STUDY OF RESISTANCE TO ROSE ROSETTE DISEASE UTILIZING FIELD RESEARCH, MOLECULAR TOOLS, AND TRANSMISSION METHODS

A Dissertation

by

MADALYN KIRSTEN SHIRES

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Chair of Committee,	Kevin Ong
Committee Members,	Herman Scholthof
	Olufemi Alabi
	David Byrne
Head of Department,	Leland S. Pierson

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ABSTRACT

Rose rosette virus (RRV) is a (-) ssRNA virus that is vectored by an eriophyid mite, *Phyllocoptes fructiphilus,* and has an exclusive plant host, roses. Roses become infected with the virus through vector feeding on plant tissues. The virus produces a wide array of symptoms on the plant, with the most common one being witches' broom, or rosette, giving the infection on plants the name of Rose Rosette Disease (RRD). Currently there is no known resistance to RRV in roses, however there are approaches being taken to discover tolerance, resistance, or immunity to the virus, such as resistance screening of currently available cultivars. In addition, the creation of new seedlings from parents with varying degrees of resistance, discovery of affordable treatments for the vector, and research on effective treatments for the virus are all being utilized for better management of RRD. These approaches have resulted in some preliminary data to inform/guide future focus of this area of study.

One area of focus for this research has been RRD resistance screening field trials. Through field trials in north Texas, over 200 cultivars of roses have been screened for resistance since 2016, with some possible RRV tolerant cultivars identified. Correct diagnosis of RRV in infected plants is another important factor in finding a source of resistance. Through development and optimization of sensitive RRV extraction and detection protocols, RRV detection has been greatly improved, allowing for more rapid screening of trial plants. The third methodology that is being investigated are alternative transmission pathways for RRV virus infection. Anecdotal evidence suggests that RRV

may be spread through roots of infected plants to healthy plants. Additionally, other typical methods of viral transmission such as seed, grafting, and mechanical will be examined in regard to RRV transmission. All preliminary data gained through this study on RRV has been used to contribute knowledge and improve understanding of RRD with the goal of finding RRD resistance.

DEDICATION

This dissertation is dedicated to my Aunt Margie, Marjorie Shires Price, who passed away due to Coronavirus less than two months before this document was finished. She was a huge inspiration in my life and showed me what it was like to be a pioneer in a chosen career field. She supported me through every part of my educational journey, and I wish that she could have seen the finished product.

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Contributors

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All work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

RRV	*Rose rosette virus*
RRD	Rose Rosette Disease
WSMV	*Wheat streak mosaic virus*
EMARaV	*European mountain ash ringspot-associated virus*
HPWMoV	*High Plains wheat mosaic virus*
AAP	Acquisition Access Period
IAP	Inoculation Access Period
PPSMV	*Pigeon pea sterility mosaic virus*
LBI	Liquid Biosynthesis Inhibitor
RNA	Ribonucleic Acid
ORF	Open Reading Frame
RdRP	RNA-dependent RNA polymerase
nt	Nucleotide
ATP	Adenosine Triphospahte
DMB	Double Membrane-bound Bodies
mRNA	messenger RNA
ER	Endoplasmic Reticulum
FMV	*Fig mosaic virus*
RLRV	*Redbud leaf roll virus*
PPSMV-1	*Pigeon pea sterility mosaic virus 1*

PPSMV-2	*Pigeon pea sterility mosaic virus 2*
AcCRaV	*Actinia chlorotic ringspot-associated virus*
rRNA	ribosomal RNA
vRNA	viral RNA
I-20	Interstate 20
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RT	Reverse Transcription
cDNA	complementary DNA
RT-PCR	Reverse Transcription PCR
qPCR	Real-Time Quantitative PCR
EtBr	Ethidium Bromide
C_t	Cycle Threshold
SDT	Simple Direct Tube Extraction
MDA-C	Modified Direct Antigen-Capture
TMV	*Tobacco mosaic virus*
CaCV	*Capsicum chlorotic virus*
RSDaV	*Rose spring dwarf virus*
RYVV	*Rose yellow vein virus*
BCRV	*Blackberry chlorotic ringspot virus*
PNRSV	*Prunus necrotic ringspot virus*
ApMV	*Apple mosaic virus*

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CHAPTER I

INTRODUCTION

Overview of Rose Rosette Disease System

Rose rosette disease (RRD) has been documented in the United States for almost 80 years but became a national problem over the last 30 to 40 years. The disease has been documented on all types of roses (wild, landscape, garden), however a large body of the RRD research has been done on *Rosa multiflora* plants. The disease is vectored by the eriophyid mite, *Phyllocoptes fructiphilus*, and it was thought that the toxigenic effects from mite feeding were causing the abnormal symptoms (Allington, et al., 1968). However, in the 1950's and 1960's, it was determined through grafting studies that a virus (tentatively named *Rose rosette virus*) was causing the symptoms (Allington, et al., 1968). In the 1980's, scientists saw round, double membrane bound virus-like particles in plant cells using electron microscopy (Gergerich & Kim, 1983). In 2011, the causal agent of RRD was confirmed to be a virus, *Rose rosette virus* (RRV), a (–) ssRNA (Babu, et al., 2017a) virus in the genus *Emaravirus* (Babu, et al., 2017b; Laney, et al., 2011).

Multiflora Rose

When RRD was first discovered in Canada in 1940, it was infecting species roses such as *Rosa woodsii* (Connors, 1941; Epstein & Hill, 1999). However, over time, it was found that RRD infected and was lethal on the noxious weed, *Rosa multiflora* (Epstein, et al., 1999). Multiflora rose was introduced into the United States for rose breeding purposes in the latter part of 1866 (Kurtz & Hansen, 2013). It was a desirable

rose to improve hybrids with because it is hardy, produces large amounts of seed and vegetative growth each year, and is tolerant to some diseases such as black spot (Epstein & Hill, 1999). This rose was also highly desirable because it worked well as a living fence and as refuge for small wildlife and birds (Kurtz & Hansen, 2013). This was especially important during the Dust Bowl era when living fences served as barriers to helped prevent erosion.

Once introduced to the United States, and especially after the 1930's, Multiflora rose became an invasive pest, being registered as a noxious weed in many states. It produces abundant fruits, so it is successfully spread via birds and mammals (Epstein & Hill, 1999). To manage the multiflora rose noxious weed problem, the idea that RRD could be introduced into areas with high populations of this invasive rose species was suggested (Amrine, 2002). It was already known that the disease (thought to be viral) could be transferred to other plants by inoculating the plant with mite infected tissue (Epstein & Hill, 1999).

Researchers had also found that eriophyid mites could overwinter on the roses under bud scales, thus maintaining the mite population and allowing for RRD to spread naturally each year. Consequently, RRD infected roses would die within 5-6 years. (Epstein & Hill, 1999). Thus, it was believed that inoculating multiflora plants with mites which spread RRD would be an effective biological control agent for Multiflora rose (Epstein & Hill, 1999). While some researchers looked into this bio-control opportunity, and the U.S. Forest Service recommended it for a short period of time, the

method was never heavily used, due to the possibility that RRD could affect ornamental roses (Amrine, 2002).

Eriophyid Mites

Phyllocoptes fructiphilus, an eriophyid mite, was identified in the 1940's on roses displaying symptoms of RRD. Early studies thought that the symptoms (i.e. excessive vegetative canes and rosetting) expressed on plants were due to mite feeding (Allington, et al., 1968). Pathogen vectors are known to modify the plant during epidermal feeding (Nault, 1997) through emitting phytotoxic substances, called toxicogenic feeding, which produces toxemia symptoms in plants (Green & Capizzi, 1990). The toxemia symptoms can be present at a localized feeding point or can be systemic causing reduced growth and chlorosis (Green & Capizzi, 1990). Common toxemia symptoms are stippling, leaf curling, leaf and stem distortion, and general plant decline (Green & Capizzi, 1990). Other eriophyid mites had been found to affect their host in this way (Allington, et al., 1968), so it was reasonable to assume that most RRD symptoms were results of mite feeding.

However, transmission studies conducted in the 1950's through the 1980's proved that RRD symptoms could be replicated in uninfected plants by grafting (Allington, et al., 1968; Di Bello, et al., 2017; Epstein, et al., 1997), so the symptoms became associated with the "virus-like disease" (Gergerich & Kim, 1983) that was later confirmed to be *Rose rosette virus*, not mite feeding. Researchers also found that the eriophyid mite was the viral vector and the main mode for viral spread. It was believed in the 1960's that the mite was isolated to mountain and rural regions of the United

States and would be limited in spread, preventing the disease from becoming an issue in urban and metropolitan areas (Allington, et al., 1968).

Eriophyid mites are the primary viral vector for members of *Emaravirus* genus. The *Eriophyidae* family of mites vector many economically important diseases on a variety of crops, such as Erinea on maples and *Wheat streak mosaic virus* (WSMV) on cereal crops (Elbeaino, et al., 2013). Eriophyids are microscopic mites with 2 pairs of legs (Epstein & Hill, 1999). Many members of the *Eriophyidae* family are free-living and commonly balloon in the air to move from plant to plant (as the RRV vector does), which causes initial field infections to appear as a random pattern before becoming a more organized pattern (Windham, et al., 2014). Additionally, a smaller number of eriophyid mites live in protected areas of the plant (typically node regions and reproductive structures), such as the mite that vectors *High Plains wheat mosaic virus* (HPWMoV). The eriophyid mite that vectors *European mountain ash ringspot-associated virus* (EMARaV) induces galls to form on host plants; these galls serve as a protected feeding spot for mites.

Transmission studies have been done with some of the mite vectors of other *Emaraviruses* to determine the acquisition access period (AAP) and the inoculation access period (IAP). Kulkarni, et al. (2007) conducted vector transmission studies using the eriophyid mite (*Aceria cajani*) that vectors *Pigeon pea sterility mosaic emaravirus* (PPSMV). The AAP of PPSMV found to be a minimum of 10-15 minutes of feeding activity while the IAP was found to be 60-90 minutes of feeding (Kulkarni, et al., 2007). The study also found that there is no latency period between AAP and IAP, suggesting

potential for immediate transmission following acquisition. The PPSMV study also concluded that the virus was transmitted in a semi-persistent manner (Kulkarni, et al., 2007). Semi-persistent transmission occurs when PPSMV is transmitted only for a small period of time from the mite to the plant. The mite eventually stops transmitting the virus after a period of time; it is suggested to be two weeks (Epstein & Hill, 1999). The lack of a latency period and semi-persistent transmission suggests that PPSMV does not propagate in the mite before transmission, however there has been a study done on the mite vector of EMARaV that shows the virus enters the mite's gut area and infects areas of the mite, suggesting that at least EMARaV is propagative in the mite (Mielke-Ehret, et al., 2010). Other applicable knowledge that both studies found was that the respective *Emaravirus* in question was not transmitted to subsequent generations through eggs of infected females (oviparous transmission) (Kulkarni, et al., 2007; Mielke-Ehret, et al., 2010)

RRV Transmission Studies

Transmission studies using *Phyllocoptes fructiphilus* and RRV have been attempted on a few occasions. Di Bello, et al. (2017) explored more into the transmission aspect of RRV and found that the mite vector must feed on an infected plant for at least 5 days before becoming viruliferous, thus the AAP for RRD is reported to be five days. This finding seems to agree with the work done on EMARaV and its vector, which suggested that the virus is propagative in the mite and there is a latency period between the AAP and IAP. This also leads to the conclusion that RRV is transmitted by the mite in a propagative, persistent manner. Di Bello, et al. (2017) also found that the IAP for

mites was as short as one hour, with the transmission efficiency increasing the longer the mite was allowed to feed.

There has been limited information on preventative treatment of plants to prevent mite population establishment which has been observed to be effective, though very costly[1]. The study conducted by Windham et al. found that weekly applications of miticides could protect plants from having established mite populations. The cost to do this is very high, especially if high numbers of plants are being treated; one rose grower said applications cost a minimum of $50,000 with each application[2]. There is also some concern if the miticide is labeled to be applied that often and at the rates used. As with many other organisms, mites can become resistant to chemical control, so the repeated use of a miticide can allow this to occur.

Preliminary data has suggested that drench application of miticides may be more effective than foliar application. This is thought to be due to the fact that systemic drenched chemicals are taken up through the roots and successfully move throughout the entire plant, whereas systemic foliar applications do not move below the areas that they are applied (Krauskopf, 2007). Applications of the miticide Kontos® (active ingredient: Spirotetramat), has successfully eliminated mites that were found in a greenhouse Kontos® can be applied through foliar application or drench, is a systemic miticide, and has a mode of action of lipid biosynthesis inhibitor (LBI) (Bayer Environmental Science, 2014). LBI mode of action is in the IRAC Group 23; this group is specific to the

[1] Windham, M. Personal Communication, October 2017.
[2] Waterhouse, D. Personal Communication. Combating RRD Conference, October 2018

treatment of mite pests (Cloyd, 2017). Group 23 miticides are effective against mites because they cause degradation of fatty acids, thus preventing the development of nymphs and larvae; reproduction is also thought to be affected by LBI (Cloyd, 2017).

Systemic miticides are desirable in the treatment of epidermal-feeding eriophyid mites (Nault, 1997) due to the fact that the pesticide is translocated in the plant through the phloem to most plant parts and can be ingested by the feeding mites. While many insecticides are contact only (must touch the insect when applied to kill), several of the miticides that are available are systemic or a mixture of both systemic and contact (Krauskopf, 2007). Therefore, a systemic treatment has a better chance to reach the niche areas of the plant that mites tend to be located and eliminate the feeding mites before the five-day AAP is fulfilled.

Research is being done to study barrier plants to slow the spread of the mite through the wind (Windham, et al., 2014). Windham, et al. (2014) found that *Miscanthus sinensis* could serve as an effective barrier to reduce the amount of mites ballooning to or from a plot of roses. While this barrier doesn't prevent RRD from reaching roses, it does slow mites down and reduce some incidence of RRD (Windham, et al., 2014).

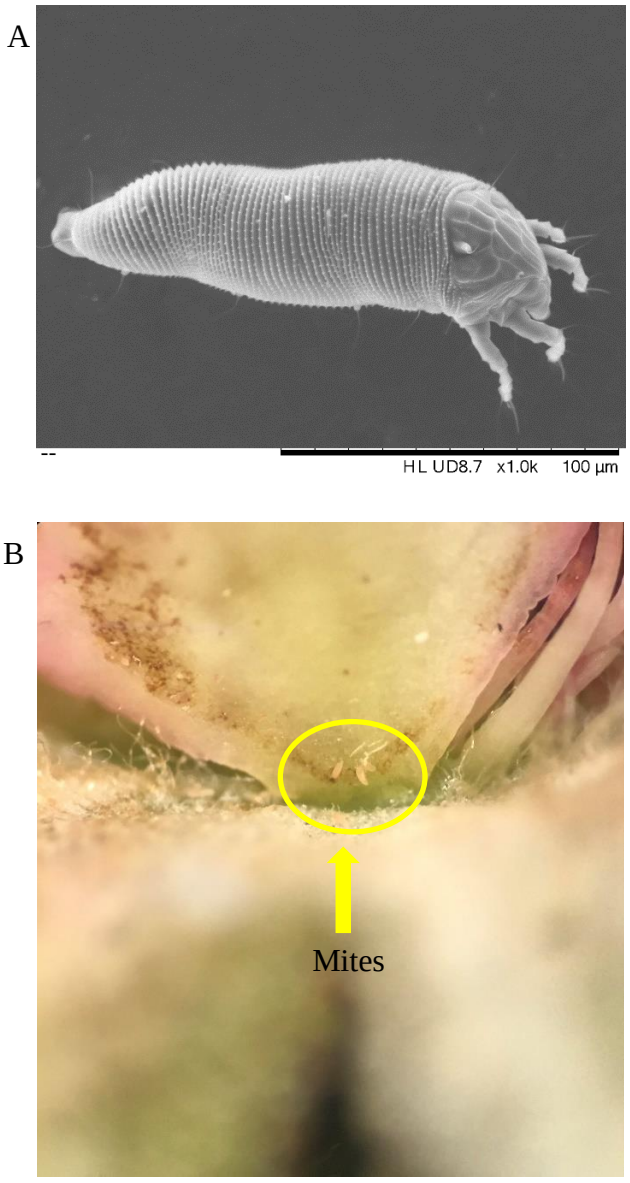

Figure 1.1. Examples of mites under an electron microscope and a dissecting microscope. (A) Electron microscope image of *Phyllocoptes fructiphilus* that was collected on Texas A&M Campus in August 2018[3]. (B) The view of mites under a 40x magnification on a dissecting scope shows the importance of using higher magnifications when checking for mites.

[3] Ron Ochoa, USDA Beltsville, August 2018 (Image A)

Rose Rosette Viral Genome

Rose rosette virus (RRV) was initially reported to be a negative sense, single stranded RNA virus with four segments in 2011 (Laney, et al., 2011). However, three additional genome segments were identified and described for the virus in 2015 (Di Bello, et al., 2015). RRV RNA 1 is 7026 nt long and has one open reading frame (ORF) that encodes the RNA-dependent RNA-polymerase (RdRP replicase) (Laney, et al., 2011). RNA 2 is 2245 nt long and has one ORF that is responsible for putative glycoprotein precursors (Laney, et al., 2011). RNA 3 is 1544 nt long and encodes the nucleocapsid protein. RNA 4 is 1541 nt with one ORF and is believed to be responsible for cell-to-cell movement. RRV moves cell-to-cell by threading through plasmodesmata in an ATP-dependent manner (Laney, et al., 2011). RNA 5 is 1665 nt long with one ORF (Di Bello, et al., 2015), the function is not known. RNA 6 is 1402 nt and has 2 ORFs. P6a, is thought to be involved with secretory pathways (Di Bello, et al., 2015) and has been found to interact with p2 (encoded by RNA 2) and p5 (encoded by RNA 5) along with having binding motifs (Di Bello, et al., 2015). RNA 7 is 1649 nt long with one ORF; p7 is responsible for forming homodimers and interacting with glycoproteins and p5 (Di Bello, et al., 2015).

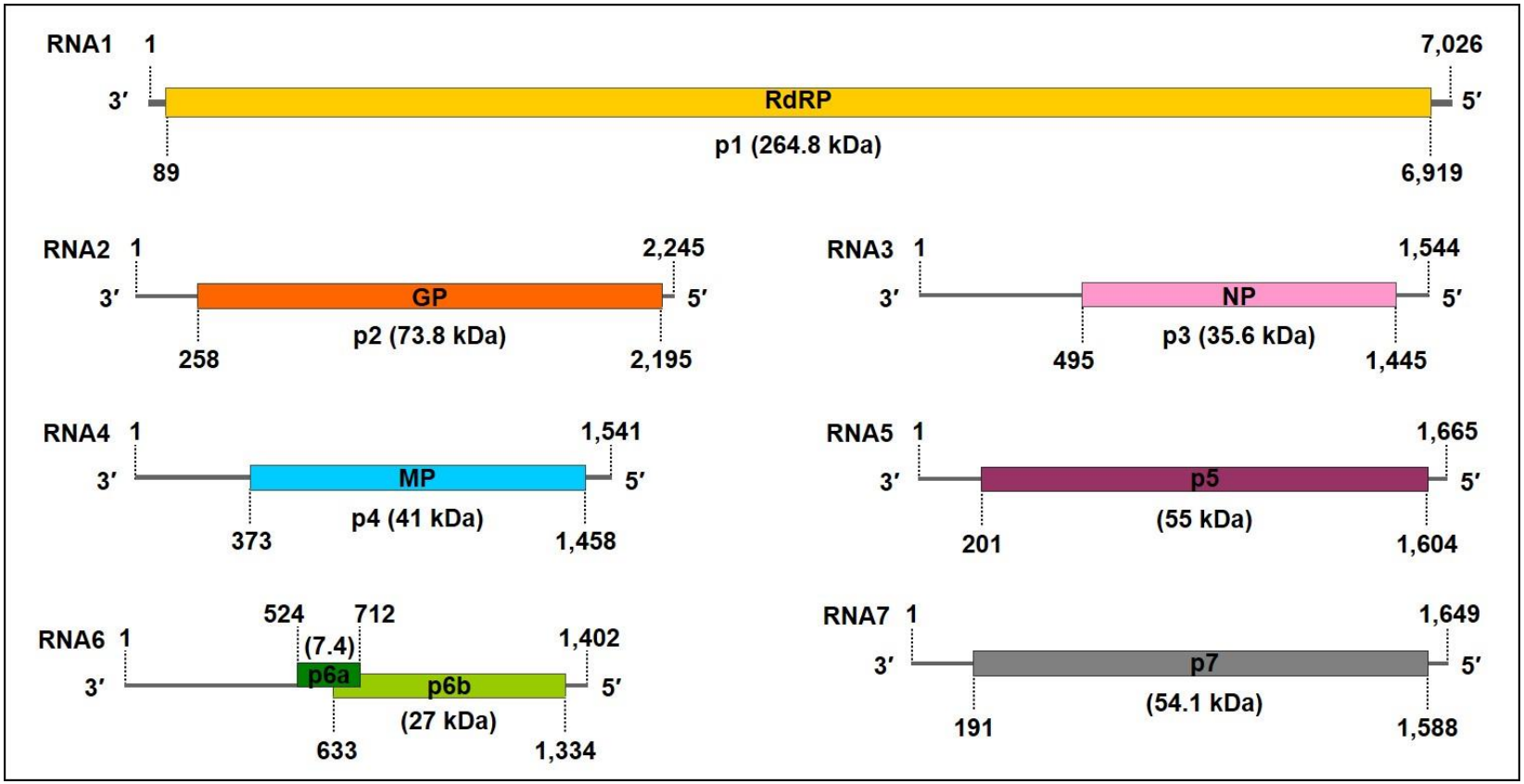

Figure 1.2. Annotated genome maps of the seven RNA segments of *Rose rosette emaravirus*. RdRP, RNA-dependent polymerase p1; GP, putative glycoprotein p2; NP, putative nucleocapsid p3 ; p4, putative movement protein; p5, protein of unknown function; p6, proteins putatively involved in secretory pathways; p7, forms homodimers and interacts with p2 and p5. The genome length of each RNA segment, the spans of the encoded genes, and the molecular weights of each protein are shown. The maps, produced in SnapGene® Viewer v.5.1.7, were not drawn to scale. GenBank accession numbers for the RNA segments are HQ871942-945 (RNA1-RNA4) and KM007081-083 (RNA5-RNA7).

Emaravirus genus

There are currently nine virus species classified in the *Emaravirus* genus. The viruses in this genus have a segmented genome with at least 4 negative RNA's surrounded by a double membrane-bound particle, and are vectored by eriophyid mites (Mielke-Ehret & Muhlbach, 2012). All viruses in this genus have spherical virions called double membrane-bound bodies (DMBs) (Elbeaino, et al., 2018; Mielke-Ehret & Muhlbach, 2012). The DMBs are typically found near the endoplasmic reticulum (ER) and the Golgi apparatus in the plant cell (Mielke-Ehret & Muhlbach, 2012). The DMBs found in RRD infected plants were first identified in the 1980's as virus like particles (Gergerich & Kim, 1983) and have since been measured in comparison to other *Emaraviruses* and are found to be of medium size (Mielke-Ehret & Muhlbach, 2012).

