulence is unclear with the microchip analysis. While the microchip analysis results could be due to the mycovirus, I cannot overlook the possibility that the reduction of rRNA is due to the killing reaction of anastomosis. Future studies would profit by doing further work to elucidate the cause of the rRNA reduction and also the effects that reduction has on the phenotype of the fungus.

The results of this study strengthens how powerful of a tool a TaqMan probe with RT-PCR can be in the detection and identification of nucleic acid. The pervasiveness of *R. solani* among different agricultural crops has a large impact on the farming industry. Due to its ability to cause such devastating crop death this plant pathogenic fungus is of great agricultural interest. Being able to quickly identify the specific blight affecting the crops will lead to faster treatment and less crop loss. My study has shown that, when optimized, a specific isolate of *R. solani* can be positively identified within 3 RT-PCR cycles. This means that a positive identification can be made within approximately 15 minutes. My study has also shown further characterization of the relationship between isolates and how a mycovirus may be affecting *R. solani.* In conclusion this was a successful study with results which have an important impact on the understanding of *Rhizoctonia solani* and future studies.

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