While all *Emaraviruses* cause symptoms that vary with the host that they infect, from ringspots and chlorotic spots to mosaic and leaf/seed deformity, they have sequence similarities and thus are grouped into a genus. This genus of viruses is in the family *Fimoviridae*, which is also closely related to *Tospoviridae* and *Peribunyaviridae*, as they all fall into the same order (*Bunyavirales*) (Elbeaino, et al., 2018). The similarities in the three families are that members of the families all are negative sense, single stranded RNA viruses with four to eight segments in the genome, enveloped virions, and stretches of nucleotides that are almost identical in varying segments of the virus (Elbeaino, et al., 2018). Species in the *Fimoviridae* family are translated through capped mRNA's, which results from cap-snatching of host mRNA's. (Elbeaino, et al., 2018). Additionally, segment 1 (RdRP), segment 2 (glycoprotein), and segment 3

(nucleocapsid) of *Emaravirues* all have high sequence identity to similar proteins of other viruses in the *Bunyavirales* order (Elbeaino, et al., 2018).

In 2008, the first *Emaravirus* that was identified, described, and assigned in the genus was *European mountain ash ringspot-associated emaravirus* (EMARaV); the virus has four segments (Di Bello, et al., 2015; Mielke-Ehret & Muhlbach, 2012). Other viruses shared similarities to EMARaV and were also classified as members of the genus. *Fig mosaic emaravirus* (FMV), a virus with six segments that has symptoms documented since the 1930's, was made a member of the genus in 2009 (Elbeanino, et al., 2009). *Raspberry leaf blotch emaravirus* (RLBV), which has eight segments and causes Raspberry Leaf Blotch Disorder, was confirmed as a member of the genus in 2012. *Pigeon pea sterility mosaic emaravirus* 1 (PPSMV-1) had symptoms that were described in the early 1930's, however the virus was not identified until 2013; the virus has five segments (Elbeaino, et al., 2014). *Pigeon pea sterility mosaic emaravirus* 2 (PPSMV-2) was identified as a member of the genus in 2015; the virus has six segments (Elbeaino, et al., 2015). *Rose rosette virus* (RRV) was identified as a member having seven segments in 2011 (Laney, et al., 2011; Di Bello, et al., 2015). *Redbud yellow ringspot-associated emaravirus* (RYRaV), a virus with five segments, was identified as a member of the genus in 2016 (McGavin, et al., 2012). *Actinidia chlorotic ringspot-associated emaravirus* (AcCRaV) was classified as an *Emaravirus* in 2017; the virus has five segments (Zheng, et al., 2016). *High Plains wheat mosaic emaravirus* (HPWMoV) infecting both wheat and maize has symptoms that have been described since the early

1930's; the virus has eight segments and was identified as a member of the genus in 2013/2014 (Elbeaino, et al., 2018; Mielke-Ehret & Muhlbach, 2012).

European mountain ash ringspot-associated virus (EMARaV)

As the first virus that was classified in the *Emaravirus* genus, in 2007, this virus played a role in the development of primers to study all other members of the genus. EMARaV has four segments, thought to be of negative sense, which are aligned closely in function with the first four segments of RRV (Mielke-Ehret, et al., 2010). Attempts at graft transmission has been unsuccessful and the virus has been shown to not be seed or pollen transmitted (Mielke-Ehret, et al., 2010). This virus is vectored by an eriophyid mite, *Phytoptus pyri* (pear leaf blister mite), which induces galls on ash leaf surfaces that it then lives in. This mite commonly feeds on plants in the Rosaceae family, including ash trees (Mielke-Ehret, et al., 2010). The most common symptom associated with viral infection is ringspots on leaves. The mite was confirmed as the vector after ribosomal RNA (rRNA) and viral RNA (vRNA) were extracted from mite samples and tested positive with PCR detection (Mielke-Ehret, et al., 2010). In the same study, it was also found that there was no significant accumulation of the virus in the oviduct of females (Mielke-Ehret, et al., 2010) which assuming RRV localizes similarly in the vector, means that oviparous transmission of the virus to subsequent generations from an infected female is unlikely. EMARaV segment sequences were used by Laney, et al. (2011) to assemble RRV primers for detection and sequencing. The previous work done on EMARaV allowed for characterizing and assigning functions to RRV viral segments, showing the close relationship of viruses in the genus. The work done on the EMARaV

may also be why Laney, et al. (2011) suspected that RRV would only have four segments, since the two viruses seem to be similar.

High Plains wheat mosaic virus (HPWMoV)

HPWMoV is a viral disease that cause mosaic symptoms on various cereal crops, with the most damage caused on wheat and maize. The disease was described in the United States in 1993 and has since been reported in several other grain producing countries such as Australia and Brazil (Louie, et al., 2006). This virus produces DMB's, similar to RRV (Louie, et al., 2006). HPWMoV is vectored an eriophyid mite, *Aceria tosichella* Keifer, also known as the wheat curl mite. This mite is a free-living mite (meaning it does not live in a gall), similar to *Phyllocoptes fructiphilus*, that has a primary mode of dissemination through wind gusts (Louie, et al., 2006). The mite has a broad host range, unlike *Phyllocoptes fructiphilus*, including grain producing monocots and monocot weeds as well (Murugan, et al., 2011). However, similarly to the RRV vector, these mites tend to be found in protected areas of plants such as the leaf whorl or sheath (Murugan, et al., 2011). The wheat curl mites are also known for vectoring another serious wheat virus, *Wheat streak mosaic virus*, and it is common for crops that are fed on by the mite to be co-infected by both viruses. The virus has been sequenced and characterized as a member of the *Emaravirus* genus based on its similarity to the first four segments of all other viruses in the genus (Elbeaino, et al., 2018). The DMB's produced by the virus and the eriophyid mite vector are both used as support for the virus placement in the genus. The virus also causes a mosaic on leaves of infected plants, which is similar to effects that other *Emaraviruses* have on their plant host.

Symptoms of RRD

Many symptoms have been described for RRD from diseased rose plants. They vary from cultivar to cultivar, so it is difficult to say what symptoms are caused by RRD and what are related to other pests/herbicide damage. Three stages of infection have been outlined in literature (Epstein & Hill, 1999). Stage 1, veins redden and there is excessive lateral growth, shoots become more succulent, and leaves and blooms appear crinkly or otherwise distorted. In stage 2, rosettes begin to form and the plant shows visible injury from cold damage. In stage 3, there are many rosettes, the internodes are shortened, and the plant ultimately dies usually due to cold damage. These stages were developed based on multiflora rose observations, and it was also noted they can vary when observing a hybrid/ornamental cultivar (Epstein & Hill, 1999). Some of the differences that have been observed so far is that many cultivars display crinkled leaves and chlorosis as initial symptoms. Preliminary data has shown that very few (less than 25) of the varieties observed/tested over the past three years have displayed reddened veins[4]. Additionally, some varieties like 'Home Run' do not display reddened leaves/canes until the final stage of infection[5].

[4] M. Shires, Preliminary Observation Data.
[5] M. Shires, Preliminary Observation Data.

A

B

Figure 1.3. Common RRD symptoms. (A) Rosettes, which are created by excessive stems growing from one node, are one of the most common symptoms of RRD. This sample also demonstrates the deformed leaves and hyper-thorniness that can be observed in RRD infection. (B) Hyper-thorniness or excessive spines are also one of the most common symptoms of RRD. Abnormally pink canes and pink leaf veins can also be observed in some cultivars that develop symptoms of RRD.

Rose Rosette Disease Epidemiology in Texas

When Rose Rosette Disease was first discovered on multiflora rose rootstock in Smith County in the early 1990's (Pemberton, et al., 2018), it was believed that the disease would not become a significant problem because of the low number of natural stands of multiflora roses present in Texas. In 1996, the disease was reported on multiflora roses in Grayson County (Ong, et al., 2015). The first time that RRD was observed on non-multiflora plants in the Dallas-Fort Worth metroplex (DFW) was in 1998. DFW was just starting an exponential growth in the late 1990's, which meant more developments, thus more houses and businesses utilizing roses in the landscape. In the early 2000's, the disease was becoming a minor problem in many areas of the metroplex. It was believed at that time RRD would never move south of the Interstate 20 (I-20) line, due to disease distribution that others had observed in the southeastern United States (Solo, 2018). The disease appeared to stay along and north of this interstate line in several other states, so that was inferred for Texas. This "I-20 line" concept has appeared to hold true in Georgia, Alabama, and Mississippi, even 20 years later, based on data from Solo et al. (Solo, et al., 2020); however, that was not the case in north Texas. While RRD appeared to stay close to this boundary for more than a decade, as general public awareness and extension outreach has increased, so have the incidences in new counties. The disease is now in the metroplex areas of Houston, San Antonio, and Austin and it has recently been reported as far west as Odessa. Documenting and following the spread of RRD has allowed for the creation of a more accurate map of disease distribution in Texas (EddMaps, 2020).

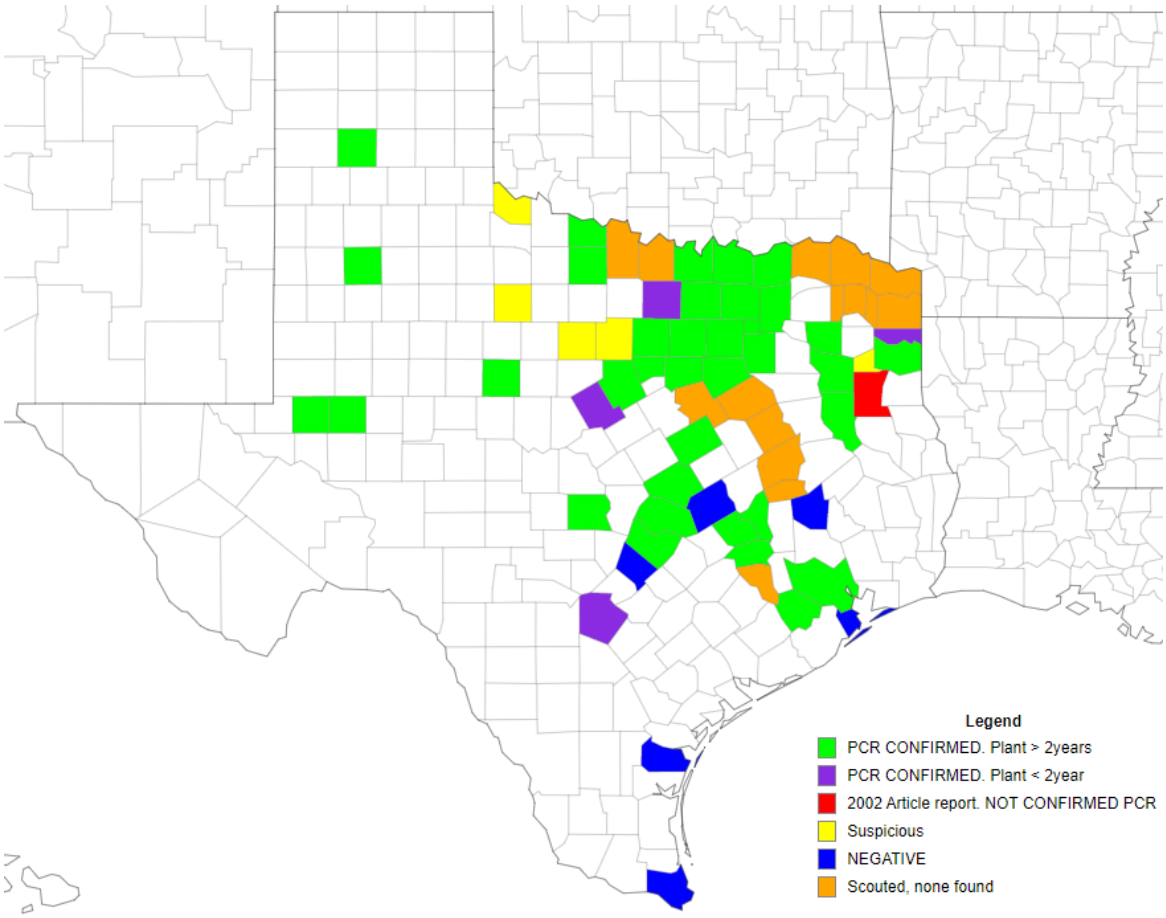

Figure 1.4. RRD spread reported in Texas. This figure is a representation of the RRV spread in Texas as of 5/12/2020[6].

[6] Bugwood, Texas Distribution Data, May 2020.

Strategies for Awareness, Outreach, and Citizen Science Activities

One way that documentation of RRD has been improved is through extension outreach and Master Gardener trainings. Extension outreach is a way to bring the science to the people; this is especially important with RRD because those who are educated about RRD can assist with gathering disease incidence information. Master Gardener trainings that are offered through extension outreach programs allow for accurate disease information to be disseminated to trained individuals who have a passion for plants and plant health. Master Gardeners are also actively involved in citizen science (general public learning scientific principles). Through citizen science, Master Gardeners are made aware of and educated on field trial development, design, and data collection, and they engage others to be involved in these tasks. Additionally, because roses are a niche crop, RRD directly impacts industry, nurseries, and homeowners so public RRD education meetings allow for the interested general public to be made aware of and trained on accurate disease information. Public meetings also allow many people to be engaged in citizen science activities. One such activity is reporting plants that are displaying symptoms to roserosette.org, which was developed as a source of RRD educational information and a reporting platform for the general public to report RRD symptoms. Citizen science activities, such as public meetings and the reporting website, have increased the number of educated general public reporting RRD symptoms, allowing for a national distribution map to be developed over the last three years (Byrne, et al., 2018; Pemberton, et al., 2018).

The following chapters are the results of attempts to gain a better understanding of RRD and the pathogen that causes it. Through attempts to find RRD resistance in cultivated roses and to increase science-based awareness of RRD to the general public, field trials were conducted where trained volunteers were utilized to assist in the scientific process. The results from these various field trials, which screened 200 cultivars, contributed to the collective information regarding susceptibility of over 1000 rose cultivars; this large collective has been built by several collaborators. A key element in the ability to study RRD is the ability to detect the pathogen which causes the disease, RRV. Several improvements to RRV diagnostics have been made over the last five years. The diagnostics study contributed a more efficient approach to RRD diagnostics via molecular detection methods, optimized these methods for use in a general plant disease diagnostic lab, as well as showing the methods could be utilized in early detection of RRD. The primary mode of RRD transmission is the insect vector *P. fructiphilus*. Historically, RRD has also been demonstrated to be graft transmissible (Epstein, et al., 1997). Grafting experiments were conducted to confirm this work and to further explore the movement of the pathogen through bud grafting. Seed, mechanical, and root transmission of RRV were also explored through this study. It is hoped that the information gathered in this study contributed to a better understanding of RRV in roses and enhanced ability to detect this virus effectivly.

CHAPTER II

SCREENING OF NAMED AND UNNAMED ROSE VARIETIES FOR RRV RESISTANCE THROUGH NATURAL INOCULATION IN FIELD TRIAL SETTINGS

Introduction

The utilization of field trials in plant science research is a useful tool that allows for plants to be placed in a normal growing environment rather than the controlled environment of a greenhouse or growth chamber. Exposure to a normal growth environment allows for a more thorough evaluation of plants characteristics including reactions to changes in weather, temperature and exposure to non-target diseases. The preceding characteristics are more controlled in a greenhouse or growth chamber; however, this results in data differences in the same cultivar between controlled and natural environment experiments. One example of this occurring within the rose rosette disease system is with the cultivar 'Stormy Weather.' This cultivar was screened for disease resistance in a controlled growth environment and did not develop symptoms during the experiment (Di Bello, et al., 2017). Once this variety was planted in several natural environment field trials in Delaware, Oklahoma, and Texas, the variety was reported with symptoms of RRD and tested positive for RRV within eight months[7].

The use of randomization in field trials also allows for a better picture of the effects of the environment and diseases. Due to the sizes of roses, shading and

[7] M. Shires. Field observations.

competition can affect how a plant responds to disease spread. With this in mind, as well as variations in moisture and soil types, the use of randomization for plot organization ensures that each plant cultivar is allowed equal exposure to the conditions of the field trial (Davis, et al., 2017). Field trials were completed using two different screening methods. The first method is randomized complete block (RCB) replicated trials. RCB replicated trials focus on a set number of cultivars that are randomized in placement, in equal amounts and multiple replications of each plant in the trial. This design was selected because there were many potential sources of variation, with the largest being mite spread virus inoculum (Davis, et al., 2017). The second method is non-replicated randomized screening. This method focused on utilizing the randomization and equal placement elements of RCB, the exception in this type of screen is that plants within each row of the field were not replicated. The use of some RCB elements encouraged uniformity within the trial, but due to no replication, did not allow each cultivar an equal chance of being infected when inoculum was introduced into the plot.

The primary focus of this set of experiments is screening of cultivars with unknown susceptibility or resistance to RRV. More than 1000 rose cultivars have been screened in a nationwide search for possible resistance to RRV, with most being found as RRV susceptible. However, there are some cultivars that have shown promise to be tolerant or possibly resistant to the virus. For the scope of this project, susceptibility to RRV is the development of obvious RRD symptoms and a positive RRV test; tolerance is defined as PCR positive for RRV but lacking RRD symptoms for at least two years;

resistance is defined as RRV and RRD symptom free for a minimum of two years (Pagan & Garcia-Arenal, 2018).

These field trials, located in multiple locations, provide for an opportunity to engage the general public, allowing for dissemination of knowledge about RRD through citizen science. Citizen science is broadly defined as the involvement of the general public in scientific research activities (Irwin, 2018). In many field trials, Texas Master Gardener volunteers were actively involved in field trial plot designs and data collection, which allowed those involved to learn the principles of field research such as randomization and disease symptomology. Additionally, it is hoped that these volunteers gained an appreciation of the scientific process and the importance of replications and sound experimental design for reliable interpretation and conclusions. Master Gardener involvement allowed large amounts of observational data to be collected for analysis on disease incidence and general plant performance.

Project Objectives and Aims

These field-based experiments are designed to evaluate disease development in a natural setting of both named and species rose cultivars for RRD resistance screening. The main objective of these field experiments was to identify potential RRD resistance and/or tolerance in cultivated roses. In this study, over 200 rose cultivars were screened through both randomized, replicated and non-randomized, non-replicated plot designs to provide some information of the performance of these cultivars against RRD. Additionally, the general public were engaged to participate in citizen science and to learn science-based information about RRD. This partnership with the volunteers

allowed for the magnification of the ability to gather observational data in multiple locations while providing science-based information (awareness-outreach) and education through volunteer training.

Materials and Methods

Purpose and General Set-up

Field trials were set up in several locations across north Texas (Figure 2.1), with the goal of allowing for natural movement and infection of RRV. Trials were in areas with medium to high disease pressure. For the purpose of annotating disease pressure risks at test locations, I described the risk of RRD associated with the test location based on existing plants displaying symptoms in the trial locations as well as barriers around the plot. While many locations were in an open environment (no natural or physical barriers to block wind), some trials were significantly more isolated. Disease pressure risk was described at all trial locations based on barriers that could prevent mite spread and proximity of other diseased roses to the trial sites, as these are theorized as factors that could influence the rapidity of disease onset and development (Windham, et al., 2014).

Trial Locations Preliminary Disease Pressure Risk Profile	
Location	**Disease Risk Profile**
	None- No symptomatic plants and many physical barriers
	Low (L)-Presence of physical barriers and low numbers of symptomatic plants
Johnson Co. (2017)	Low-Medium (LM)- Presence of physical barriers and higher numbers of symptomatic plants, though far from the location
	Medium (M)- Less physical barriers and infected plants in closer proximity to the plot location
Dallas Co. (1) (2016) Denton Co. (2017) Dallas Co. (2) Non-Replicated (2018) Johnson Co. Non-Replicated (2018)	Medium-High (MH)- Few physical barriers and more than 20 symptomatic plants in close proximity to plot location
Dallas Co. (2) (2017) Dallas Co. (3) (2018) Dallas Co. (1) Non-Replicated (2018) Archer Co. Non-Replicated (2017)	High (H)- No physical barriers and high amounts of symptomatic plants close to plot
Archer Co. (2016) McLennan Co. (2019)	Very High (VH)- No physical barriers, high amounts of symptomatic plants, plot planted in previously infected rose beds

Table 2.1. Preliminary Profile of Disease Pressure for Replicated and Non-Replicated Trial Locations. Most trials were determined to have a higher chance at developing RRD. The Johnson county location was an exception when compared to other plot as its disease risk profile was much lower than other trials.

2016 Replicated Trials

Two trial locations were selected for 2016 trials: Archer Co. (Wichita Falls, Texas) and Dallas Co. (1) (Dallas, Texas). Ten cultivars were selected for the 2016 trial; these cultivars were selected based on previous research showing that they may show some resistance or tolerance to RRV. One positive control, 'The Double Knock Out© Rose', was selected due to the cultivar's ability to display noticeable and typical RRV symptoms upon infection. The other cultivars that were used in 2016 trials were: 'Chuckles', 'Basye's Purple Rose', 'Caldwell Pink', 'Belinda's Dream', 'Home Run', 'Hot Cocoa', 'Shreveport', 'Nearly Wild', and 'Pink Home Run'. Six replications of each cultivar were planted in Archer Co.; four replications of each cultivar were planted in Dallas Co. 1. Both plots were planted in early June 2016. Weather (environmental) conditions were similar in both locations during planting with average low temperatures of 24ºC and average highs at 34ºC.

Observational and plant testing data for each trial was collected in different frequencies. Volunteers were utilized at both locations to assist in data collection for these trials. All volunteers (citizen scientists) were provided with two or more hours of training to increase awareness and basic understanding of RRD and RRD symptoms prior to being tasked with data collection for this project. The Archer Co. plot had data collected on a bi-monthly basis; citizen science observations were done by the homeowner, the Wichita County Extension Agent and Master Gardeners. Data was not collected during the winter months of December to March, when roses were dormant. Data from the Dallas Co. 1 plot was collected on a monthly basis by citizen scientists

from the Dallas County Master Gardeners group. After the initial presence of symptoms was noted, the observation interval changed to every two weeks to better monitor disease onset in other rose plants. Data was not collected in December-February each year due to slowed plant growth with cool temperatures. Data collected by all trained volunteers included presence/absence of RRD symptoms, general observed plant health, and other potential stressors, such as drought, pruning, or leaf spot symptoms.

2017 Replicated Trials

Three trial locations were selected for 2017 plot locations. Johnson Co. (Cleburne, Texas), Denton Co. (Denton, Texas), and Dallas Co. 2, (Farmers Branch, Texas). Twenty cultivars for each 2017 trial were selected based on three factors: the variety was not currently being screened in other locations, reports or observations of possible tolerance, and the roses were better suited for Texas environment. Three cultivars were also carried over from the 2016 trials because of the lack of disease presence after one growing season; those were 'Basye's Purple', 'Caldwell Pink', and 'Chuckles'. Additionally, 'The Rainbow Knock Out © Rose' served as the positive control for all 2017 trials. The positive control variety was used due to the obvious symptoms that it displayed upon infection. The other cultivars selected for the trial plots were: 'Jean Teresa', 'Tupelo Honey', 'Mrs. R. M. Finch', 'Lafter', 'Margaret McDermott', 'Miranda Lambert', 'Basye's Blueberry', 'Rhodologue Jules Gravereaux', 'Carefree Spirit', 'Cherokee Rose', 'Fires of Alamo', 'Repeat Blooming Swamp Rose', 'Emmie Gray', 'Smokin' Hot', 'Rouletti' (Denton Co. and Dallas Co. 2 only), 'Naga Belle' (Denton Co. and Dallas Co. 2 only), Serena x Basye's Thornless (Johnson Co.

only), and *Rosa arkansana* (Johnson Co. only). Six replications of each selected cultivar were planted in Johnson Co., four replications in Denton Co., and one replication in Dallas Co. 2. All trials were planted in March 2017; therefore weather (environmental) conditions during planting were similar for all sites with temperature ranging from 10º C for lows to 21ºC for highs. The Dallas Co. 2 plot was not replicated, due to space constraints, so it was used a screening site for 2017 trial cultivars due to low amounts of disease pressure in replicated plot locations. As all cultivars in the trial were the same as other replicated trials, the data from this plot is included in this section.

Data was collected on a bi-monthly basis at both the Denton Co. and Johnson Co. field trials. Data collection and plant care was completed by cooperators at Hill College in Johnson Co. as well as the Johnson County Extension Agent and Master Gardeners. Data Collection for the Denton Co. plot was completed by Denton County Master Gardeners. Data on the Dallas Co. 2 plot was completed on a bi-annual basis and was done by a City of Farmers Branch cooperator. No data was collected in the winter months of December through March at plots due to plant dormancy. Similar to the previous years, all citizen scientists were provided with RRD training and collected information about RRD presence/absence, general plant health, and other stressors.

2018 Replicated Trials

One trial location was identified for the 2018 RRV trials; that location was in north Dallas at the Texas A&M AgriLife Research Center. As in previous years, cultivars were selected based on if they had been screened in another RRD trial and if the variety had shown potential tolerance in one or more growing seasons. There was

one variety carried over from 2017, ‘Lafter’, due to multiple reports of no symptoms in several trials. Two positive control cultivars were utilized; those were ‘Home Run’ and ‘Old Blush’. These cultivars were used due to the symptoms displayed when infected. Three additional varieties, ‘My Girl’, ‘Easy Elegance Kiss Me’, ‘Top Gun’, were documented as RRD susceptible, but were needed for additional experiments at the research center. The additional 14 cultivars that were selected are: ‘The Sunny Knock Out © Rose’, ‘Purple Pavement’, ‘John Davis’, ‘Winnipeg Parks’, ‘Moje Hammarberg’, ‘Little Buckaroo’, ‘Blanc de Coubert’, ‘Topaz Jewel’, ‘John Cabot’, ‘Morden Fireglow’, ‘Morden Centennial’, ‘Hansa’, and ‘The Coral Knock Out © Rose’. Five replications of each cultivar were planted in the plot in April 2018 where weather (environmental) conditions had a temperature range of 14ºC for lows and 26ºC for highs. There was also a partial trial of twelve 2018 trial plants put in Rowlett, Texas, however due to plant death, there was no data collected from this location.

Data was collected from the plot at three months, seven months, and 18 months after planting. Data collection was done in-person.

2019 Replicated Trials

One location was selected for an RRD trial in 2019, Woodway, Texas at the Carleen Bright Arboretum. Cultivars for the trial were selected based on previously suspected tolerance and if the cultivar had been screened previously. Additionally, at the request of the plot manager, roses were also selected for the bloom color of white, pink, and red as well as hardiness in the Texas heat. The variety that was carried over from previous trials was ‘Lafter’. The positive control for the plot was the cultivar ‘Belinda’s

Dream'. This variety was selected because it displays very noticeable symptoms once infected. The 10 additional varieties that were screened in the plot were: 'Texas Centennial', 'Fellenburg', 'Borderer', 'Mrs. Sam Houston', 'Miss Lillian', 'Felicia', 'Kirsten Poulsen', 'Marichoness of Londonberry', 'Francis Dubriel', and 'Old Baylor'. Three replications of each of the 12 cultivars were planted at the plot in March 2019, where weather (environmental) conditions with low temperature at 11ºC and high temperatures at 20ºC.

Data was collected by McLennan County Master Gardeners on a monthly basis. This plot was much further south than other plots, which allowed for data collection to occur year-round because of a lack of plant dormancy. All citizen scientists were trained about RRD and collected observations similar to previous years, with the addition of plant measurements.

Non-Replicated Trials

Non-replicated screening trials were also designed and randomized in 2017 and 2018. These trials focused on screening as much potential germplasm at a time, so only one copy of each plant was placed in the trial.

The location that was selected for the 2017 non-replicated trial was Archer Co., Texas. This trial was located in the same location as the 2016 Archer Co. trial. The 2017 trial plants were inter-planted with 2016 trial plants to maximize disease screening of germplasm. Plants were planted in March (average low: 9ºC, average high 20º C) after threat of last freeze had passed. Plants that were used for this screening trial were germplasm from the Texas A&M Rose Breeding Program as well as numerous name

cultivars that had not been screened for resistance in Texas. In total, 105 plants were planted in the trial. The positive control for the screening trial were the remaining 2016 trial plants, which were still developing RRD symptoms.

The 2018 locations for the screening trial were chosen for their disease pressure risk and amounts of symptomatic plants present. The first location was the existing Dallas Co. 1 plot from 2016; the second location of a non-replicated trial was the existing Dallas Co. 2 location; the third location was Johnson Co., also at the existing plot. All plants were planted in March therefore, weather (environmental) conditions were all similar at all locations with temperature ranging from 10º C for lows to 21ºC for highs.

Similar to the 2017 non-replicated trials, most roses for Dallas and Farmers Branch locations were germplasm developed by Texas A&M Rose Breeding Program graduate students. A total of 31 rose seedlings were planted in the Dallas plot and 15 seedlings were added the Farmers Branch plot. Four additional named cultivars were added to the Dallas plot location; those were 'Top Gun', 'Stormy Weather', 'Bull's Eye', and 'The Sunny Knock Out © Rose'. The Johnson Co. location was planted with several replications of the cultivar 'Top Gun'.

Observational Data Collection

Observational data was collected by the volunteers from each field trial. Volunteers were trained to capture observations at select intervals for the presence/absence of RRD symptoms and overall plant health. Overall plant health ratings were determined by assessing the overall condition of the plant and noting if

other rose disease were present, if the plant appeared stunted in growth or blooms, and if the plant had symptoms of abiotic stresses; this information was used to determine if plant death was due to stress or RRD. Intervals for the observations completed by volunteers were determined with input from the plot manager at each location. The collected information was compiled and analyzed to provide an overview of the incidence (and severity) of RRD and the effect of the multiple locations based on the disease risk profile. In the replicated field trials, RRD symptom observations were used to calculated the percent of disease incidence by cultivar, where the number of RRD suspect observations within a cultivar were divided by the total number of observations completed for each cultivar to provide a comparison of incidences between the field trial locations by rose cultivar.

Tissue Sampling for RRV detection

The protocol that was used for tissue sampling from plants in both replicated and non-replicated trials is as follows. Two to five grams of leaf material was sampled from a given plant. This tissue was maintained at room temperature when sampled and then stored at 4° C until PCR testing was performed. Leaf tissue sampling was performed on selected, possibly RRD symptomatic plants in plots to confirm that RRV was present in the plot. In addition, whole plot testing for all locations occurred two times, meaning that all plants in the plot were leaf sampled (except Dallas County 3). Root sampling was performed by collecting five to 10 grams of secondary roots from each plant. These samples were stored in the same manner as leaf tissue. Root sampling only occurred at plot termination or when RRD positive plants were removed (dependent upon the plot

management plan). The percentage of RRV detection was determined by dividing the number of PCR positive plants of a cultivar by the total number of plants for that cultivar. Detection data is not provided for non-replicated trials as most plants were only tested one time, at the date of plot removal. Detection of RRV from plant tissue provided confirmation that the plant was infected. Furthermore, these results allowed for the comparison of molecular detection testing to the observational data. The percent of RRV detection by cultivar enabled us to speculate on the potential susceptibility of that cultivar to RRV infection. To make a final designation of Susceptibility (S), Tolerant (T), Resistant (R), or Unknown (U) for each cultivar in replicated and non-replicated trials, both symptom observations and testing data were considered. A "Susceptible" (S) label was assigned to cultivars that developed recognizable RRD symptoms and resulted in al least one positive RRV PCR test for the cultivar. A "Tolerant" label was assigned to a cultivar if no recognizable RRD symptoms were seen for two years, but the cultivar had at least one positive RRV test. A "Resistant" label was assigned if the plant had no symptoms and no positive PCR tests for two years, and the "Unknown" label was assigned if the variety was not screened for the two-year minimum needed to meet the definitions for tolerance or resistance.

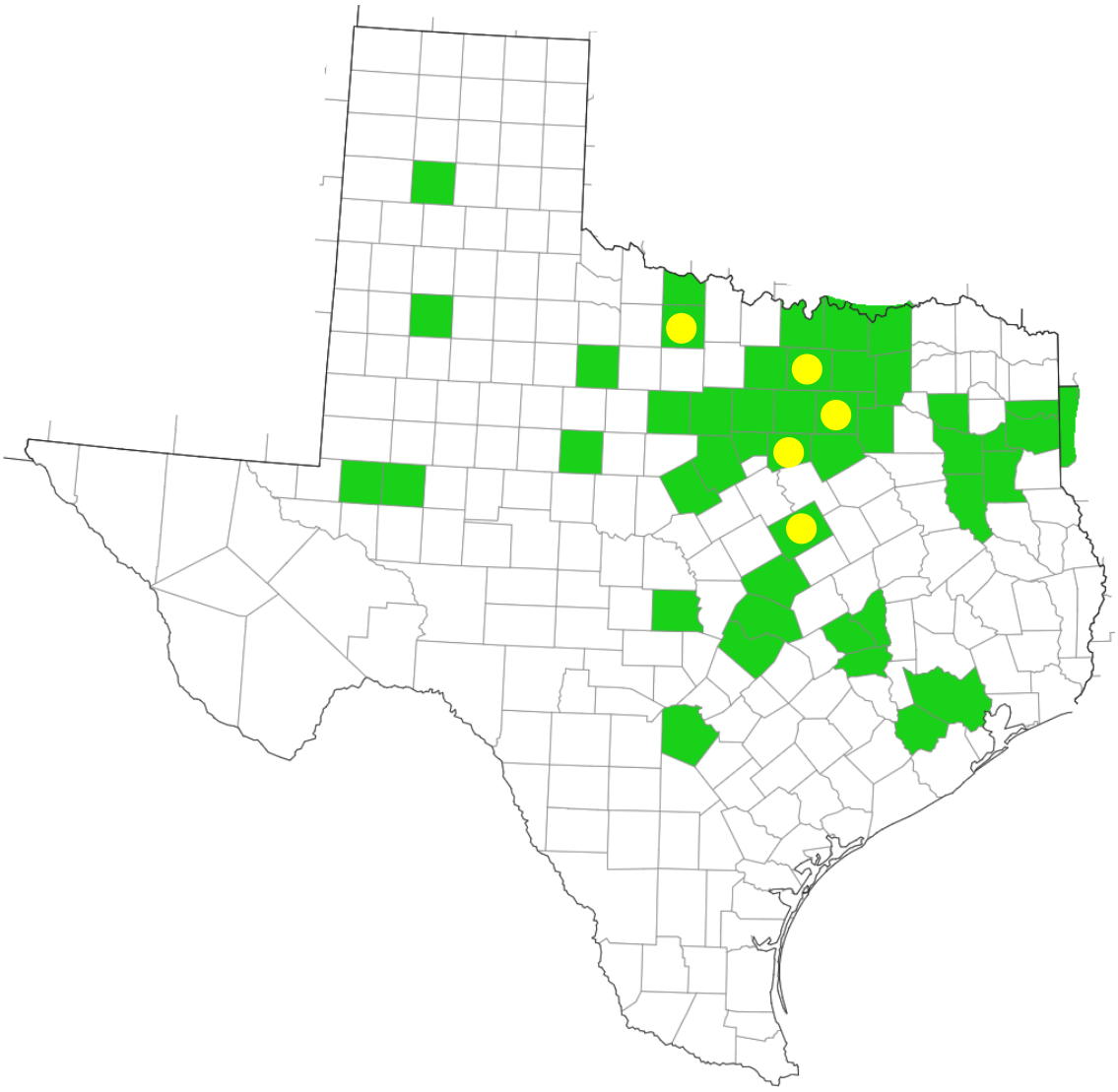

Figure 2.1. Spatial distribution of field trials throughout north Texas. A graphical representation of the five counties that RRV screening plots were located **(EddMaps, 2020)**. The yellow dots denote the five counties; one county, Dallas, had three plots in the same county.

Results

More than 60 rose cultivars were screened through the seven replicated plot locations; over 150 cultivars were screened through the non-replicated field trials. Table 2.1 summarized the results from the field trials showing both observational data and PCR testing (RRV detection assay) data by location. To maintain information integrity, susceptible plants reported by citizen scientists were confirmed observationally, in-person, and which cultivar (at least one plant) yielded a positive RRV detection (PCR) assay. One observation from this table, is that several plants had positive RRV tests, but did not develop symptoms over a two-year period. In most plots that were characterized as High or Very High RRD risk had symptoms initially developing on positive control plants. At the Dallas Co. (3) plot, poor management contributed to high plant mortality resulting in reduced numbers of plants where observations can be made, hence the observational results are susceptible to bias. This table also summarizes RRV testing data collected for cultivars in the replicated trials. All roses in Dallas County 1, Johnson County, Denton County, and Dallas County 2 were screened for two or more years, meeting the minimum time period that was placed to allow for identification of whether plants are “susceptible”, “tolerant”, or “resistant”. No cultivars in these plots were designated with a R because all evaluated plants had reports of symptoms and/or at least one positive RRV test. There were 14 cultivars designated as T. The more common commercial cultivars such as those in the Knock Out© Rose family and the Home Run rose family developed symptoms quickly and yielded many positive RRV PCR tests.

Four species roses as well as four roses with *Rosa rugosa* parentage were some of the cultivars that were determined to be “tolerant” to RRD.

Replicated plots in Archer County, Dallas County 3, and McLennan County as well as all non-replicated trials (Table 2.2) did not meet the two-year threshold, so were not assigned T or R. Therefore, only S (if symptoms developed) or U (PCR positive, asymptomatic or PCR negative, no symptoms); 17 replicated trial cultivars in replicated trials received a U designation. Cultivars that received a U designation need further screening to make a determination about “susceptible”, “tolerant”, or “resistant”.

Replicated Field Trials Observational. Testing, and Ranking Data															
Rose Cultivar	**Ranking**	**Archer Co. (30 RRD Observations)**		**Dallas Co. (1) (220 RRD Observations)**		**Johnson Co. (84 RRD Observations)**		**Denton Co. (64 RRD Observations)**		**Dallas Co. (2) (6 RRD Observations)**		**Dallas Co. (3) (20 RRD Observations)**		**McLennan Co. (12 RRD Observations)**	
		RRD (%)	**RRV (%)**	**RRD (%)**	**RRV (%)**	**RRD (%)**	**RRV (%)**	**RRD (%)**	**RRV (%)**	**RRD (%)**	**RRV (%)**	**RRD (%)**	**RRV (%)**	**RRD (%)**	**RRV (%)**
'Basye's Blueberry'	Tolerant	-	-	-	-	1	33	1	75	0	0	-	-	-	-
'Basye's Purple'	Tolerant	0	2	0	50	1	33	1	25	0	0	-	-	-	-
'Belinda's Dream'	Susceptible	76	100	66	25	-	-	-	-	-	-	-	-	0	33
'Blanc d Coubert'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'Borderer'	Unknown	-	-	-	-	-	-	-	-	-	-	-	-	0	0
'Bull's Eye'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'Caldwell Pink'	Tolerant	7	17	0	100	0	50	11	100	0	100	-	-	-	-

Table 2.2. Totals and final designation for all cultivars screened in replicated trials. Cultivars used as positive controls are highlighted in yellow. Binary observation were completed by citizen science volunteers for the following plots: Archer Co., Dallas (1) Co., Johnson Co., Denton Co., McLennan Co. The first six months of volunteer collected observational data (RRD) was excluded from results to account for human error that can occur in learning to distinguish new growth versus RRD symptoms. Observations at all other plots were completed in-person. The percentage of RRD was calculated based on the counts of possible symptoms in each cultivar divided by the total number of observations made on the cultivar in each plot. The percentage of RRV was calculated using the number of plants from the individual cultivar that tested positive for RRV at least one time during the plot time; this number was divided by the total number of plants of the cultivar. The ranking was determined based on the observational data and testing percentages for each cultivar.

Rose Cultivar	Ranking	Archer Co. (30 RRD Observations)		Dallas Co. (1) (220 RRD Observations)		Johnson Co. (84 RRD Observations)		Denton Co. (64 RRD Observations)		Dallas Co. (2) (6 RRD Observations)		Dallas Co. (3) (20 RRD Observations)		McLennan Co. (12 RRD Observations)	
		RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)
'Carefree Spirit'	Susceptible	-	-	-	-	0	17	22	50	0	0	-	-	-	-
'Cherokee'	Tolerant	-	-	-	-	0	17	6	25	0	0	-	-	-	-
'Chuckles'	Tolerant	3	33	0	25	1	50	1	50	0	100	-	-	-	-
'Emmie Gray'	Susceptible	-	-	-	-	6	33	3	75	0	0	-	-	-	-
'Felicia'	Unknown	-	-	-	-	-	-	-	-	-	-	-	-	0	0
'Fellenburg'	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	11	33
'Fires of Alamo'	Susceptible	-	-	-	-	2	17	2	100	100	100	-	-	-	-
'Francis Dubreuil'	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	0	33
'Hansa O/R'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'Home Run'	Susceptible	72	100	83	100	-	-	-	-	-	-	0	0	-	-
'Hot Cocoa'	Susceptible	72	100	61	75	-	-	-	-	-	-	-	-	-	-
'Jean Teresa'	Tolerant	-	-	-	-	0	17	1	75	0	100	-	-	-	-

Table 2.2 (Continued)

Rose Cultivar	Ranking	Archer Co. (30 RRD Observations)		Dallas Co. (1) (220 RRD Observations)		Johnson Co. (84 RRD Observations)		Denton Co. (64 RRD Observations)		Dallas Co. (2) (6 RRD Observations)		Dallas Co. (3) (20 RRD Observations)		McLennan Co. (12 RRD Observations)	
		RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)
‘John Cabot'	Susceptible	-	-	-	-	-	-	-	-	-	-	10	20	-	-
‘John Davis'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Kirsten Poulsen'	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	100	67
‘Kiss Me'	Susceptible	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Lafter'	Tolerant	-	-	-	-	1	33	18	75	0	0	-	-	0	33
‘Little Buckaroo'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Marchioness of Londonberry'	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	0	0
‘Margaret McDermott'	Tolerant	-	-	-	-	1	0	15	100	0	0	-	-	-	-
‘Miranda Lambert'	Susceptible	-	-	-	-	1	17	0	75	0	0	-	-	-	-
‘Miss Lillian'	Unknown	-	-	-	-	-	-	-	-	-	-	-	-	0	33
‘Moje Hammarberg'	Unknown	-	-	-	-	-	-	-	-	-	-	0	20	-	-

Table 2.2 (Continued)

Rose Cultivar	Ranking	Archer Co. (30 RRD Observations)		Dallas Co. (1) (220 RRD Observations)		Johnson Co. (84 RRD Observations)		Denton Co. (64 RRD Observations)		Dallas Co. (2) (6 RRD Observations)		Dallas Co. (3) (20 RRD Observations)		McLennan Co. (12 RRD Observations)	
		RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)
‘Morden Centennial'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Morden Fireglow'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Mrs. R. M. Finch'	Susceptible	-	-	-	-	0	33	7	75	67	100	-	-	-	-
‘Mrs. Sam Houston'	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	17	33
‘My Girl'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Naga Belle'	Susceptible	-	-	-	-	-	-	38	75	100	100	-	-	-	-
‘Nearly Wild'	Susceptible	74	100	80	75	-	-	-	-	-	-	-	-	-	-
‘Old Baylor'	Unknown	-	-	-	-	-	-	-	-	-	-	-	-	0	0
‘Old Blush'	Susceptible	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Pink Home Run'	Susceptible	31	50	85	100	-	-	-	-	-	-	-	-	-	-
‘Purple Pavement'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
‘Repeating Swamp Rose'	Tolerant	-	-	-	-	0	50	2	100	17	0	-	-	-	-

Table 2.2 (Continued)

Rose Cultivar	Ranking	Archer Co. (30 RRD Observations)		Dallas Co. (1) (220 RRD Observations)		Johnson Co. (84 RRD Observations)		Denton Co. (64 RRD Observations)		Dallas Co. (2) (6 RRD Observations)		Dallas Co. (3) (20 RRD Observations)		McLennan Co. (12 RRD Observations)	
		RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)
'Rhodologue Jules Gravereaux'	Tolerant	-	-	-	-	1	50	2	50	17	0	-	-	-	-
Rosa arkansana	Tolerant	-	-	-	-	0	0	-	-	-	-		-	-	-
'Rouletti'	Susceptible	-	-	-	-	-	-	7	100	100	100	-	-	-	-
Serena x Basye's Thornless	Tolerant	-	-	-	-	2	17	-	-	-	-	-	-	-	-
'Shreveport'	Susceptible	55	17	80	75	-	-	-	-	-	-	-	-	-	-
'Smokin' Hot'	Tolerant	-	-	-	-	0	0	0	25	0	0	-	-	-	-
'Texas Centennial'	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	0	33
'The Coral Knock Out© Rose'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'The Pink Double Knock Out© Rose'	Susceptible	100	100	41	75	-	-	-	-	-	-	-	-	-	-

Table 2.2 (Continued)

Rose Cultivar	Ranking	Archer Co. (30 RRD Observations)		Dallas Co. (1) (220 RRD Observations)		Johnson Co. (84 RRD Observations)		Denton Co. (64 RRD Observations)		Dallas Co. (2) (6 RRD Observations)		Dallas Co. (3) (20 RRD Observations)		McLennan Co. (12 RRD Observations)	
		RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)	RRD (%)	RRV (%)
'The Rainbow Knock Out© Rose'	Susceptible	-	-	-	-	9	17	3	100	83	100	-	-	-	-
'The Sunny Knock Out© Rose'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'Top Gun'	Susceptible	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'Topaz Jewel'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-
'Tupelo Honey'	Tolerant	-	-	-	-	1	0	7	100	0	0	-	-	-	-
'Winnipeg Parks'	Unknown	-	-	-	-	-	-	-	-	-	-	0	0	-	-

Table 2.2 (Continued)

Final Designation on RRV- Non-Replicated Trials	
Cultivar	**S/U**
16072-N001	S
10FA3	S
10FA6	S
10FA7	S
11FA6	U
11FA7	S
12FA6	U
13FA6	U
13FA7	U
14FA3	S
14FA6	S
14FA7	S
15003-N001	S
15003-N002	S
15003-N003	S
15021-N001	S
15023-N003	S
15045-N002	S
15FA3	U
15FA7	S
16009-N006	U
16009-N038	U
16009-N043	S
16009-N045	U
16009-N059	U
16009-N066	U
16009-N071	S
16009-N076	S
16009-N078	S
16009-N083	U

Table 2.3. Final designation of RRV susceptible, tolerant, resistant, or unknown status for non-replicated trials. This table encompasses the susceptibility decision on cultivars that were screened through non-replicated trials. No decisions about tolerance or resistance were made in the non-replicated trials due to no trials meeting the two-year threshold for those determinations to be made.

Rose Cultivar	**S/U**
16009-N128	S
16009-N130	U
16009-N133	U
16009-N136	S
16009-N163	S
16009-N167	S
16009-N169	S
16009-N170	U
16009-N171	U
16009-N174	U
16009-N175	U
16009-N177	U
16009-N178	U
16009-N182	U
16057-N004	S
16058-N001	U
16059-N001	S
16059-N002	U
16065-N001	U
16067-N004	S
16072-N004	U
16073-N002	U
16081-N007	U
16083-N004	U
16085-N004	U
16086-N002	U
16201-N001	U
16201-N003	S
16401-N011	S
16401-N015	U
16407-N014	U
16407-N016	S
16407-N019	U
16407-N024	S
16409-N003	U

Table 2.3 (Continued)

Rose Cultivar	S/U
16FA3	U
1FA2	S
1FA3	U
1FA5	S
1FA6	U
2FA2	U
2FA3	U
2FA6	U
2FA7	S
2FA8	U
3FA3	S
3FA5	S
3FA6	S
3FA7	U
3FA8	S
3FA9	S
4FA2	S
4FA3	S
4FA5	S
4FA6	U
4FA7	S
5FA5	S
5FA6	U
5FA7	S
6FA3	U
6FA6	U
6FA7	U
7FA3	S
7FA5	S
7FA7	S
8FA3	S
8FA5	S
8FA6	U
8FA7	S
9FA3	S

Table 2.3 (Continued)

Rose Cultivar	S/U
9FA6	U
‘About Face’	S
‘Above and Beyond’	S
‘Apple Dapple’	S
‘Apricot Drift’	S
‘BougainFeelYa’	S
‘Bull's Eye’	U
‘Carefree Spirit’	S
‘Cinco de Mayo’	S
‘Como Park’	S
‘Doubleloons’	S
‘Drop Dead Red’	S
‘Easy Does It’	S
‘Easy Going’	S
EE1130	U
EE1139-N002	U
‘Electron’	S
‘Eureka’	S
‘Eyeconic Lemonade’	S
‘Eyeconic Lychee Lemonade’	S
‘Eyeconic Pink Lemonade’	S
‘Fame’	S
‘High Voltage’	S
‘Hydrangealicious’	S
‘In the Mood’	S
‘Intrigue’	S
‘Just Joey’	S
‘Ketchup & Mustard’	S
‘Kiss Me’	S
‘Lemon Splash’	S
‘Linda Campbell’	S
‘Michelangelo’	U
‘Mystic Fairy’	S
‘Oh My’	S
‘Old Timer’	S

Table 2.3 (Continued)

Rose Cultivar	S/U
‘Purple Splash’	S
‘Red Drift’	S
‘Screaming Neon Red’	U
‘Showbiz’	S
‘Stormy Weather’	U
‘The Sunny Knock Out© Rose’	U
‘Sunshine Daydream’	S
‘Tamango’	S
‘Tequila Sunrise’	S
‘Top Gun’	S
‘Topaz Jewell’	U
‘Traviata’	S
‘Valentine's Day’	S
‘Whimsy’	S
‘Yellow Brick Road’	U
‘Yellow Submarine’	U

Table 2.3 (Continued)

Discussion

While more than 200 named, species, and research rose cultivars were screened for resistance to RRV, in this study, no cultivars met the criteria to be considered resistant (no symptoms and no positive test for a minimum of two years). For the scope of this project, tolerance was defined as a plant with positive RRV detection test but lacked the development of symptoms for a minimum of two years; 14 cultivars met the criteria to be considered tolerant to RRV. While tolerant plants may still allow for transmission of RRV through mite feeding, these cultivars could be a solution in areas where RRD symptomatic plants are widespread for consumers to continue to enjoy roses. While the genotypic makeup for what may be conferring tolerance in these cultivars is unknown, the ability to recommend a tolerant rose to consumers is beneficial. Tolerant roses would give consumers a solution to the disease problem while allowing them to have roses in their landscape. Tolerant plants may also provide another set of rose genetics to explore and work towards resistance to RRV.

Through non-replicated screening trials, several sets of germplasm from rose breeders were quickly and effectively screened for susceptibility to RRV. As these trials were developed to be a rapid screen, the cultivars were not replicated and did not meet the minimum of a two-year screen, plants were reported as susceptible or unknown. These results were reported back to the respective plants breeders, which allowed the breeder to identify these plants as having no resistance to RRD.

Through the cooperation of extension personnel, Master Gardeners, and a private homeowner, data was collected from various trials through participation in citizen

science. Several workshops were presented, reaching over 500 people, and teaching attendees how to setup field trials, how to collect usable data, why plots are randomized, recognition of early RRD symptoms in various cultivars, and tracking disease progression. While I did not collect output and outcome data from individuals participating in these citizen science activities, it is hoped that volunteers that were trained gained a better understanding and appreciation of the scientific methods and the work that is invested in an experiment with scientific soundness. Anecdotally, it was observed that these trained volunteers (Master Gardeners) were able to share their knowledge with others that supported County Extension Agents monitoring efforts.

Through these field experiments, I have identified and confirmed more previously unknown rose cultivars susceptible to RRD. Unfortunately, this study did not reveal a resistant rose cultivar. Based on information collected over a period of two or more years, rose cultivars with tolerance to RRD were identified. Tolerant plants may eventually develop symptoms and become susceptible, as has been observed in the rose cultivar 'Brite Eyes' in field trials in other states[8]. Such observations of symptom development latency have prompted researchers to suggest only designating cultivars that have been in trials for four or more years. In field experiments, the following cultivars exhibit tolerance to RRD based on a two year screening period: 'Bayse's Blueberry', 'Bayse's Purple', 'Caldwell Pink', 'Cherokee', 'Chuckles', Serena x Bayse's Thornless species rose, 'Jean Teresa', 'Lafter', 'Margaret McDermott', 'Rhodologue

[8] M. Windham, personal communication. Fall 2018.

Jules Gravereaux', 'Repeating Swamp Rose', 'Smokin' Hot', 'Tupelo Honey', *R. arkansana* species rose. The non-replicated trials have also shown that putting plants in areas of high disease pressure can be an effective way to quickly screen new germplasm sources for RRV susceptibility or tolerance with symptoms and/or RRV detection capable as early as four months.

While this study has identified cultivars that are potentially tolerant to RRD, it may be useful to use molecular studies to map potential genetic linkages to RRD tolerance traits for breeding purposes. Disagreements of how to designate a tolerant cultivar among researchers, whether after two or four years of field trials, may necessitate further work to better our understanding of the virus latency period in cultivated roses. The addition of recruiting and utilizing volunteers provided both additional help and burden. It would be beneficial to consider measuring potential outcomes and outputs, such as adoption of best management practices for RRD to better understanding/recognition of a reliable and effective RRD management practice, in future studies involving volunteers.

These field experiments continue to confirm that no cultivated rose cultivars are resistant. However, tolerance to RRD appears to be present. Mechanisms contributing to the tolerance effects is not known and should be considered in future studies to provide a better understanding on how the rose plant can live in the presence of RRV and may lead us into a future where roses can be further enjoyed in spite of the *Rose rosette virus*.

CHAPTER III

EVALUATION, VALIDATION, AND OPTIMIZATION OF CURRENT DIAGNOSTIC METHOD(S)

Introduction

Accurate and sensitive plant disease diagnostics is crucial for identification and management of plant diseases that affect both ornamental and food crops. Plant disease diagnostics is traditionally completed through a process called conventional diagnostics (Miller, et al., 2009). Conventional diagnostics entails visual inspection of the pathogen, culturing and growing the unknown pathogen, biochemical tests, and observations of disease symptoms (Miller, et al., 2009). This method of diagnostics can be useful for identification of bacterial and fungal pathogens, given that they are not obligate parasites. Problems can arise when performing conventional diagnostics on obligate parasites, as it is not possible to culture these pathogens outside of a living host. Conventional diagnostics can also be time and resource intensive, making testing of thousands of plant samples very difficult to accomplish. Therefore, utilization of molecular plant diagnostics has become very popular in plant pathology over the last ten years as more effective and accurate molecular testing methodology has been made available and affordable for plant diagnosticians to utilize (Miller, et al., 2009).

One of the most popular forms of molecular plant disease diagnostics is the utilization of polymerase chain reaction (PCR), which is a process that enables a target sequence of a pathogen genome to be amplified through binding of sequence specific forward and reverse primers, allowing for detection of that pathogen. This specific

sequence of genetic information must be unique to that exact pathogen, as non-specific primer binding can result in a false positive result. PCR allows for pathogen detection by utilizing a very small amount of genetic material and can be performed within one to two days whereas conventional diagnostics can often take two weeks to one month. The PCR process was developed in the 1980's, after the discovery of a stable Taq DNA polymerase made automation of DNA replication possible using a thermocycler (Bartlett & Stirling, 2003). Quantitative real-time PCR (qPCR), which is a process that utilizes a next generation polymerase, was developed in 2009 and has quickly become one of the most popular PCR processes due to its speed for detection (Bartlett & Stirling, 2003).

The main steps of the PCR cycle are denaturation (heating of DNA to dissociate the double stranded helix), annealing (lower temp to allow primer binding at complementary site on the genome), and elongation (higher temp to allow polymerase to make new, short segments of DNA). In the case of RNA viruses, an extra step is required before the PCR process can occur: a reverse transcription (RT) (generation of DNA from an RNA template) to convert RNA to complementary DNA (cDNA).

The most utilized PCR processes in plant disease diagnostics are conventional PCR and qPCR. The main PCR protocols for RNA viruses are one-step or two-step protocols; typically, these protocols determine if the RT step is performed in a separate tube (two-step) or in the same tube as PCR (one-step). Conventional Reverse Transcription (RT)-PCR for RRV involves performing a one-step protocol PCR to produce a product that can then be visualized using gel electrophoresis. After electrophoresis is performed, the presence of a pathogen can be determined based on

sample fluorescence in gel visualization. Fluorescence and visualization are possible through the use of Ethidium Bromide (EtBr) or other chemistries, such as gel red. The use of qPCR is more rapid for pathogen detection and can often be more sensitive than RT-PCR. The process of qPCR involves the same step as RT-PCR; however, visualization of the PCR product is done at the end of each PCR cycle to give a numeric measurement of the amount of pathogen contained in a sample. Visualization of qPCR products is possible using an additional oligo that is fluorescent tagged, called a probe. The probe binds to a specific sequence within the target amplicon and fluoresces at a known wavelength of light (specific to the fluorescence on the probe) allowing the thermocycler to measure pathogen amounts in each cycle which results in a cycle threshold (C_t) value for the amount of pathogen. C_t value is a measure of PCR target concentration (applied biosystems, 2016). While qPCR is quick and generally effective, it is not always efficient to be used for plant diagnostics due to equipment and supply costs.

The extraction of RNA or DNA from a plant for use in PCR or qPCR is another process that must be simple and cost efficient, yet sensitive enough to not contribute to false negative results. Qiagen© RNeasy Plant Mini Kit is an example of a silicon-based spin column (filtration) technology. The kit utilizes a small amount of fresh plant tissue, typically new growth followed by freezing the tissue, beating the tissue to lyse cells, and then using a series of washes and buffers to produce a stable RNA extract. This kit can be cost prohibitive and difficult to teach; however, it produces an extract of high quality. Another extraction method that can be utilized is the Simple Direct Tube (SDT)

(Suehiro, et al., 2005) or Direct Antigen-Capture (Babu, et al., 2017b) process. In this process, plant tissue is smashed to aid in cell lysis, the lysed material is then added to a polypropylene tube to allow virions to bind directly to the tube walls through adsorption. This method is quick and cost efficient, however it may not yield a clean, RNase free extract as the column-based technology because of the process being completed in one tube and does not utilize specific buffers to prevent RNA degradation, like column-based extraction kits.

Molecular diagnostic capabilities for *Rose rosette virus* (RRV) became possible with the development of the first primer set in 2011 (Laney, et al., 2011). This conventional RT-PCR primer set targets segment 1 of RRV, which encodes for the replicase segment. The first set of qPCR adapted RRV primers were published in 2015 and target segment 3 (nucleocapsid) (Dobhal, et al., 2016). Subsequent qPCR primers sets were published in 2017 that targeted both segments 2 (glycoprotein) and segment 3 (Babu, et al., 2016; Babu, et al., 2017). Three more RT-PCR primer sets were published in 2017 that target segment 3 and 4 (movement protein) (Bratsch, et al., 2017; Di Bello, et al., 2017). These primer sets target the four segments of RRV that have a known function.

Project Objectives and Aims

In order to be able to effectively study the nature of RRV and the disease it causes, reliable detection of the virus is critical. The project objective was to identify, optimize and implement an effective, reliable, consistent, and inexpensive method(s) for RRV detection. Time and cost are two important factors in the operation of a general

plant disease diagnostic lab. My goal was to identify a reliable genomic extraction method that is sufficient, easy to execute, and inexpensive for use in a general plant disease diagnostic lab. Several PCR-based RRV detection methods have been previously published; therefore, I planned to evaluate these protocols, specifically evaluate primer sets, and optimize the method(s) for use in diagnostic lab situations. Currently that is no reliable methods to effectively detect early RRV (asymptomatic) infection; my goal was to evaluate and optimize some primer sets for their ability to be used in asymptomatic detection of RRV in the rose plant.

Materials and Methods

Extraction

The primary focus of this study was to optimize an RNA SDT extraction method and verify its stability for use in diagnostics. The first study is to compare a standard extraction method (Qiagen© RNeasy plant mini kit) utilized in general plant diagnostic labs to a rapid one tube extraction (Modified Direct Antigen-Capture (MDA-C)) method. These two extraction methods were compared for extraction effectiveness as well as costs inputs required to complete them. Further studies were completed using both extraction methods to verify temperature stability of the MDA-C method. Two additional studies were performed utilizing only the MDA-C method. The first focused on determining if adsorption was the mechanism that enabled antigen trapping; the second was focused on the universality of MDA-C for other viruses.

RNA Extraction Process Comparison

In this project, RRV extraction was completed using two different extraction methods, Qiagen© RNeasy Plant Mini Kit and MDA-C. Qiagen© yields a stable (low RNA degradation), total RNA extraction. The MDA-C is a one-tube antigen trapping extraction method which yields a possibly less stable extraction (due to more RNA degradation). A cost comparison was performed between these two approaches to evaluate the financial cost that is incurred by these methods. Cost of consumables was calculated on a one sample basis and labor was calculated at $16.75/hour rate.

Qiagen© RNeasy Plant Mini Kit

This extraction is performed per manufacturer instruction.

1) Plant tissue is sampled, with preference given to the newest/freshest tissue; 0.05 grams of tissue is chopped and weighed out for extraction. Chopped tissue is placed in a 1.4MM homogenizing bashing bead lysis tube.

2) Lysis tube in immersed in liquid nitrogen for 1 minute and processed through a bead beater for 40 seconds. Lysis tubes are returned to liquid nitrogen for 1 minutes and then to the bead beater for another 40 seconds. Lysis tubes are spun down in a centrifuge for 30 seconds at 8,000rcf.

3) 450µl of RLT buffer is added to each lysis tube; tubes are then vortexed vigorously and centrifuged at 14,000rcf for 1 minute. Lysate is collected from lysis tube, taking care to avoid the tissue pellet in lysis tube.

4) Lysate is placed in a QIAshredder spin column that is in a 2 ml collection tube. The spin column-tube is centrifuged at 14,000rcf for 2 minutes.

5) Supernatant of the column flow through is transferred to a new 1.5ml microcentrifuge tube; this is done without disturbing the cell-debris pellet in the previous 2ml collection tube. Amount of supernatant collected from 2ml tube is estimated and 0.5 volumes of 96-100% ethanol are added to the supernatant tube and mixed by pipetting the volume several times. This lysate solution is then transferred to a RNeasy spin column, which is placed in a 2ml collection tube.

6) The tube is then centrifuged for 15 seconds at 10,000rcf. Flow-through and collection tube are discarded and RNeasy spin column is placed in a new 2ml collection tube. Transfer, spin, and discard steps may need to be repeated if the lysate amount is over 700µl upon adding ethanol.

7) After column is transferred to new collection tube, add 700µl of RW1 buffer to the RNeasy spin column. Centrifuge for 15 seconds at 10,000rcf. Discard flow through but retain the 2ml collection tube and column.

8) Add 500µl of RPE buffer to the RNeasy spin column and centrifuge for 15 seconds at 10,000rcf; discard the flow through but retain column and collection tube.

9) Add 500µl of RPE buffer to RNeasy spin column and centrifuge for two minutes at 10,000rcf; remove the RNeasy column and place in a 1.5ml micro centrifuge tube.

10) Add 50µl of RNase free water directly on the spin column membrane and centrifuge for one minute at 10,000rcf. Discard the column and retain the eluted RNA in the micro centrifuge tube.

Modified Direct Antigen-Capture (MDA-C) RNA Extraction (Figure 3.1)

1) Sample two grams of plant tissue from sample, with preference being given to material in the best condition. Any tissue type (leaves, roots, non-woody stems, petals, hips) can be used, but fresher tissue allows for the best extraction.

2) Place tissue in an ELISA samples mesh bag (Agdia. IN) and add 3.5ml of PBST/ 1 gram of tissue. PBST is prepared at rate of 1 gram of PBST powder to 100ml of H_2O. Homogenize tissue and buffer in mesh bag to achieve cell lysis. Homogenization can be done using a drill press or pestle.

3) Transfer 1500µl of lysed cell/plant tissue mixture to 1.5ml micro centrifuge tube. (May need to add more PBST to mesh bag mixture if too thick for pipetting.)

4) Incubate lysis mixture on ice for two minutes, then remove and pour lysis mixture out of tube.

5) Rinse tube with 1ml of PBST and pour out; repeat this rinse and pour process four more times, for a total of five rinses.

6) Pop any remaining bubbles in tube (using a pipette tip or by flicking the tube) and then heat tube for one minute at 95° C; close tube lid if it pops open during heating.

7) Remove from heat and place tube in -20° C for 1 minute.

8) Add 30µl of H_20 and 2µl of RNasin to the tube to elute the viral particle retained in the tube.

9) Vortex tube vigorously to collect all viral particles that are on wall of tube. Complete extraction by centrifuging tube at 10,000rcf for 10 seconds.

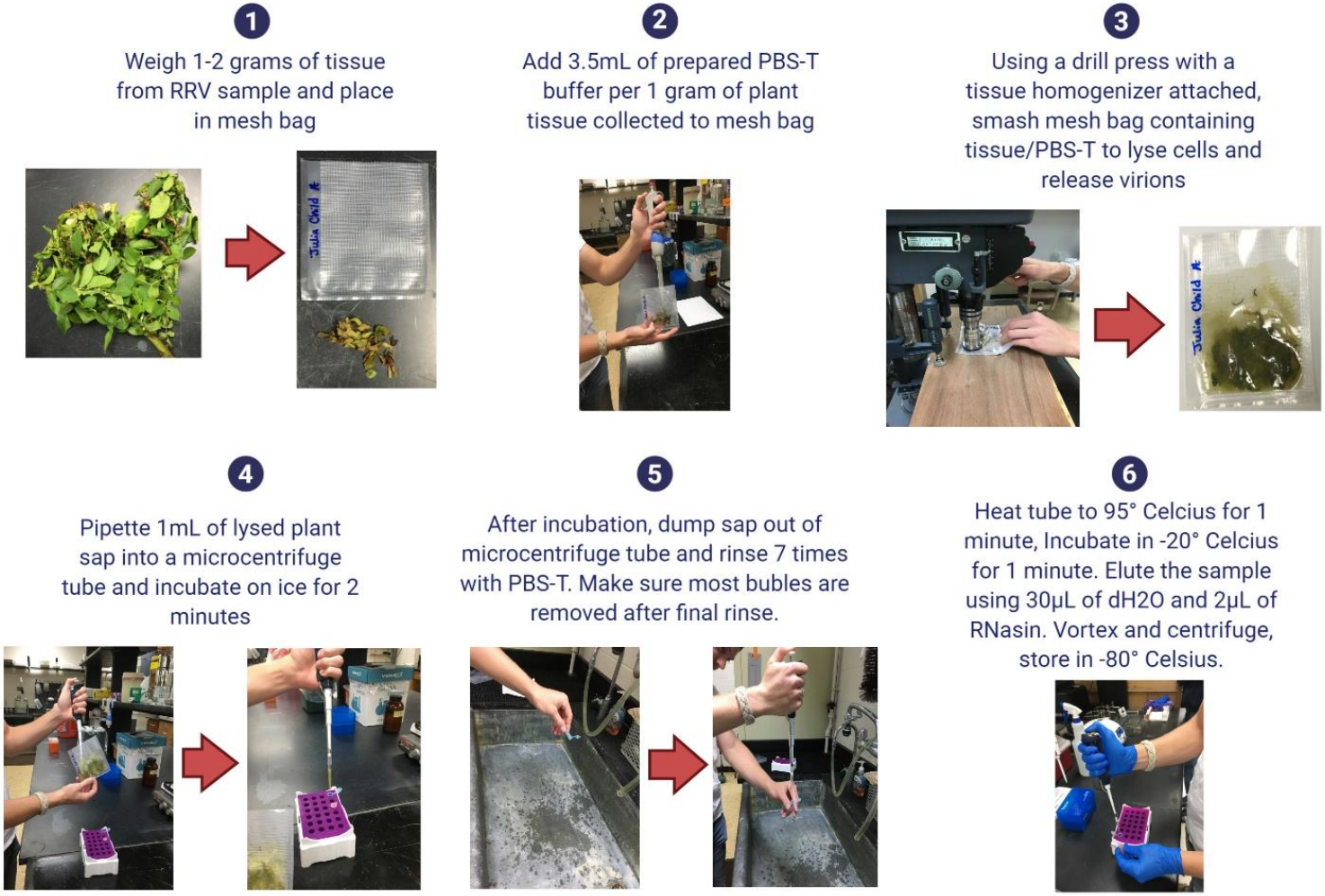

Figure 3.1. MDA-C Extraction Process. This figure shows the full MDA-C protocol using the most common equipment.

Stability of MDA-C Extracted RNA

Stability of RNA extracts subjected to freeze-thaw cycles

Extracts from each of the previously described extractions methods were compared to determine if extracts from Modified Direct Antigen-Capture method were as temperature stable as Qiagen© extracts. Samples were taken from a single symptomatic plant and four extractions of each method were prepared according to the protocol for extraction. Extracts from MDA-C and Qiagen® were quantified and normalized to similar RNA concentrations. All extracts were subjected to multiple thawing and freezing cycles. Repeated heating and cooling can impact unstable RNA extracts by allowing RNase to become active and degrade RNA (Brustein, 2015). Thawing was done by removing the extracts from -20° C and allowing them to warm to room temperature. Testing, using one-step qPCR protocols, was done before the experiment to establish a baseline (all samples were in strong positive range) and subsequently completed after two, four, six, and 10 thawing and freezing cycles. Freezing and thawing experiments were repeated with MDA-C extracts that had higher C_t values (less positive for RRV). The same method was followed, except that Qiagen© extracts were not from the same plant as MDA-C, however they were normalized to a similar concentration as the MDA-C extracts.

Extraction Comparison of Extreme Temperatures

Extractions were performed from a single symptomatic plant utilizing both RNA extraction methods. An initial RRV detection assay was performed, and samples were determined to be in the strong positive range (high titer). The quantified and

normalized extracts were subjected to varying temperature increases, 24 hours at 22-25° C, 72 hours at 22-28° C, and 24 hours incubated at 32° C. The RRV qPCR detection assay was performed, and C_t values were compared among the two extraction methods.

Secondary Study- Mechanism of MDA-C RNA Extraction & Exclusivity of RNA Extract

A brief experiment was designed and performed to assess if viral particles binding to microcentrifuge tubes by plastic could be inhibited by exogenous proteins competing for the same binding sites. A second experiment was designed and performed to determine if MDA-C RNA extraction is exclusive for use on RRV detection, or if it could be used for detection of other viruses.

Prevention of Adsorption Experiment

Due to natural, non-specific binding ability of polypropylene plastic, experiments were done to attempt to prevent binding of viral particles to plastic micro-centrifuge tubes. Two substances high in protein, 1% Bovine Serum Albumin (BSA) and 10% Skim Milk, were chosen to prevent binding of viral particles by outcompeting particles for binding sites. Once both substances were prepared according to manufacturer instructions, they were stored in 4° C to chill. In the first of two experiments, 18 1.5ml tubes were labeled for BSA, 18 for Skim Milk, and 18 for H_2O (Negative Control). 1000µl of each substance was added to the respective tube and incubated for five minutes, 15 minutes, 30 minutes, 1 hour, 12 hours, and 24 hours. After each incubation step was completed, tubes were rinsed with 1000µl of PBST and stored in -20°C. Plant tissue was collected from a single symptomatic plant and extracted

according to the MDA-C protocol. The treated tubes were utilized for MDA-C throughout the RNA extraction. The extracts captured in the treated tubes were then tested using the one-step qPCR protocol. In the second binding experiment, 24 for skim milk treatment, and 24 for H_2O (negative control) treatment. 1000µl of each substance was added to the respective tube and incubated for a set amount of time. Intervals used were 15 minutes, 30 minutes, two hours, six hours, 12 hours, 24 hours, 48 hours, and 72 hours. After each incubation period tubes were rinsed with dH_2O and stored at -20° C. Tissue collection, utilization of tubes, and testing were done following the steps of the previous coating experiment. One modification that was made to the extraction process was the use of H_20 to rinse tubes instead of PbST. This change was made to determine if PbST removed all proteins from the coated tubes, enabling RRV binding to occur. The coating times were increased in this experiment to determine if a longer incubation time would prevent RRV binding.

Evaluation of MDA-C RNA extracts for detection of other Viruses

After high success with detection of RRV using the MDA-C method, detection of other RNA viruses using the method was questioned. Rose material infected with *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV), *Blackberry chlorotic ringspot virus* (BCRV), *Rose spring dwarf virus* (RSDaV) and *Rose yellow vein virus* (RYVV) was collected from a rose collection from Florida Southern College. Tissue was sampled and extracted according to the MDA-C RNA Extraction protocol. Detection testing was done following the Taqman One-step qPCR protocol and using primers and probes that were specific for the virus. *Tobacco mosaic virus* (TMV) and

Capsicum chlorosis virus (CaCV) infected material was obtained from TPDDL and extracted following the MDA-C protocol; detection was performed using the one-step RT-PCR protocol. Gel electrophoresis was performed following the previously described protocol.

Detection Approaches

Three RRV detection approaches were compared for effectiveness to detect this virus: conventional RT-PCR utilizing a one-step kit, and two real-time PCR approaches (Taqman Quantitative One-Step PCR and Taqman Quantitative Two-Step PCR).

Conventional One-Step RT-PCR Detection of Rose rosette virus

Qiagen© One-Step RT-PCR Kit was used in this study. Master Mix (as prepared on a per sample basis) is: 14.5µl of H_2O, 5µl of 5X one-step RT-PCR buffer, 1µl of 10mM dNTPs, 1µl of enzyme mix (Omniscript reverse transcriptase, Sensiscript reverse transcriptase, and HotStar Taq DNA Polymerase), 1.25 of 10µM RRV Forward (F) and Reverse (R) primers (F and R primers are mixed to provide ease-of-use), and 0.25µl of 50mg/ml BSA. Reagents are added to sterile 1.5ml master mix tube, tube is inverted several times by hand, then spun in centrifuge for 20 seconds at 8000rcf. Transfer 23µl of master mix to a 0.2ml PCR tube. (Keep master mix and PCR tubes at -4° C during PCR preparations). 2µl of RNA template is added to the PCR tube for a total volume of 25µl in 0.2mL PCR tube. PCR tube is vortexed and centrifuged for 10 seconds at 8000rcf; pop any bubbles that are in the bottom or on the sides of the PCR tube by gently tapping the tubes on the counter. PCR tube is places in thermocycler and run with the following parameters: Lid Temperature 99° C; 30-minute incubation at 50° C; 15-

minute incubation at 95° C; 40 cycles of 30 seconds at 94° C, 30 seconds at 51°C, 60 seconds at 72° C Final Extension for 10 minutes at 72° C. After PCR is completed, products are stored at -20° C. Controls used in PCR are H20, RRV negative rose (healthy control), RRV positive rose.

PCR products were visualized on a 2% TAE-agarose gel. Dye used for samples was 6X dye; 5µl of PCR product was dyed with 1µl of dye. After dyeing, samples were loaded into prepared gel. Samples were ran at 5V/cm for 60 minutes. After electrophoresis was completed, gels were visualized using BioRad UV chamber and ImageLab software. A band at the appropriate size for the primer set indicates a positive detection result for RRV.

Taqman Quantitative (Real-Time) One-Step PCR Detection of Rose rosette virus

Reagents used for qPCR, as prepared on a per sample basis, are: 11.5µl of RNase free water, 5µl of Taqman™ Fast Virus 1 Step Master Mix (Thermofisher), 1µl of 10µM Forward and Reverse RRV primer (primers are mixed for ease-of-use), and 0.5µl of 10µM TaqMan RRV(2) Probe. All reagents are added to a sterile 1.5ml micro centrifuge tube, inverted by hand several times, and centrifuged for 20 seconds at 8000rcf. After centrifuging, 18µl of master mix is added to PCR well. (PCR tubes are Applied Biosystems MicroAmp Reaction tubes). Additionally, 2µl of RRV sample extract is added to the PCR tube for a total volume of 20µl. After capping all PCR wells, tubes are vortexed and centrifuged for 8-10 seconds at 8000rcf; any remaining bubbles in the bottom or sides of mix are removed. PCR is performed on sample with the following parameters on an Applied Biosystems QuantStudio™ 6 Flex Fast Real-Time PCR

Machine. Thermocycler settings: Experiment is set to standard curve, block is selected as Fast 96 well, Instrument run is set as Fast, and Reagents are set as Taqman. Further definitions for Reporter/Quencher settings for probe are: RRV2 is set to FAM/None, Passive Reference is set to ROX. Cycling Parameters are: reverse transcription for five minutes at 50° C; 20 seconds at 95° C (data collection off); 40 cycles of three seconds at 95° C (data collection off), 30 seconds at 60° C (data collection on).

Taqman Quantitative (Real-Time) Two-Step PCR Detection of Rose rosette virus

This method utilizes the RRV cDNA Synthesis: Promega GoScript™ Reverse Transcription System. The first step to generate cDNA is as follows: prepare denaturation mix by adding 2µl of RNase free water and 1µl of Random Hexamer primers per sample to a 0.2ml tube; add 2µl of RRV RNA to the tube for a total volume of 5µl. Heat tubes to 70°C for 5 minutes; chill on ice for 5 minutes; centrifuge at 2000g for 10 seconds and store on ice. Prepare reverse transcription master mix by aliquoting (per sample) 7µl of RNase free water, 4µl of GOScript™ 5X Reaction Buffer, 1µl of $MgCl_2$ (25mM), 1µl of PCR nucleotide mix (10mM), 1µl of Recombinant RNasin© Ribonuclease Inhibitor (40µ/µl), 1µl of GoScript™ Reverse Transcriptase for a total of 15µl of master mix per sample. Vortex, centrifuge at 2000g for 10 seconds and then add the 5µl of denaturation mix previously held on ice to the 15µl of reverse transcription master mix; vortex and centrifuge at 2000g for 10 seconds. cDNA thermocycler settings for mix are anneal for 25°C for 5 minutes; extend at 42°C for 60 minutes, incubate at 70°C to inactivate reverse transcriptase, hold at 4°C.

The second step of this method utilizes the RRV qPCR using TAKARA Premix Ex Taq™ protocol. To complete PCR utilizing the cDNA made in the first step, prepare a Master mix on a per sample basis as follows: 2.5µl of RNase free water, 10µl of TAKARA Premix Ex Taq (2X), 0.8µl PCR primer F/R RRV_2 (5µM), 0.4 µl Rox Ref Dye II (50X), 1.6µl BSA, 2µl PVP 40 (100mg/ml). Vortex and centrifuge at 2000g for 20 seconds; aliquot 18µl of master mix into each sample tube; add 2µl of previously made cDNA to each corresponding well; vortex and centrifuge 2000g for 10 seconds. Perform qPCR using the following parameters: Presence/absence experiment, 95°C for 30 seconds, 40 cycles (95°C for 3 seconds followed by 60°C for 30 seconds), 60°C for 30 seconds.

Interpretation of qPCR Value Results

C_t values are generated to indicate when positive detection of the target occurred. Lower C_t values mean a higher infection titer. C_t threshold for detection is often set at 37.0 as non-specific amplifications and contamination is suspected to cause late detection. Typical range for positive detection is C_t values between 5 and 37. A negative sample will result in a value of 0 or Undetermined. C_t values that are equal to or less than 29 are considered strong positives and samples that are 30-37 are considered to be moderate to weak positives (WVDL, 2013). There are several types of variations that can occur with each qPCR run that affect the C_t value that is yielded. In the TPDDL, we accept the variation of a sample to be +/- 3.5 C_t values. Some of the factors that can cause variation are differences in master mix preparation, detection efficiency of the specific run, and small amounts of contamination (appliedbiosystems, 2016). While all

efforts are taken to minimize the effects of these external factors, due to nature of a diagnostic clinic, variation is expected.

Primer Evaluation and Optimization

RRV Primer Selection

Nine primer sets for RRV detection were evaluated in this study. These primer sets were published between 2011 and 2018. Multiple RRV primers were released/reported throughout the duration of this project and were incorporated to this study (Table 3.1).

Available RRV Primers					
Designation	**Sequence**	**Length of Amplicon (BP)**	**RNA segment targeted**	**Type of PCR**	**Reference**
RRV	F: 5′-CAGAATGAACCATAGATGTC-3′ R: 5′-AATGGTCTGCTCGAGATT-3′	319	1	RT-PCR	(Laney, et al., 2011)
RRV3	F: 5'-AGACTTGAATGAGTCTGATGTTCT-3' R: 5'-GCATCATCGAAAGGAGTAGTCTC-3'	411	3	RT-PCR	(Bratsch, et al., 2017)
RRV4	F: 5'-CGTGACAGGCTCACTTGATT-3' R: 5'-CCTGACAGTGCAGAGCTTAAT-3'	475	4	RT-PCR	(Bratsch, et al., 2017)
RRV (db/271)	F: 5′-GCACATCCAACACTCTTGCAGC-3′ R: 5′-CTTATTTGAAGCTGCTCCTTGATTTCC-3′	271	3	RT-PCR	(Di Bello, et al., 2017)
RRV2	F: 5'-TGCTATAAGTCTCATTGGAAGAGAAA-3' R: 5'-CCTATAGCTTCATCATTCCTCTTTG-3' PROBE: 5'-TGCTAGAGACATTGGTACAACAAGCAA-3'	104	3	qPCR	(Dobhal, et al., 2016)
RRV_2-1	F: 5'-CCACAGACAGTTGCAGTAGTT-3' R: 5'-TGGAGCCGTTGAATGCTTAG-3' PROBE: 5'-ACAGCTGAAGCCATCATGAACCTT-3'	117	2	qPCR	(Babu, et al., 2016)

Table 3.1. Published primers for RRV Detection. Primers have been developed to target various sections of the first four segments of the virus. The first four primer sets listed are optimized for RT-PCR. The final five primer/probe sets listed are optimized for use in qPCR.

RRV_2-2	F: 5'-TGGAGCCGTTGAATGCTTAG-3' R: 5'-TTGGCTCTACCCTTTCTTTCC-3' PROBE: 5'-TGAACAAGGGTGGACCATTCCACA-3'	100	2	qPCR	(Babu, et al., 2016)
RRV_3-2	F: 5'-ACACTCTTGCAGCTGATACTG-3' R: 5'-CTGATACTGTTATCATCGAGCTG-3' PROBE: 5'-AGCTTCGGGTCCTCAAGTTGACAA-3'	117	3	qPCR	(Babu, et al., 2016)
RRV_3-5	F: 5'-CTGATACTGTTATCATCGGAGCTG-3' R: 5'-TCTGAATCTCAGGCTTCACTA-3' PROBE: 5'-AGCTTCGGGTCCTCAAGTTGACAA-3'	94	3	qPCR	(Babu, et al., 2016)

Table 3.1 (Continued)

Primer set sensitivity assay

Primer set sensitivity was evaluated using RNA template from one symptomatic plant source, extracted using the Qiagen© RNeasy kit. The resulting extract was quantified to determine the RNA concentration; concentration of RNA was below 100ng/µl. A five-fold dilution series (1µl template + 4µl dH_20) with 10 dilution steps was completed. A five-fold dilution series was performed due to a lowered RNA concentration in the RNA extract. The concentration of primers used in both conventional and qPCR protocols was 0.5 µM. The initial sample and the resulting dilutions were subject to each primer set utilizing the appropriate PCR protocol. One-Step RT-PCR protocol was used with primer sets RRV, RRV3, RRV4, and RRV(db/271). One-Step qPCR protocol was used with primers RRV2, RRV_2.1, RRV_2.2, RRV_3.2, and RRV_3.5. Results of each PCR test were evaluated based on the dilution step that the primer set was able to amplify. MDA-C extracts were originally used, however due to the low RNA quantity that results from adsorption, all detection efficiency was lost within one dilution in the series (data not shown).

Primer set detection effectiveness

The nine primer sets in Table 3.1 were evaluated for sensitivity to be able to detect RRV. RNA template used for this experiment was extracted using the MDA-C extraction method. Primer sets developed for qPCR were subjected to evaluation using Taqman qPCR One-Step Protocol and primer sets developed for conventional PCR were subjected to Qiagen© One-Step RT-PCR Kit (as previously described above). To compare effectiveness in RRV detection, 50 RRV samples were selected based on their

initial C_t value using the plant diagnostic lab standard primer set (RRV2). Each sample was subject to the primer set utilizing the appropriate PCR protocol. Conventional Qiagen© One-Step RT-PCR protocol was used with primer sets RRV, RRV3, RRV4, and RRV(db/271). Taqman qPCR One-Step protocol was used with primer sets RRV2, RRV_2.1, RRV_2.2, RRV_3.2, and RRV_3.5. The template was not normalized for this experiment, rather all samples selected were in the strong (< 29 C_t value) range. The ability of the primer set to detect RRV was recorded.

Validation for Early Detection

Numerous RRV suspect plants were utilized for this study. These plants came from both field trials (work covered in chapter 2) and greenhouses (work covered in chapter 4). Two primer sets were used in this test; RRV(db/271), developed for RT-PCR and RRV2, developed for qPCR. All samples were extracted using the MDA-C protocol; detection protocols that were used are One-Step RT-PCR and One-Step qPCR. No RNA concentrations were recorded as only presence/absence data was collected. Through the duration of this study, asymptomatic plants that were suspected to be infected with RRV were tested utilizing the previously outlined extraction and detection processes. Data for this study was collected over a period of 18 months. The total number of asymptomatic plants tested with each primer set was recorded. The percentage of asymptomatic, infected plants detected by the given primer set was determined by dividing the number of positive samples by the total number of samples tested with the primer set. The number of samples tested with each primer varies, as more samples were tested using the RRV(db/271) primer. All samples tested with the RRV2 primer set were also tested

using the primer set RRV(db/271). Some PCR products from RRV(db/271) were sequenced for verification that asymptomatic detection was occurring. This study focused on determining primer ability to detect RRV in more than 300 asymptomatic cultivars with the assumption that interference with detection by rose physiology and chemistry is negligible.

Results

The purpose of this study was to develop and/or optimize RRV extraction and detection protocols that would allow multipurpose plant disease diagnostic labs, such as those that are members of NPDN, to be able to perform accurate and consistent RRV diagnostics in a reliable and reproducible fashion.

Extraction-Primary Studies

The first step in any RRV molecular detection protocol is the extraction of genomic template from a test subject. In a general/multipurpose plant disease diagnostic lab, typical of most NPDN member labs, extraction methods should be inexpensive, shelf stable, and easy to perform. Current standard method for RNA extraction in many NPDN labs is the commercially available Qiagen® RNeasy kit. In this study, I compared this approach with MDA-C, which was cheaper to run than Qiagen® RNeasy.

A cost comparison study was performed on both RNA extraction methods on a per sample basis, with findings that the MDA-C method costs $8.39 compared to Qiagen© extraction kit cost at $20.80 (Table 3.2).

Two symptomatic plants were used in the analysis (Table 3.3) to compare the ability to detect RRV between the two extraction methods. Samples GH1-2 returned a C_t range of 17.532-18.363 from the Qiagen® extraction, while the MDA-C extracts returned a RRV detection C_t value range of 24.954-27.772. Qiagen® samples GH3-4 returned a C_t value range of 16.261-18.397, while MDA-C extracted samples GH3-4 returned a C_t value range of 21.684-25.383. Samples GH1-2 showed an increase in the C_t range of MDA-C extracts by 7.422-9.353. Similarly, samples GH3-4 indicated an increase in C_t value for RRV detection from the MDA-C extract of 4.423-6.986 suggesting that the RRV titer in the MDA-C extract is reduced by at least 10000-fold compared to the Qiagen® extraction. Multiple extraction performed from a single plant utilizing both extraction methods for a comparison of detection abilities showed there were differences in detection between the extraction methods, but MDA-C still yielded results that were acceptable for routine diagnostic processes (Table 3.3).

Price Comparison Data	
Qiagen© RNeasy Plant Mini Kit Extraction	**Modified Direct Antigen-Capture Extraction**
Kit: $8.20/ Sample	Mesh Bag: $0.34/ Sample
	PbST: $0.05/ Sample
	rRNasin®: $2.40/ Sample
Labor (45 min/sample): $12.60	Labor (20 min/sample): $5.60
Total Cost (per sample): $20.80	**Total Cost (per sample): $8.39**

Table 3.2. Price and time comparison for two RNA extraction protocols. On a strict cost basis, excluding labor, there is a $5 dollar difference in material costs between the two extraction methods. When adding labor into the cost analysis, there is a $12.41 difference in the two extraction methods.

Comparison of Two Extraction Methods		
Sample Name	**Qiagen© (C_t value range) (n=6)**	**MDA-C (C_t value range) (n=6)**
GH 1-2	17.532- 18.368	24.954-27.722
GH 3-4	16.261-18.397	21.684-25.383

Table 3.3. Comparison of MDA-C versus Qiagen detection sensitivity. The results showed there were differences in extraction efficiency, likely due to the adsorption mechanism that only traps virions on certain spots of the tube wall, whereas Qiagen© is a total RNA extraction.

The need for a rapid extraction protocol allowed for the identification and optimization of the MDA-C extraction method. Further examination of the MDA-C extraction method was done to determine its repeated use and temperature stability. The stability of the MDA-C extraction method to multiple freeze/thaw cycles was evaluated. Extract products from both Qiagen® and MDA-C were subjected to two, four, six, and ten freeze/thaw cycles (Tables 3.4 and 3.5). In tests utilizing extracts from highly infectious plants (Table 3.4), there was little change in the C_t value generated (C_t value= <1) over the different number of freeze/thaw cycles. This indicates that repeating these temperature cycles does not appear to affect the quality of the RNA for detection purposes when RRV titer is high. The same test was conducted using samples with low RRV titer that initially tested, using the diagnostic lab standard protocols, in the moderate to weak positive range (Table 3.5). The results show that Qiagen® and MDA-C samples which were moderate and weak positives for RRV were more likely to see a rise in C_t value with repeated freezing and thawing when compared to strong positive samples. Most changes in C_t value was within the expected range of variation (+/- 3.5),

however by cycle 10, most samples exceeded this range and experienced degradation. This shows that repeated use of less positive samples can yield a false negative result.

Comparison of Qiagen® and MDA-C extracts exposed to prolonged increased temperature was also evaluated to simulate samples left on lab benches for extended time periods. Results from this test show little change in the Qiagen® extracts at all test periods (24 hours 22-25°C, 72 hours 22-28°C, and 24 hours 32°C). However, MDA-C extracts showed an increased C_t value of almost 2 cycles when time is prolonged for 72 hours at 22-28°C and when temperatures were elevated to 32°C for 24 hours.

MDA-C Extraction Stability				
	Qiagen© (C_t)		MDA-C (C_t)	
Cycle	**High Titer (n=2)**	**Low Titer (n=2)**	**High Titer (n=2)**	**Low Titer (n=2)**
Initial	18.397, 18.138	31.747, 36.166	24.971, 22.661	31.078, 35.753
2 Cycles	18.268, 17.715	32.360, undetermined	24.722, 21.961	31.888, 38.370
4 Cycles	18.270, 17.932	33.695, undetermined	24.696, 21.943	32.174, 36.354
6 Cycles	18.368, 17.799	31.801, 38.141	24.896, 21.684	30.348, 36.196
10 Cycles	17.945, 17.532	35.336, undetermined	24.954, 21.879	32.934, undetermined

Table 3.4. MDA-C extract stability. The results show that Qiagen© and MDA-C samples which were low titer for RRV were more likely to see a rise in C_t value with repeated freezing and thawing when compared to strong positive samples. Most of the samples were within in the acceptable range of variation (+/- 3.5) that is established in the TPDDL, however most samples in cycle 10 exceeded that number.

MDA-C Extraction Stability Extremes		
Lab Extreme	**Qiagen© (C_t) (n=2)**	**MDA-C (C_t) (n=2)**
Initial	18.033, 18.444	24.602, 21.936
24 hours @ 22-25° C	16.261, 18.299	25.454, 22.719
72 hours @ 22-28° C	16.373, 18.397	27.585, 24.476
24 hours @ 32° C	16.293, 18.21	27.722, 25.383

Table 3.5. MDA-C extracts after being exposed to 24 and 72 hours of room temperatures. When samples were left at room temperature for 24 hours, some small C_t changes were noticed in the MDA-C samples, however the samples remained in the strong positive range. When samples were left at room temperature for 72 hours, another small increased in C_t value was noticed in MDA-C extracts, however, detection was not greatly impacted. After samples were heated for 24 hours, C_t values remained like the results yielded in the previous experiment.

Extraction- Secondary Studies

Two experiments were completed to determine the mechanism of MDA-C that allowed for RNA extraction. (Tables 3.6 and 3.7). The suspicion is that non-specific binding, also called adsorption, which could be interfered with the addition of proteinaceous material. Theses brief experiments seem to indicate that the use of highly proteinaceous substances, such as BSA and skim milk, prevent adsorption of RRV from occurring. The first of these experiments utilized samples that yielded C_t values in the strong positive range and incubation periods of skim milk and BSA at two minutes, two hours, four hours, six hours, and 24 hours. No incubation period with either substance yielded C_t values that were outside of the acceptable range of variation (+/- 3.5) from the untreated control (Table 3.6). As no change was found in the first experiment of prevention of adsorption, it was decided to repeat the experiment using only skim milk and longer incubation periods (Table 3.7). The additional incubation periods that were

added are 48 hours and 72 hours. The results of this experiment show that skim milk, when rinsed with dH2O does increase the C_t values outside of the acceptable range of variation. This is different from the original experiment and thought to be due to the change to dH2O as the rinsing agent instead of PbST. Increased incubation times did not cause a rise in C_t value.

Work was completed utilizing the MDA-C protocol for seven additional RNA and DNA viruses. All extracts yielded a similar C_t value or band when compared back to the lab standard extraction kits (data not shown). It is believed that the extraction method works well for most viruses due to viruses being composed of proteins that can non-specifically bind to polypropylene plastics.

Prevention of Adsorption Experiment		
Coating Time Interval	**BSA C_t Value Range (n=3)**	**Skim Milk C_t Value Range (n=3)**
No Coating	27.951-29.488	25.228- 28.955
2 Minute Incubation	26.662- 27.178	25.904-29.072
2 Hour Incubation	24.734-27.844	26.519-29.294
4 Hour Incubation	24.491-29.892	26.539-31.682
6 Hour Incubation	25.389-27.905	23.993-29.572
24 Hour Incubation	24.432-27.924	24.714-28.247
24 Hour H_2O Coated Control	25.291-26.492	24.527-26.253

Table 3.6. Prevention of binding experiment using BSA and Skim Milk. Results of this study showed that BSA had no effect on preventing binding of RRV, while skim milk had a very small effect when compared back to the uncoated control. It appears that four hours is the ideal length of incubation as this is when the largest increase of C_t value was seen.

Prevention of Adsorption Experiment		
Coating Time Interval	**H_2O C_t Value Range (n=3)**	**Skim Milk C_t Value Range (n=3)**
No Coating	23.403-25.631	24.869- 25.473
2 Minute Incubation	24.859- 25.733	27.515- 30.986
2 Hour Incubation	25.757- 25.835	30.117- 32.986
4 Hour Incubation	25.640- 27.094	26.330-30.444
6 Hour Incubation	23.838- 24.162	27.152- 30.620
24 Hour Incubation	24.474- 26.565	29.944- 38.919
48 Hour Incubation	24.432- 25.703	28.394- 32.025
72 Hour Incubation	26.368- 26.671	28.606-29.434
24 Hour H_20 Coated Control	23.551- 24.224	22.901-24.800

Table 3.7. Prevention of binding experiment using Skim Milk and increased time intervals. The results showed that 24 hours had the most impact on prevention of RRV binding. This was not consistent when compared to the original experiment.

Detection

To improve the diagnostic capabilities for RRV, several improvements were made in the RRV detection protocols. The first study in this section of results focused on identifying weaknesses of the established RRV protocol. One-Step RT-PCR and Two-Step qPCR were compared to each other when the same MDA-C extracts yielded differing RRV detection results (Table 3.8). This comparison showed that One-Step Detection was more sensitive than the Two-Step detection protocol. After identifying a suitable One-Step qPCR protocols, the same samples were ran on both protocols (Table

3.9). The results showed that One-Step qPCR was more sensitive than the previous Two-Step method.

Comparison of Conventional vs qPCR Detection Methods	
Detection Protocol	**Number of Samples Detected**
One-Step RT-PCR	13
Two-Step qPCR	4

Table 3.8. Comparison of One-Step Conventional RT-PCR and Two-Step qPCR Protocols. The results show that the One-Step RT-PCR protocol detected more samples than the Two-Step PCR protocol.

Comparison of One-Step and Two-Step qPCR Detection Methods	
Detection Protocol	**Number of Samples Detected**
One-Step qPCR	6
Two-Step qPCR	0

Table 3.9. Comparison of One-Step and Two-Step qPCR protocols. The One-Step qPCR protocol detected all six heavily symptomatic samples, whereas the Two-Step protocol did not detect any of the samples as RRV positive.

Primer Evaluation and Optimization

To further improve diagnostics, primer sensitivity and detection efficiency tests were completed (Table 3.10). A review of the reliability of the various primers sets to detect RRV was conducted as several RRV primers were published during the scope of this experiment. Each primer set was observed to have different PCR results when used on either One-Step protocol. All available primers were compared using the same 50 extracts and the appropriate PCR protocol. This study showed the base of detection efficiency for all primer sets using heavily symptomatic material. The qPCR tests yielded C_t values in the strong positive range (less than 30) for most samples, with few falling in the moderate positive range (30-34). Most primers were capable of detecting all samples, except RRV (Laney, et al., 2011) and RRV4. These two primer sets

detected 47 and 49 samples, out of 50 respectively; RRV4 also amplified non-specific bands. The results from efficiency and sensitivity tests demonstrated that primers adapted for the third segment of the RRV genome were generally the most sensitive and the most accurate for detection. Evaluation of sensitivity and detection efficiency of published RRV primer sets allowed for primers to be identified that are most sensitive for each type of PCR used in plant diagnostics.

Sensitivity Assay	
Primer	**Detection Limit (pg/reaction)**
RRV	2000
RRV_2.1	16
RRV_2.2	0.64
RRV2	16
RRV3	16
RRV_3.2	0.64
RRV_3.5	0.128
RRV(db/271)	0.64
RRV4	2000

Table 3.10. Primer sensitivity testing. The results for the sensitivity experiment showed that RRV and RRV4 were the least sensitive primers. The results also showed that RRV_3.5 was the most sensitive primer, detecting RRV to the 0.128pg/reaction.

Validation for Early Detection

Finally, primer detection tests revealed that detection of asymptomatic, infected plants was possible (Table 3.11). The screening of primers also enabled the identification of primer sets that are capable of detecting asymptomatic, RRV infected plants.

Asymptomatic Detection			
Primer	**Number Tested**	**Number Detected**	**% of Samples Detected**
RRV2	1083	66	6.1%
RRV(db/271)	1304	342	26.23%

Table 3.11. Asymptomatic detection of RRV. Overall, the percentages show that RRV(db/271) appeared to be the more efficient primer for asymptomatic detection. However, there were differences in asymptomatic samples detected by each primer set, where one primer would detect an asymptomatic sample and the other primer set would not.

Discussion

Throughout the research on optimization and adaptation of RRV diagnostic protocols, several improvements were made. A major improvement that was made to the RRV diagnostic process was the adaptation of a rapid RNA extraction method, the Modified Direct Antigen-Capture. While this process had been published before, it was not adapted for use in a diagnostic lab and did not work well for extracting large amounts of samples. By making a few changes to the process, such as utilizing mesh bags and 1.5ml tubes, the modified extraction protocol enabled the mass processing of samples as well as being plant disease diagnostic lab friendly. Both the extraction method and improved diagnostic protocols have been shared with several other research and plant disease diagnostic labs across the United States. Most people utilizing the protocols as written have had no issues with repeatability of the extraction method and generally have good detection results.

RNA extraction methods must be both teachable and cost effective. The Qiagen© method, while it yields an exceptionally clean and stable extract, can be very cost prohibitive. It also takes more time to complete the extraction on a per sample basis. In

addition, the Qiagen© method takes more time inputs for training of unskilled labor. The MDA-C protocol yields a stable, clean extract, that works very efficiently for presence/absence diagnostic tests.

Further research into the MDA-C method was needed to validate the stability and mechanisms that enable the extraction method to work. As the MDA-C is a quick and non-pure RNA method, it was thought that RNA extracts would not be stable at the same C_t values over time because of RNase activity. After two experiments that involved thawing the extract to room temperature and then freezing it several times, very little change in the C_t value was observed. When compared to Qiagen© extracts, the changes in C_t were similar and within expected C_t value range meaning that the amount of viral RNA was not degrading through repeated temperature changes and uses. While this lack of degradation means the extracts are stable short-term, it was observed that the MDA-C extracts can degrade over a long storage period, even in -80°C temperatures (data not shown). The MDA-C extraction method is not recommended for long-term storage, but is efficient for short term storage and routine diagnostics. Stability of MDA-C was also tested through temperature extremes. The MDA-C extracts did not degrade when left out for 24 or 72 hours in room temperature, suggesting that the extract is stable. There were some changes in C_t values when the extract was heated to 32° C to simulate temperatures that could be experienced in shipping, suggesting that prolonged exposure to warm temperatures could degrade the RNA. This information enabled us to primarily utilize the MDA-C protocol for most samples tested throughout the scope of this study

with the knowledge that detection of RRV presence/absence was not affected by the MDA-C method.

To explore the mechanism of antigen trapping in the MDA-C extraction method, BSA and skim milk were utilized as those substances have large amounts of proteins. Substances with high protein amounts were important because it is believed that viral particles are captured in polypropylene tubes through adsorption, which is the non-specific binding of proteins to plastics. Tubes were coated with the high protein substances and incubated. After the incubation, the coated tube was used to capture viral particles. As both experiments show, skim milk nor BSA were able to outcompete the viral particles. BSA coated tubes did not have a differing C_t value from untreated tubes and this substance was not used in the second experiment. Skim milk, which was used in both experiments, did raise the C_t value of extracts when compared to the untreated and H_2O treated tubes, but it was not able to fully outcompete viral particles for binding sites on the plastic tube. Literature does suggest that plastics can affect RNA and DNA extraction, so it is likely that adsorption to polypropylene plastic is the trapping mechanism. However, more experiments would need to be done to confirm that adsorption is occurring.

For an extraction method to be adopted for use in plant disease diagnostic labs, it is desirable for the method to be applicable for more than one pathogen. Research samples were collected from ongoing rose virus research and after following the MDA-C extraction and pathogen-specific detection protocols, it was found that the MDA-C extraction method also worked for (+)ssRNA viruses. To ensure that the method wasn't

specific to only rose viruses, the extraction was also done for TMV ((+)ssRNA) and CaCV ((-)ssRNA). These extracts were as efficient as the standard extraction protocols for the viruses. RYVV, a DNA virus, has also been utilized in the MDA-C extraction method and the detection results were comparable to the lab standard extraction. This work shows that the MDA-C extraction protocol is applicable to several plant viruses and would be very effective for plant disease diagnostic labs, since many of the common plant viruses are (+)ssRNA.

One of the most notable improvements made to the diagnostic process is the adaptation of a one-step protocol for both RT-PCR and qPCR. The change to a one-step protocol improved diagnostic efficiency and reduced the numbers of false negative samples. This is believed to have occurred because one-step qPCR protocols are more sensitive for detection of lower RRV titer samples. It has been shown in literature that a two-step qPCR protocol was more efficient at detection of highly expressed genes (higher concentrations) and was five cycles higher than one-step qPCR on lower expressed genes (lower concentrations) (Wacker & Godard, 2005). Less false negatives allow for quicker removal of plants and helps build trust with stakeholders that submit samples for diagnostic testing.

Screening of available RRV primers allowed for comparison of all published primers on the same set of samples. While several primers identified all highly positive RRV samples, several had issues with non-specific amplification when further comparison work was done. The results show that primers adapted for segment three of the virus tend to perform better than those developed for segments one and four. The

primers that were optimized for segment three are in various location of the genome which could explain why the primers do not equally detect all samples. The results also show that the most sensitive primers were those developed for use in qPCR, which is expected due to the third primer that functions as probe, providing better primer coverage.

Through the screen of primers, it was realized that asymptomatic detection may be possible. RRV2 and RRV(db/271) were consistent in amplifying asymptomatic plants as RRV positive. These “asymptomatic positives” were confirmed for RRV through sequencing of extracts. Early detection of RRV is crucial to disease management as asymptomatic, PCR positive plants can be removed before serving as a source of inoculum to infect other plants. As the results show, RRV(db/271) seems to be more efficient at detecting asymptomatic plants, however, there are several extracts that were positive with RRV2 and not RRV(db/271). The unequal amplification of asymptomatic positives between the two primer sets is something that needs more research, but is likely due to the position of the primer sites on the viral genome. After the discrepancy in asymptomatic detection between the two primer sets was noticed, both primer sets were used on research samples to ensure that no false negative tests results occurred from only utilizing one of the primer sets.

Overall, this research improved the RRV extraction and detection protocols, as well as identifying the best published RRV primer for use in diagnostic work. Protocols developed with the work were disseminated to other labs to ensure that the most efficient RRV testing is being performed. Future work in this objective should be focused on

better, more sensitive primer sets as well as determining why “asymptomatic positive” sample vary in detection efficiency, dependent upon the primer being used.

CHAPTER IV

DETERMINATION OF ALTERNATE RRV TRANSMISSION METHODS

Introduction

Transmission of plant pathogens is an important aspect to understand with respect to a disease system. While many pathogens are transmitted through an insect vector, there are several other vegetative-based methods that can cause the spread of a pathogen. One of the best examples of a vegetative-based disease spread is the spread of Oak wilt disease. Oak wilt is a disease caused by the fungal pathogen *Brietziella fagacearum*. This fungus is spread to new hosts through sap-feeding beetles known as Nitidulidae, and is also spread through root grafting of an infected plant to adjacent healthy plants (Gibbs & French, 1980), extending the spread of the pathogen from a single infected plant. Determining if there are multiple ways for a disease to spread from a single plant is crucial in disease management. Disruption of transmission pathways would greatly reduce and/or eliminate disease incidence.

Mechanical, graft, and root transmission have been documented as methods of disease spread of fungal and viral rose pathogens (Golino, at al., 2007; Silva, et al., 2018). *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) have been shown to be graft transmitted when an infected bud was grafted onto healthy rootstock, as well a clean bud becoming infected after grafting onto infected rootstock (Silva, et al., 2018). An example of viral root transmission that occurred in roses can be found in a study that focused on the transmission of PNRSV and ApMV. Researchers found that virus symptoms were still being observed (on previously healthy plants), even

after excluding all other possible methods of spread, leading to the hypothesis that root transmission was responsible for the lingering symptoms. In an experiment to test this hypothesis, symptomatic plants infected with PNRSV and ApMV were spot treated with a herbicide (glyphosate). Between 10% and 50% of the plants developed herbicide related symptoms, suggesting root grafting between roses (Golino, Sim, Cunningham, & Rowhani, 2007). This study suggested that root transmission of one of more viruses can occur in roses, especially when planted in close proximity of each other, such as mass landscape rose plantings.

Emaraviruses have been shown to have multiple methods of virus transmission, though eriophyid mites remain the primary vector for disease spread (Mielke-Ehret & Muhlbach, 2012). While some viruses in the genus, such as *European mountain ash ringspot-associated virus* (EMARaV), *Fig mosaic virus* (FMV), and *Pigeon pea sterility mosaic virus* (PPSMV) (Divya, et al., 2005), have been detected in the seed coat of various hosts, there has not been evidence to suggest that they are seed transmitted to the resulting embryo (Mielke-Ehret & Muhlbach, 2012). However, *High Plains wheat mosaic virus* (HPWMoV) has been reported to have a very low seed transmission rate (0.008%) under greenhouse conditions (Forster, et al., 2007; Mielke-Ehret & Muhlbach, 2012). Most viruses in the genus have been shown to be propagated through grafts or cuttings. Additionally, HPWMoV has been found to be transmitted to the natural host through mechanical transmission methods (Forster, et al., 2007; Mielke-Ehret & Muhlbach, 2012). There have been attempts to mechanically transmit PPSMV and FMV

to tobacco plants; these experiments resulted in a 10-40% success rate, showing the respective viruses can be mechanically transmitted (Mielke-Ehret & Muhlbach, 2012).

Due to the symptoms that are produced by Rose Rosette Disease (RRD) being consistent with toxicogenic feeding by eriophyid mites (Slykhuis, 1980), there have been grafting experiments to determine if RRD symptoms will occur without mite feeding. In the 1950's, it was found that RRV could be transmitted from an infected Multiflora rose to a healthy Multiflora rose through the use of grafts (Allington, et al., 1968). This transmission method worked very efficiently, while also proving that RRD symptoms were not related to mite feeding. Additional experiments were done by Amrine, et al. (1988) graft transmission was again proven to transmit *Rose rosette virus* (RRV) to plants, though it was noted to occur at a lower rate than mite transmission, however this work did again confirm that RRD symptoms were due to the virus, not mite feeding (Amrine, 1988). Graft transmission of RRV was a recommended practice for control of Multiflora roses for several years, however it was not heavily utilized outside of research experiments (Amrine, 2002).

Two additional methods of RRV transmission without mite feeding have been explored. The first method that is hypothesized to transmit RRV is mechanical transmission. In previous studies, mechanical transmission of RRV was shown to possibly occur when mechanically inoculated into Nicotiana species (Rohozinski, et al., 2006). Additionally, mechanical transmission of RRV to tobacco and other roses through the use of an air gun is being evaluated further to demonstrate that this form of RRV transmission is possible (Verchot, et al., 2020). The second method is root

transmission of RRV, which was suggested in the 1950's (Allington, et al., 1968), but was not believed to be an important method of spread because roses were not utilized on a mass scale for landscaping at that time. However, as the rose industry has evolved and RRV has spread across the United States, root transmission is being questioned once again.

Project Objectives and Aims

The objective of this study was to gain a better understanding of other potential avenues of transmission and to confirm some previous work on transmissibility of the disease. Previous studies suggest that seed transmission is unlikely to occur (Epstein & Hill, 1995; Mielke-Ehret & Muhlbach, 2012), suggesting that a healthy plant could be recovered from seeds collected from an infected plant, which may be a useful approach for breeders to maintain RRD-free germplasm. As RRV continues to spread across the country, infection of rose breeding blocks is occurring. Seed transmission studies were conducted to determine if RRV could be moved vertically from parent to embryo. Previous reports from Allington et al. (1968) and Epstein et al. (1997) demonstrated that RRD was graft transmissibile which was a factor in deducting that RRD may have been caused by a viral agent. The aim of the graft transmission experiment is to confirm that RRV is graft transmissible in cultivated roses that are currently in the market. Additionally, some RRD tolerant cultivars will be used in this graft transmission study, using a combination of infected/non-infected scion and rootstock, to determine if symptom expression by these cultivars is possible when exposed to only RRV without mite transmission. Epstein & Hill (1995) indicated that mechanical transmission of

RRD is negligible in Multiflora roses. The mechanical transmission experiment was conducted to evaluate if the previous conclusion was also true for cultivated roses. Root transmission of RRV was suggested as a possible transmission method by Allington et al. (1968). Observations made in field trial experiments where roses planted into areas with residual active infected crown and root tissue, indicated a more rapid development of symptoms (less than four months) when compared to other trial location that were not previously planted in roses (nine or more months). The goal of the root transmission study was to confirm that RRD is root transmitted. The knowledge gained from these transmission experiments would help to provide a better understanding of how and if there are alternate transmission methods, which would lead to potential development of better RRD management practices.

Materials and Methods

Seed Transmission

To evaluate if healthy plant can be recovered from infected/symptomatic plants, hips were harvested from infected plants. Symptomatic plants showing at least two RRD symptoms were utilized in the 2019 experiment; asymptomatic, PCR positive plants were utilized in the 2020 experiment due to the difficulty of finding symptomatic hip producing plants. 2019 hips were collected from three cultivars, 'Carefree Spirit', 'Screaming Neon Red' and 'Purple Pavement.' The cultivars were collected from a public rose garden in Tulsa, Oklahoma in late October 2018. The 2020 hips were collected from 36 asymptomatic, PCR positive cultivars from three locations (Denton,

TX, Dallas, TX, Burleson Co., TX) in 2019. Hips were collected from asymptomatic plants for this study due to the difficulty of identifying symptomatic hip producing plants. Additionally, based on the positive results from the 2019 seed study, a negative control cultivar was necessary to verify that resulting seedlings were not causing an interaction with RRV testing. Plants used in both studies were grown in a natural garden setting, meaning that the pollen parent for the subsequent seeds is unknown, due to open pollination that can occur in roses.

Seeds were extracted from collected rose hips using a seed extraction protocol used by the Texas A&M Plant Breeding Program. Samples containing less than 5 hips: rose hips were cut open using a scalpel and allowed to dry on a paper towel for no longer than one week. Samples containing more than 5 hips: rose hips were placed in a blender with 500mL of water and macerated until seeds were released (usually three to five minutes of blending). The subsequent seed slurry was strained and placed on a paper towel to dry for a time no longer than one week. Seeds were collected, counted and stored in 4°C temperatures until planting. Seeds that were significantly smaller than apparent "mature" seeds were considered to be immature and were discarded. Collected seeds were stored for one week to four months, depending on when the hips were collected.

Seeds harvested for the 2020 experiments were surface sterilized prior to cold stratification to minimize contaminants that might be occurring on the rose seed surface, including potential RRD deposition on the seed. These seeds were surface sterilized by being soaked in a 20 percent bleach mixture (100µl bleach + 400µl H2O) for three

minutes. Seeds were then rinsed with RO water and soaked in a RO water bath for an additional three minutes. At the end of the RO wash interval, seeds were placed on paper towels to dry for no longer than 24 hours. Seed samples were returned to 4° C cold storage after sterilization. Short time intervals and bleach concentrations were chosen to prevent damaging rose seeds. It is common for seed coat to be slightly damaged, so while surface sterilization is typically done for up to 15 minutes, a short interval was chosen to prevent seed coat breakdown.

Seeds were planted in a potting media with a high amount of perlite (70-80% perlite) and soil was moistened to the point of saturation. Seed were spaced, on average, one-half to one inch. Planting trays were then placed inside a bag and sealed with tape to maintain high humidity conditions. The seed tray was placed at a constant 4° C temperatures for 10 weeks. Following this cold stratification step, planters were moved to a growth chamber (light 16 hours, temp 25° C). Rose seedlings were observed one to two weeks after being placed in a growth chamber. Seedlings remained in a growth chamber for five months and were then moved to a temperature-controlled greenhouse and subjected to environmental light and humidity conditions. The germination rate for resulting seedlings was determined by dividing the resulting number of seedlings by the total amount of seeds that were cold stratified.

In the 2019 experiment, seedlings were tested for presence of RRV using both conventional RT-PCR/RRV(db/271) and real-time qPCR/RRV2 at two, three, four, five, eight, eleven, and fourteen months. The 2020 experiment was sampled at month two only and subsequently terminated due to the COVID-19 pandemic restriction to conduct

future experimentation. New growth leaf samples (5-10 leaves) were sampled and RNA extracted using the MDA-C method. The Conventional RT-PCR One-step detection protocol/ RRV(db/271) primer set and qPCR One-step/RRV2 primer set protocol were used for RRV detection. Products from the conventional RT-PCR were visualized on 2% agarose gel. Positive RRV detection resulted in a 271 base pairs (bp). In the real-time qPCR assays, sample is RRV positive when the C_t value is <37. Testing percentages for each time point were determined by dividing the number of positive seedlings by the total number of germinated seedlings.

Graft Transmission

In the graft transmission experiment, budwood and rootstock were evaluated. Cultivars were selected for their susceptibility or tolerance to RRV for these experiments. Susceptibility is defined as RRD symptoms and a positive RRV test. Tolerance is defined as PCR positive, but lacking development of symptoms for two or more growing seasons. Five (5) tolerant cultivars (Table 4.1) were selected based on the lack of RRD symptoms with continued exposure to the disease of a period of time from 2016-2018. Eleven (11) susceptible varieties (Table 4.1) were selected because they developed symptoms and had positive RRV tests during the period of observation from 2016-2018. The negative control variety utilized was 'The Double Knock Out© Rose'. A negative control was utilized to verify that grafting occurred in the absence of disease.

Buds used were dormant buds (those that had not differentiated into leaves). Tissue that bud was extracted from was slightly hard (not new growth, not woody tissue). Buds were removed from plant tissue in two ways; the first involved cutting a

shallow rectangle shape around the bud using a scalpel and lifting the bud from underlying cambium tissue. The second method of bud removal focused on utilizing a grafting knife to slice the bud away from the cane and then separating the bud from cambium tissue. Once buds were collected, they were placed in a cup of RO water to maintain the integrity of the bud. Rootstock plants were selected from 14 cultivars (Table 4.1). These plants served as rootstock for multiple (1-5) buds. Areas used for grafting were cleared of all spines to facilitate grafting.

The T-bud grafting method was used in this experiment. Briefly, a cut is made on the rootstock into the bark layers of the cane using a scalpel. The cut was made in a "T" shape, which provided a pouch for the graft to be slid into. Buds were placed in the cut area quickly to prevent drying out of the xylem and pith layers. After bud was placed, the graft area was wrapped in parafilm to prevent drying out of the graft and rootstock. The budding process took about seven minutes per graft. Once budded, plants were placed in the greenhouse (summer/fall conditions were full sun, high humidity, temperatures ranged from 27ºC -49ºC) and observed for up to four months for bud break.

Two grafting experiments were conducted. (1) July Experiment- buds were collected from asymptomatic, RRV positive plants (Table 4.5). (2) October experiment- buds were collected from symptomatic, RRV positive plants (Table 4.5).

Leaf samples were collected for RRV detection post-grafting. Samples were taken from the rootstock, both above (A) and below (B) the graft union, as well as from the scion (G) that resulted from a successful graft union. For the (1) July experiment (asymptomatic RRV positive buds), samples were taken at three, six, and nine months

post-grafting. For the (2) October experiment (symptomatic PCR positive buds), samples were taken at three- and six-months post grafting. Leaves above and below the graft site were sampled to determine if RRV was moving throughout the rootstock via phloem tissues. Positive RRV detection from either sample would also confirm that RRV transmission from scion to rootstock occurred. However, to reduce competition for nutrients and hormones between the rootstock and emerging buds, many rootstock plants were trimmed off at the top of the graft union, resulting in lower "Above" samples through the course of the experiment. Leaves were sampled from the scion to confirm that RRV was present after grafting was performed.

All collected leaf samples were RNA extracted using the MDA-C method and PCR was completed using both one-step RT-PCR protocol and one-step qPCR detection protocol. Products from the conventional RT-PCR were visualized on 2% agarose gel. Samples that were positive for RRV resulted in a product sized at 271 bp. In the qPCR assay, C_t values less than or equal to 37 were considered to be positive. It is expected that the primer set RRV(db/271), used in RT-PCR, will be more sensitive in detection of asymptomatic infections and that primer set RRV2, used in qPCR, will be most accurate in detection of symptomatic grafts/plants. Total and adjusted percentages of graft transmission were calculated by dividing the total positive samples by the total amount of samples. The adjusted percentage is noted because some buds were grafted to previously infected rootstock as a positive bud-positive graft control group, therefore it was necessary to remove these from the total results and show an adjusted percentage.

Cultivars Selected for Use in Grafting Experiments			
Cultivar	**Designation**	**Rootstock**	**Scion**
‘Bayse’s Purple’	Tolerant	X	X
‘Lafter’	Tolerant	X	X
‘Caldwell Pink’	Tolerant	X	
‘Chuckles’	Tolerant	X	X
‘Cherokee’	Tolerant	X	
‘Belinda’s Dream’	Susceptible	X	X
‘Koko Loko’	Susceptible	X	
‘Midnight Blue’	Susceptible	X	
‘Pretty Lady’	Susceptible	X	
‘Scentimental’	Susceptible	X	
‘Hot & Sassy’	Susceptible	X	
‘Edith’s Darling’	Susceptible	X	
‘White Drift’	Susceptible		X
‘Coral Drift’	Susceptible		X
‘Sweet Drift’	Susceptible	X	X
‘The Pink Double Knock Out© Rose’	Susceptible and Negative Control	X	X

Table 4.1. Cultivars utilized in grafting experiments. Various cultivars were selected for their tolerance or susceptibility to RRV to be used in grafting experiments.

Mechanical Transmission

Three cultivars were used in the mechanical transmission experiment. These three cultivars are highly susceptible to RRD and readily develop observable symptoms when infected with RRV. Five leaves were selected in a random pattern around the plant. Care was taken to not select leaves that were from new growth or older leaves. Most leaves selected were in the mid-to-top of the plant canopy. The leaves were marked with 'X' to denote those as the virus inoculated leaf. A mock control plant was also selected, and leaves were marked with a 'M'.

Mechanical transmission (sap transmission) was tested using inoculum prepared from 5 grams of leaf tissue, which was lysed and added to a virus inoculation buffer. This buffer was prepared by adding 0.05M of Potassium Phosphate Monobasic to 1.0% Celite and filling to 100mL volume with distilled water. This protocol has been successfully used for virus inoculation into various woody hosts (Fulton, 1966). A similar protocol was utilized by Epstein et al. (1995) to prepare inoculum for RRV mechanical transmission tests. Forty microliters of virus inoculum buffer was added to the mock control plant and gently wiped across the leaf to prevent damage. The control plant was allowed to dry and then RO water was sprayed on the leaf to wash off remaining buffer. Forty microliters of the buffer-sap mixture were added to each 'X' marked leaf and was gently wiped across the leaf to spread the buffer mixture, carefully as to not cause severe leaf damage. While the goal of mechanical transmission is to make microtears in leaves to allow for viral infiltration, too much damage from the celite can cause the leaf to senesce before viral infection occurs. After this was done, the

buffer-sap mixture dried on the leaves, which were then sprayed with RO water to rinse any remaining buffer. Plants were placed in a growth chamber with parameters of 25° C, 800µmol of light, and 60% humidity. Conditions were later changed to 500µmol of light and 40% humidity to prevent black spot formation and subsequent defoliation. After one month in the growth chamber, plants were moved to a temperature-controlled greenhouse and subjected to typical environmental light and humidity conditions for an additional five months.

One month and six months after mechanical transmission was performed, leaf samples were taken from the plant. Two inoculated leaves were sampled to test for virus presence (an exception to this occurred at six months as most inoculated leaves had senesced). Another sample of random new growth (5 leaves total) on each inoculated plant was taken. Leaves were lysed and RNA extracted using MDA-C; PCR was completed using both RT-PCR One-step Detection/RRV(db/271) primer set and qPCR One-step Detection/RRV2 primer set protocols. Detection was completed on the conventional PCR products through the use of a two percent agarose gel and run parameters of 5 volts/ cm (180 volts for 60 minutes). Samples were considered positive when an amplified band at the expected size was seen in visualization; qPCR samples were positive if they were equal to or less than 37.0 C_t value. Results were analyzed based on the presence or absence of RRV, as determined by PCR testing, and determinations were made about the occurrence, or lack thereof, of mechanical transmission of RRV. The percentage of positive leaves was calculated by dividing the total number of positive plants by the total number of plants tested.

Root Transmission

Root transmission of RRV experiments were conducted in 2018, 2019 and 2020. Experiments utilize strategies to encourage root growth and interaction between rose plants, such as plant placement in planters, focused or targeted irrigation. The experiments also focus on using shallow containers to encourage horizontal root growth.

Two rose cultivars were used in 2018 experiment: 'Julia Child' and 'Ketchup & Mustard'. Infected 'Julia Child' and 'Ketchup & Mustard' plants were acquired from a grower field in 2017 and maintained in the greenhouse with regular miticide treatments before and during the experiment to ensure that plants were free from eriophyid mites. Plants were symptomless when shipped from grower field in fall of 2017 but were in a RRV infected block and developed symptoms in early 2018. Healthy 'Julia Child' and 'Ketchup & Mustard' plants were acquired from the growers in March 2018.

This experiment was conducted in a 40-gallon container. A central divider was used to physically separate the infected from the healthy plants. Symptomatic, PCR positive plants were placed in one side of a container while healthy plants were place directly opposite side of the container. The root balls were broken up to encourage lateral and spread out root growth. A piece of plexi-glass was placed between the two sets of plants to prevent foliar contact. The experiment with cultivar 'Julia Child' was terminated at 3 months; the experiment with cultivar 'Ketchup & Mustard' was terminated at 5 months. Any new symptomatic plant material on the healthy plants was sampled when first observed and tested for RRV presence. Leaves (symptomatic or asymptomatic) were sampled from all plants at the time of experiment termination. The

‘Julia Child’ experiment plants were observed daily for six weeks until the time of symptom development in healthy experiment plants, and that symptomatic plant was subsequently leaf sampled. All healthy and infected plants were leaf sampled after three months, when the experiment was terminated. ‘Ketchup & Mustard’ plants were observed for symptom development on a weekly basis for five months. As no symptoms were observed and the infected plants were in poor health, all plants in the experiment were leaf sampled. No root samples were taken from either cultivar.

The 2019 experiment used two cultivars: symptomatic ‘Sweet Drift’ and healthy ‘The Pink Double Knock Out© Rose’. A symptomatic cutting containing 3-5 nodes was made and placed in a plug cell; a healthy 3-5 node cutting was made and placed in the other side of a plug cell. These cuttings were monitored daily for root growth and interaction. No results were collected from this experiment due to experiment contamination from fungus gnats.

In the 2020 experiment, 3 cultivars were used: ‘The Double Pink Knock Out© Rose’ served as the infected plant and test plants were cultivars ‘Iceberg’, and ‘Peach Drift’. Experiment was conducted in a 3-gallon container. Soils/media were initially removed from all plants. A symptomatic plant was placed in the container and a healthy plant was placed on the opposite. In attempts to hasten root interactions, roots from infected and non-infected plants were intertwined and was wrapped in parafilm to maximize contact. All roots were covered with potting soil containing 80-90% perlite and were maintained with normal practices. Plants were observed for three and a half

months. Leaf and root samples from all plants were sampled and tested for RRV presence at the termination of this experiment

RNA extracted from leaf and root samples using the MDA-C protocol**.** PCR was completed using both the Conventional RT-PCR Detection of *Rose rosette virus* protocol and primer set RRV(db/271) and One Step Real Time PCR Detection of *Rose rosette virus* protocol and primer set RRV2. Visualization was completed on the conventional PCR products using a two percent agarose gel and run parameters of 5 volts/ cm (180 volts for 60 minutes). RT-PCR samples were considered positive if a band at the expected size was present when the gel was visualized. qPCR samples were considered RRV positive if the C_t value was less than or equal to 37.0.

Results

Seed Transmission

Testing was completed on the 2019 seedlings at several time intervals (Table 4.2). The germination rate was low for both experiments; 2.3% for 2019 seedlings, and 16.92% for 2020 seedlings. One hundred percent of resulting seedlings were tested in the 2019 experiment, however, only 89.1% of seedlings were tested in 2020 due to plant death. The 2019 experiment qPCR (primer set RRV2) (Table 4.2) resulted in 61% of seedlings that were RRV positive at two months, 7.1% at four months, 35.7% positive at five months, and 21.4% positive at 11 months. Testing performed at three, eight, and 14 months yielded no positive seedlings. The 2019 experiment, RT-PCR results (Table 4.2) yield 89.3% percent of seedlings that were positive at two months, 28.6% percent at

three months, 25% were positive at five months, and 10.7% were positive at eight months. Tests performed at four, 11, and 14 months were all negative results.

Testing was performed one time on 2020 seedlings (Table 4.3). 873 seedlings from 23 different cultivars were evaluated. RRV screening was performed at two months and then the experiment was terminated due to time constraints. When utilizing the RRV2 primer set and qPCR, no seedlings were positive for RRV, while 0.61% of seedlings were positive for RRV using the RRV(db/271) primer set. Half of the positive 2020 seedlings were collected from the ‘Chuckles’ plant cultivar.

2019 Seed Transmission Results															
Variety	**Germ**	**2 Mos.**		**3 Mos.**		**4 Mos.**		**5 Mos.**		**8 Mos.**		**11 Mos.**		**14 Mos.**	
		RRV2	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**
'Purple Pavement'	25	16	24	0	7	0	0	10	7	0	2	6	0	0	0
'Screaming Neon Red'	3	1	1	0	1	2	0	0	0	0	1	0	0	0	0
'Carefree Spirit'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total Numbers	**28**	**17**	**25**	**0**	**8**	**2**	**0**	**10**	**7**	**0**	**3**	**6**	**0**	**0**	**0**
Total Percentages (%)		**61**	**89**	**0**	**29**	**7**	**0**	**36**	**25**	**0**	**11**	**21**	**0**	**0**	**0**

Table 4.2. RRV seed transmission percentages as detected utilizing both RRV PCR assays. The germination rate for all cultivars utilized in the experiment was 2.30%. Testing results show that seedlings had the highest virus titer when first germinated and gradually decreased in virus titer over the 14-month observation period.

2020 Seed Transmission					
Number	**Cultivar**	**Germ.**	**Tested**	**RRV2 Positive**	**RRV(db) Positive**
160	'Emmie Gray'	-	-		
181	'Basye's Blueberry'	-	-		
150	'Tupelo Honey'	-	-		
148	'Naga Belle'	-	-		
174	'Rouletti'	-	-		
147	'Lafter'	10	8		
163	'Chuckles'	12	11		
161	'Naga Belle'	2	2		
171	'Fires of Alamo'	21	21		1
168	'Lafter'	2	2		
169	'Jean Teresa'	1	1		
149	'Fires of Alamo'	23	21		
178	'The Rainbow Knock Out© Rose'	-	-		
166	'Margaret McDermott'	-	-		
156	'Margaret McDermott'	-	-		
164	'Emmie Gray'	-	-		
153	'The Rainbow Knock Out© Rose'	-	-		
172	'Mrs. R. M. Finch'	-	-		
180	'Basye's Blueberry'	-	-		
152	'Basye's Blueberry'	-	-		
201	'Mrs. R. M. Finch'	-	-		
185	'Carefree Spirit'	-	-		
195	'Fires of Alamo'	21	21		
202	'Lafter'	7	5		
182	'Miranda Lambert'	12	11		

Table 4.3. Percentage of RRV seed transmission from asymptomatic, PCR positive parent plants. The results of this experiment showed that seed transmission occurred in 0.6% of seedlings from asymptomatic plants. Of the positive seedlings, three were from the 'Chuckles' cultivar.

Number	Cultivar	Germ.	Tested	RRV2 Positive	RRV(db) Positive
193	'Margaret McDermott'	-	-		
184	'The Rainbow Knock Out© Rose'	-	-		
216	'The Rainbow Knock Out Rose'	-	-		
214	'Fires of Alamo'	-	-		
187	'Rouletti"	-	-		
99	'Chuckles'	21	19		
203	'Naga Belle'	-	-		
A235	'Complicata'	8	6		
133	'Lafter'	-	-		
123	'Chuckles'	100	10		1
128	'Carefree Spirit'	-	-		
A236	'Serena'	3	8		1
126	'Basye's Blueberry'	1	1		
549	'Stormy Weather'	2	2		
523	Species Cultivar	2	1		
128B	'Carefree Spirit'	-	-		
543	Species Cultivar	1	1		
513	Species Cultivar	-	-		
504	Species Cultivar	-	-		
536	Species Cultivar	-	-		
541	Species Cultivar	3	3		
547	Species Cultivar	1	1		
255	'Chuckles'	22	21		2
501	Species Cultivar	2	1		
131	'Emmie Gray'	9	8		
553	'The Sunny Knock Out© Rose'	-	-		
130	'Cherokee'	-	-		
545	Species Cultivar	650	650		
607	'Winnipeg Parks'	1	1		
A264	Species Cultivar	4	4		1
622	'Moje Hammarberg'	-	-		

Table 4.3 (Continued)

Number	Cultivar	Germ.	Tested	RRV2 Positive	RRV(db) Positive
640	'John Davis'	1	-		
626	'Winnipeg Parks'	1	-		
639	'Blanc de Coubert'	1	1		
631	'John Cabot'	1	1		
A247	Species Cultivar	-	-		
699	'John Davis'	-	-		
632	'Winnipeg Parks'	-	-		
641	'Morden Centennial'	8	8		
621	'Morden Centennial'	11	11		
603	'John Davis'	5	3		
618	'Morden Centennial'	11	9		
A241	Species Cultivar	-	-		
Totals		**980**	**873**	**0**	**6**
Total %			**89**	**0**	**0.6**

Table 4.3 (Continued)

Figure 4.1. 2020 Seedlings that yielded a positive RRV result. While no seedlings developed obvious RRD symptoms over the observation period, most infected seedlings had abnormal growth. Most plants had abnormal leaf shape or color, while others appeared stunted.

Graft Transmission

Several plants were grafted with asymptomatic and/or symptomatic buds throughout these sets of experiments. It was observed that asymptomatic buds that had begun to swell (close to breaking dormancy) performed better than buds that were still very dormant. It was also observed that symptomatic grafts emerged much quicker and had a better initial grafting percentage than the asymptomatic buds. The faster timeline is likely because buds retrieved from the infected plants were less dormant (closer to forming leaves) than the buds collected from asymptomatic plants. However, rootstocks grafted with infected buds tended to have a higher death rate (data not shown). Successfully grafted asymptomatic buds to infected or clean rootstock were tested at three, six and nine months; successful symptomatic grafts were tested at three and six months. The three month asymptomatic bud test (Table 4.4) showed the qPCR/RRV2 primer set did not detect any positive plants, however RT-PCR/RRV(db/271) primer set detected 9.1% of grafts as positive and 5.8% of rootstocks (Below and Above) as positive for RRV.

At the six-month testing of the asymptomatic experiment, the symptomatic experiment was also tested at its three-month interval (Table 4.4), therefore the resulting percentages are for both experiments. Using the primer set RRV2 and qPCR, 22.7% of grafts were positive and 23.6% of rootstocks were positive. The primer set RRV(db/271) and RT-PCR detected 10.9% of grafts as positive, and 7.5% of rootstocks were positive. For the nine-month interval of asymptomatic/six-month interval of symptomatic (Table 4.4), results were once again combined. Primer set RRV2/qPCR detected 10.4% of

grafts and 6.9% of rootstocks as positive, while the RRV(db/271) primer set/RT-PCR detected 2.7% of grafts and 3.4% of rootstock.

Table 4.5 summarizes testing results for plants that were grafted in this experiment. Only samples that yielded a positive test result were reported, so this table is not all-inclusive of the entire grafting experiment. It is shown that all tolerant cultivars yielded at least one positive RRV test after being grafted with an RRV infected bud; none developed observable symptoms within the eight-month interval that grafted plants were retained. All other cultivars that were suspected or documented to be RRV susceptible developed symptoms when grafted with symptomatic buds, but not asymptomatic buds though the rootstock did become positive in many rootstocks after grafting.

Graft Transmission Results																		
Sampling Interval	**3-Month Asymptomatic Only**						**6-Month Asymptomatic/ 3-Month Symptomatic**						**9-Month Asymptomatic/ 3-Month Symptomatic**					
Sample Location	**(G)**		**(B)**		**(A)**		**(G)**		**(B)**		**(A)**		**(G)**		**(B)**		**(A)**	
Primer Set	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**	**RRV2**	**RRV (db)**
Total Sample Numbers	66		60		49		`110		75		21		115		87		20	
Total Positive Numbers	0	6	0	1	0	3	27	12	9	2	3	1	13	3	6	3	0	0
Total (%)	**0**	**9**	**0**	**2**	**0**	**6**	**25**	**11**	**12**	**3**	**14.3**	**5**	**11**	**3**	**7**	**3**	**0**	**0**
Adjusted (%)						**4**	**23**		**9**				**10**					

Table 4.4. Graft transmission percentages that resulted from PCR testing. The numbers of grafts increased from the second to third interval due to several dormant asymptomatic grafts finally emerging after nine months. The results showed that there was transmission of RRV from infected scion to healthy rootstock as the percentage of positive rootstock ((B) and (A)) samples increased over each testing interval.

2019-2020 Grafting Experiment Results								
Rootstock Variety	**# of Graft Unions**	**Scion Variety**	**Oct.**	**Jan.**	**April**	**qPCR**	**RT-PCR**	**Notes**
'Caldwell Pink'	2	'White Drift'	X (B)				X	
'Caldwell Pink'	2	'White Drift'	X (G)				X	
'Caldwell Pink'	2	'White Drift'	X (G)				X	
'Pretty Lady'	1	'Lafter'	X (A)				X	
'Lafter'	1	'White Drift'	X (G)				X	
'Lafter'	2	'Chuckles'	X (G)				X	
'Lafter'	1	'Bayse's Purple'	X (G)				X	
'Midnight Blue'	2	'White Drift'	X (A)				X	*
'Belinda's Dream'	2	'Lafter'	X (G)				X	
'Caldwell Pink'	1	'The Double Pink Knock Out© Rose'		X (G)	X (G, B)	X		!
'Caldwell Pink'	1	'White Drift'		X (G)		X		

Table 4.5. Successful Rootstock-Scion Grafts that resulted in a positive RRV detection assay. Only samples that yielded a positive RRV test are reported in this table. All tolerant cultivars became asymptomatic, RRV positive when grafted with a RRV positive bud. No symptoms developed on the tolerant plants. 'Bayse's Purple' cultivar was noted to abort all symptomatic buds once leaves began to emerge from the bud (data not shown). Several of the suspect RRV susceptible cultivars developed symptoms in the rootstock after being grafted with an RRV positive bud. 'Pretty Lady' cultivar aborted all positive buds, but still developed symptoms soon after grafting.

Rootstock Variety	# of Graft Unions	Scion Variety	Oct.	Jan.	April	qPCR	RT-PCR	Notes
‘Caldwell Pink’	1	‘Sweet Drift’		X (G, A)	X (G)	X	X (Jan. Scion Only)	!
‘Scentimental’	1	‘Sweet Drift’		X (G)	X (G)	X	X (Jan Scion Only)	!
‘Scentimental’	1	‘Sweet Drift’		X (G)	X (G)	X	X (Jan Scion Only)	!
‘Scentimental’	1	‘Sweet Drift’		X (G)	X (G)	X		!
‘Scentimental’	1	‘Sweet Drift’		X (G)	X (G)	X		!
‘Scentimental’	2	'White Drift’		X (G)		X		
‘Scentimental’	3	‘White Drift’		X (G, B)		X		
‘Scentimental’	2	‘White Drift’		X (B)	X (G)	X (Below Only)	X (Scion Only)	
‘Pretty Lady’	1	‘Sweet Drift’		X (G, A)	X (B)	X	X	!
‘Pretty Lady’	1	‘Sweet Drift’		X (G)	X (G)	X	X (Jan. Scion Only)	!
‘Pretty Lady’	2	‘White Drift’		X (B, G)		X		*
‘Bayse's Purple’	1	‘Sweet Drift’		X (A)	X (G)	X		!
‘Bayse's Purple’	1	‘Sweet Drift’		X (G)		X		!

Table 4.5 (Continued)

Rootstock Variety	# of Graft Unions	Scion Variety	Oct.	Jan.	April	qPCR	RT-PCR	Notes
‘Bayse's Purple’	2	‘Sweet Drift’		X (G)		X		!
‘Cherokee’	1	‘White Drift’	X (A)	X (B)		X (Below Only)	X (Above Only)	
‘Lafter’	1	‘Bayse's Purple’		X (G)			X	
‘Lafter’	1	‘Sweet Drift’		X (G, B)		X	X	!
‘Lafter’	2	‘Sweet Drift’		X (G)		X	X	!
‘Lafter’	1	‘Sweet Drift’		X (G, B)	X (G)	X	X (Jan Scion Only)	!
‘Lafter’	1	‘Lafter’		X (B)			X	
‘Edith's Darling’	1	‘The Double Pink Knock Out© Rose’		X (G)		X		* !
‘Midnight Blue’	1	‘Sweet Drift’		X (G)		X	X	!
‘Midnight Blue’	2	‘Lafter’		X (G, B)		X		*

Table 4.5 (Continued)

Rootstock Variety	# of Graft Unions	Scion Variety	Oct.	Jan.	April	qPCR	RT-PCR	Notes
‘Belinda's Dream’	2	‘The Double Pink Knock Out© Rose’		X (B)	X (G)	X	X (April Scion Only)	!
‘Belinda's Dream’	1	‘White Drift’		X (G)		X		
‘Belinda's Dream’	1	‘White Drift’		X (G)		X		
‘Belinda's Dream’	1	‘White Drift’		X (G, B)		X		
‘Belinda's Dream’	2	‘Lafter’		X (G)		X	X	
‘Belinda's Dream’	1	‘The Double Pink Knock Out© Rose’		X (G)	X (G)	X	X (April Scion Only)	!
‘Belinda's Dream’	2	‘The Double Pink Knock Out© Rose’		X (G)	X (G)	X	X (April Scion Only)	!
‘Belinda's Dream’	3	‘Chuckles’		X (G)		X		
‘Pretty Lady’	1	‘The Double Pink Knock Out© Rose’			X (B)	X		!
‘Cherokee’	1	‘White Drift’			X (B)	X		

Table 4.5 (Continued)

Rootstock Variety	# of Graft Unions	Scion Variety	Oct.	Jan.	April	qPCR	RT-PCR	Notes
'Lafter'	1	'Chuckles'			X (B)	X		
'Lafter'	1	'Sweet Drift'			X (G)	X		* !
'Lafter'	1	'Coral Drift'			X (G)		X	
'Caldwell Pink'	2	'The Pink Knock Out© Rose'			X (B)		X	
'Midnight Blue'	1	'Lafter'			X (B)	X		*
'Midnight Blue'	1	'Lafter'			X (B)	X		*

Key:
! = Symptomatic Bud (before grafting)
*** = Positive Rootstock (before grafting)**
B = Rootstock, Below Graft
A = Rootstock, Above Graft
G = Scion

Table 4.5 (Continued)

Mechanical Transmission

The ability to mechanically transmit a plant virus is desirable due to the ability to study the virus in the absence of a vector. Plants were tested at one month and six months after mechanical transmission was attempted; both inoculated leaves and new growth were sampled. All cultivars utilized are susceptible to RRV and were treated as one group when inoculated with RRV. The results from the one month experiment (Table 4.6) show that the RRV2 primer set/qPCR did not detect RRV in either type of sample, while RRV(db/271) primers/RT-PCR did detect RRV in 18.2% of inoculated leaves. When plants were tested again at the six-month interval (Table 4.6), neither primer set yielded a positive result for mechanical transmission plants.

Mechanical Transmission Experiment Results								
	One-Month Sampling				Six-Month Sampling			
Primer	**RRV2**		**RRV(db)**		**RRV2**		**RRV(db)**	
Number	**Inoculated**	**New Growth**	**Inoculated**	**New Growth**	**Inoculated**	**New Growth**	**Inoculated**	**New Growth**
592	Negative	Negative	Negative	Negative	-	Negative	-	Negative
1010	Negative	Negative	Negative	Negative	-	Negative	-	Negative
1002	Negative	Negative	Negative	Negative	-	Negative	-	Negative
1009	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
1007	Negative	Negative	Negative	Negative	-	Negative	-	Negative
1008	Negative	Negative	**Positive**	Negative	-	Negative	-	Negative
547	Negative	Negative	Negative	Negative	-	Negative	-	Negative
545	Negative	Negative	Negative	Negative	-	Negative	-	Negative
590	Negative	Negative	**Positive**	Negative	-	Negative	-	Negative
1004	Negative	Negative	Negative	Negative	-	Negative	-	Negative
1001	Negative	Negative	Negative	Negative	-	Negative	-	Negative
Totals (%)	0	0	**18**	0	0	0	0	0

Table 4.6. Results of mechanical transmission experiment. Two inoculated leaves were positive in the one-month testing interval using the RRV(db) primer set. Most inoculated leaves were not sampled at the six-month interval and no new growth from any inoculated plants was positive.

Root Transmission

The preliminary experiment, conducted in 2018, involved two cultivars, 'Julia Child' (Figure 4.11) and 'Ketchup & Mustard'. One of two healthy 'Julia Child' plants developed RRD symptoms and was confirmed for RRV six weeks post-initiation of the experiment. At experiment termination, three months post-initiation, both symptomatic plants remained RRV positive. In addition, the healthy plant that displayed symptoms was also RRV positive; the asymptomatic healthy plant remained RRV negative. After four months of observation on the 'Ketchup & Mustard' experiment, all plants were tested and in addition to both symptomatic plants yielding positive results, one healthy plant tested positive for RRV, though it was asymptomatic. Results of the 2020 experiment were collected three months post-initiation. PCR testing (Table 4.7) showed that 77.8% of symptomatic plants and roots were positive for RRV using the RRV2 primer set and qPCR. With the use of RT-PCR and the RRV(db) primer set, 11.2% of leaves and no roots were positive for RRV.

Figure 4.2. Results from 2018 root transmission of 'Julia Child' cultivar. The first picture (left) shows the experimental setup of the 2018 experiment. The second (right) shows the symptoms that developed on a healthy plant six weeks post-initiation.

2020 Root Transmission Results				
Primers	**RRV2 Leaf**	**RRV2 Root**	**RRV(db) Leaf**	**RRV(db) Root**
Symptomatic (9 Samples) (%)	78	78	11	0
Healthy (14 Samples) (%)	0	0	0	0

Table 4.7. RRV detection assay results from 2020 root transmission experiments. Primer set RRV2 detected 78% of samples, whereas primer set RRV(db/271) detected 11% of samples. No root transmission of RRV from symptomatic to healthy was observed.

Discussion

These transmission experiments focused on determining other factors that could contribute to the spread of RRV outside of the known mite vector. Details on other methods of viral transmission that can occur in the environment allows for better disease management by all parts of the rose industry. Transmission information can affect how rose breeders manage their germplasm fields, how producers' space and treat roses in the field, and how homeowners manage affected plants.

Through the exploration of potential seed transmission, new information on the RRV transmission system was derived. Seed transmission has been reported with one other Emaravirus (Gupta, et al., 2018), but had not been reported with RRV. The 2019 observations indicate that seed transmission is possible when seeds, harvested from symptomatic plants, are germinated and grown. However, the variability in the ability to detect RRV consistently over time and with one detection method is troubling. There appears to be a downward trend in RRV detection with both RT-PCR and qPCR protocols. In the 2019 experiment, RRV was not detected by either protocol 14 months after germination. One observation using primer set RRV2 and qPCR is the trend in hot

temperatures and lack of detection. While temperature does not explain the 0% detection result in month three, it does explain those results in months eight (October) and 14 (April) as the ambient temperature in Texas is very warm. There are two instances where the primer set RRV(db/271)/RT-PCR detected RRV in the seedlings when RRV2 did not, and there are two months where the qPCR primer set detected RRV when RT-PCR primers did not. It is unknown what is causing these shifts in detection, but it could show possible variability in segment three of RRV. The 2020 seeds were collected from asymptomatic plants. The results yielded showed only 0.61% of seedlings were positive; suggesting that the expression of symptoms is likely correlated with a higher virus titer and therefore, a higher percentage of infected seed. The original goal for exploring seed transmission was to verify that it is not occurring so that rose breeders have the option to recover crosses. However, though transmission rates are very low using symptomatic or asymptomatic roses, the data suggests that seed transmission may be occurring. More long-term studies need to be done, but it is likely that seeds can serve as a pathway for further spread of RRD. An area of concern is that Multiflora plants regularly produce more than one million seeds each year. Even though only a small percentage of these seeds harbor RRV, these infected seeds can result in an infected plant that serves as a virus reservoir. As Multiflora roses are ubiquitous with the middle and eastern United States landscape, the rampant spread of RRV in these areas could have been aided by seed transmission. Seed transmission also makes removal of infected plants more crucial in order to manage the disease in a landscape.

Graft transmission of RRD has been previously reported in Multiflora roses by Epstein et al. (1997). The study sought to confirm and record this transmission using cultivated shrub roses and to focus on symptom expression in tolerant plants (Table 2.4). Graft transmission experiments confirmed through both PCR testing and symptom observations that graft transmission of RRV is occurring in modern-day cultivated roses. It was also discovered that infected buds from heavily symptomatic plant can be successfully grafted, despite being lower quality tissue, and can cause RRV infections as well as symptoms in the rootstock cultivars. It was previously unknown if buds from heavily symptomatic roses would be appropriate for grafting due to the fleshy nature of symptomatic material. The studies showed that 75% of successful grafts of symptomatic buds developed leaflets. However, it was observed that rootstocks grafted with these symptomatic buds appeared to be more likely to abort the graft (data not shown).

To address the question of symptom expression in tolerant plants, buds from asymptomatic, positive plants and symptomatic plants were grafted onto four potential RRV tolerant cultivars; those were ‘Bayse’s Purple’, ‘Caldwell Pink’, ‘Lafter’, ‘Cherokee’. The results showed that both types of buds successfully transmitted RRV to the rootstock cultivar. However, unlike other susceptible rootstock cultivars that developed symptoms after grafting, none of the potential tolerant rose root stock plants developed symptoms. In this experiment, it was common to see the known susceptible plants develop symptoms in the foliage of the rootstock, as well as the graft displaying symptoms. This was not the case with tolerant roses, as no tolerant rootstock developed symptoms even though the graft had symptomatic leaves. These observations further

support the idea of roses that can remain tolerant to RRV. While tolerant varieties may still contribute to the spread of RRV, they do offer options to rose consumers that live in areas heavily affected by RRD.

Results from attempts at mechanical/sap inoculations utilizing inoculum prepared from diseased/symptomatic leaves suggests that sap inoculation is not occurring. While RRV was detected (using RT-PCR/RRV(db/271)) in 18.2% of inoculated leaves at the one-month time period, it is suspected that this was potential localized deposition of the RRV. No RRV was detected in any of the new growth at one- or six-month time intervals. There are several possibilities to these failed attempts, such as inadequate inoculation technique that resulted in poor infiltration or uptake of the virus into the plant. It has been documented that Emaraviruses are typically difficult to transmit mechanically and this may be the case for RRV as well. One reason that mechanical transmission of RRV may be difficult is that RRV is a (-)ssRNA virus and needs a reverse transcription step to start infection. The extra step may affect how the virus can infect cells on the surface of leaves. The experiment could be changed to use a more aggressive silicate in the inoculum buffer and to rub leaves harder to get better infiltration. Additional work that has been completed by Verchot, et al. (2020) demonstrated a successful mechanical transmission of RRV into Nicotiana utilizing an infectious clone of RRV. This work indicates that mechanical transmission of RRV is possible, however the methodology used for inoculation is different from the approach we utilized.

The question of root transmission of RRV was raised early into the broader RRD project when plants developed symptoms within six weeks of being planted into beds with active infected crown tissue and roots. Root transmission was questioned further through additional field trials and another project that suggested an infected plant at close spacing infected all plants adjacent of it. The 2018 root transmission experiments suggested that root transmission may occur as early as 6 weeks, which was similar to earlier observations. However, one limitation to this study is that because "healthy" plants were not displaying symptoms, they were assumed to be RRV free. It is possible that the plants were asymptomatic positive for RRV and that is how symptoms developed in such a short time frame on the 'Julia Child' plants. At the time of experiment initiation, the RRV(db) primer was not available and the concept of asymptomatic positive plants was not one that was known. The 'Ketchup & Mustard' healthy plants did not develop symptoms, but did test positive after four months, which is near the time that the use of RRV(db) primer began. It is possible the plants were already infected and did not have root transmission occur from contact with infected plant roots. The 2019 root experiments were not completed due to several issues, including infestation of fungus gnats. Due to time restrictions, there was not time to replicate these experiments, however it does seem possible that this set of experiments would provide quick data on root transmission. The 2020 root experiments yielded inconclusive results. While it is believed that root transmission is a possible way for RRV to be transmitted to a new host, the 2020 repeat experiment did not produce results that would confirm the 2018 observations. Additional work needs to be performed to

confirm if root transmission is occurring. Adjustments that could be made to improve this experiment is to use smaller, more shallow pots and to use infected plants with a more vigorous root system. The infected plants were cuttings from other symptomatic plants and the roots systems were not as vigorous as roots systems in the 2018 experiments. Had symptoms been seen in healthy plants, the infected plants would have been treated with an herbicide to determine if herbicide damage symptoms developed in the healthy plants.

Future work that could be conducted with seed transmission is to test various parts of RRV suspect seeds to determine where the virus localizes in the seed. RRV infected seedlings could also be tested more specifically (days instead of months) to determine if seedlings are positive immediately after germination or if it takes an undetermined amount of time for viral levels to build. A more long-term (two plus years of continuous testing data) seed experiment is needed to determine if viral titer is eventually reduced to undetectable levels or if the plants remain positive and asymptomatic long-term. A future grafting study that should be conducted with tolerant varieties grafted with symptomatic buds is the maintenance of successful graft unions for two years to meet the threshold for symptom development. Future experiments for root transmission could involve doing more specific testing of plant roots to see if there are certain roots that are RRV positive when symptoms developed in healthy plants.

In exploration of the various ways that RRV can be transmitted in the absence of the eriophyid mite vector, I looked at seed transmission, graft transmission, mechanical transmission, and root transmission. Seed transmission experiments do suggest that seed

transmission might be occurring, but there is a need for a long-term study. Grafting experiments confirmed previous work on RRD graft transmissibility, and further showed that RRV is transmitted via bud grafting. Using a combination of infected/non-infected scion and rootstock, the utility of tolerant rootstock was shown where no RRD symptoms were observed up to eight months after grafting. A quick attempt at mechanical/sap inoculation resulted in conclusions that are like previous reports that RRD is not easily transmitted through mechanical means. However, new information from other researchers suggest/demonstrated that mechanical transmission can occur. Root transmission, based largely on observations from field trials in high disease pressure locations, brought about questions on the ability of RRV to move from plant to plant via the roots. The initial root transmission experiment indicated that this was possible, however, this result was not reproduced in subsequent years. While it is still believed that root transmission is possible, additional work will need to be done to confirm this suspicion.

CHAPTER V

CONCLUSIONS

Summary

Rose Rosette Disease (RRD) was identified as a virus-like plant disease in the 1940's (Pemberton, et al., 2018). It was not until 2011 that RRD was confirmed to be caused by the *Rose rosette virus* (RRV) (Laney, et al., 2011). Since the initial characterization of four viral segments was completed (Laney, et al., 2011), three other segments have been identified (Di Bello, et al., 2015) and eight different primer sets (Babu, et al., 2017a; Babu, et al., 2017b; Bratsch, et al., 2017; Di Bello, et al., 2017; Dobhal, et al., 2016) have been published to aid in identification of viral infection. It has also been found that no known commercial rose cultivars are resistant to RRV, however it is possible that sources of resistance may be found in wild rose species, especially those that are native to the United States (Byrne, et al., 2018).

In this study, I contributed to the knowledge about possible RRD resistance through the development of seven replicated field trials, planted in various locations across north Texas. Each of these field trials contained plants that had not yet been screened for RRD, which provided more information on the resistance of cultivars. Based on the data collected, I was also able to create a definition for RRD tolerance; plants that remain asymptomatic while infected with RRV for two or more years. This definition of tolerance provides possible cultivars that can be recommended to landscapers and homeowners in areas where RRD is endemic to the location. The trials

also offered the opportunity for better education of the general public and Master Gardeners about RRD symptoms and the entire RRV disease system.

Furthermore, through work in this project, I was able to refine RRV diagnostic processes through the modification of a rapid RNA extraction method published method by Babu et al. (2017b). In addition, sensitivity screening of all published RRV primer sets was performed and primers that target segment three were recommended for the most sensitive PCR detection. Finally, the concept of asymptomatic, RRV positive plants was developed through further primer screening work that was performed on roses in field trials. Overall, this work improved and streamlined RRV diagnostic processes and allowed for more clarity on which primer sets to utilize based on the type of PCR that is being performed.

Another aspect of this project was vegetative-based transmission studies. The first transmission method that was explored was vertical RRV transmission from infected parent to seeds. It was found that seed transmission of RRV is occurring at a very low percentage. Graft transmission was revisited utilizing commercial rose cultivars; it was found that graft transmission of RRV does occur with a range of rose cultivars. Graft transmission projects also showed that grafting buds from RRD symptomatic roses did not produce symptoms in rootstock plants from previously identified tolerant cultivars. Mechanical/sap transmission was attempted; however, it warrants more research. The last transmission method that was examined is root transmission, which was found to occur on a limited basis, however this result has not yet been replicated to verify this finding.

One trend that was noted throughout all project objectives is the tendency of RRV titer to go below detectable levels in the hot Texas summer months. This change is accompanied by little or no symptom expression and improvements in plant health. Upon further research, it is proposed that a "heat masking" phenomenon (Szittya, et al., 2003) is occurring in summer months (May-August). There are several instances of this effect occurring throughout the project. In field trials, when roses would be PCR tested in March, many times the plants would yield C_t values in the strong positive range for RRV. However, when tested in August or November, very few of the roses would yield a positive result. In the same set of experiments, light RRD symptoms would occur in cooler months (November-March), but would disappear when the temperature began to rise. In transmission experiments, the same effect was noticed, however it was more extreme due to much higher temperatures in greenhouses. The differences in RRV has also been noted in primer sets. I have observed that the RRV(db) primer set (Di Bello, et al., 2017) tends be more sensitive in samples taken in August-March, whereas the primer set RRV2 (Dobhal, et al., 2016) yields more sensitive results on samples in April-September.

While heat masking refers to more external factors, it is also related to changes in RNA silencing (Chung, et al., 2016). In extreme heat, RNA silencing by plants tends to be more effective against viruses, reducing the number of viral particles in plants. RNA silencing is also what provides the heat masking effect as it can allow asymptomatic leaves to emerge at higher temperatures (Chung, et al., 2016). It is proposed that higher temperatures affect a possible suppressor of silencing that is produced by RRV, allowing

the host plant's RNA silencing machinery to be more effective in reducing viral loads in plants. Heat related RNA silencing is also proposed to be part of why known susceptible cultivars of roses remain infected with RRV, yet asymptomatic, for more than 18 months.

Overall, this project yielded new data about levels of resistance which were observed in roses planted in field and greenhouse conditions. It also yielded several diagnostic improvements to better the accuracy of RRV detection. Results were gathered that suggested seed transmission and graft transmission do occur on a limited basis. All topics contributed to or confirmed information about RRV, helping to bridge knowledge gaps about the Rose Rosette Disease system as a whole.

Extended Work Collaborations

The ability to utilize a non-destructive and rapid diagnostic tool that can provide accurate results about diseases, stress, or herbicides is desired by the plant industry as a whole. Raman Spectroscopy has the potential to meet some of those demands. For the rose industry, field diagnostics technology is desired by plant breeders, growers, and nurseries to ensure that plants are clear of diseases or other stressors when sold to the consumer. Raman Spectroscopy has shown the potential to detect RRV in both symptomatic and asymptomatic plants. It is hoped that this field-based screening tool can be used to reduce the number of roses that are sold to consumers with biotic or abiotic issues and to determine which plants need to receive further disease testing by a plant diagnostic lab. Work that was done to address the use of this technology for

detection has been previously published by Farber et al. (2019). In this work, I selected plants from southern Oklahoma and several locations in Texas that were asymptomatic, PCR positive or symptomatic, PCR positive plants. I then identified a PCR negative rose. All rose cultivars were 'The Double Knock Out© Rose' or 'The Pink Double Knock Out© Rose'. After RNA extraction using MDA-C and PCR testing using RT-PCR and RRV(db/271) primer set, plants were separated into classifications based on symptomology (or lack thereof) and PCR testing results. Raman Spectroscopy was then performed on 30 leaves from each classification, with a total of 808 spectra being collected from the five classes of leaves (Farber, et al., 2019). Bands in each spectra were analyzed and assigned to common plant components such as cellulose, glucose, lignin, and so on. Two bands appeared to be distinct indicators of RRV presence and infection: $1610cm^{-1}$ (lignin) and $1720cm^{-1}$ (stress response) (Farber, et al., 2019). Overall, this work showed preliminary results that this technology can be used for RRV screening and detection in a rapid, non-destructive way.

To further utilize Raman Spectroscopy in a field-based setting, studies were completed to determine if molecular changes in plants that are associated with herbicides can be detected. RRD symptoms are commonly confused with symptoms of herbicide damage. and Few herbicides are labeled for use on or around roses, which leads to herbicide damage occurring from misuse or drift. Through herbicide application experiments utilizing both label and diluted rates, herbicides with active ingredients of Glyphosate, Dicamba, and/or 2,4-D have all been proven to cause serious damage or death to roses whenever applied directly to the plant. These herbicides are optimized for

broadleaf plants and negatively affect roses through the active ingredient's mode of action. In preliminary results, Roundup (active ingredient: glyphosate) has been found to produce a specific and recognizable fingerprint when Raman Spectroscopy is performed on treat plant leaves. In the dilution experiment, Roundup treated plants yield symptoms very consistent with what is typically observed in the field on herbicide damaged plants. The shortened internodes, rosette grow, and generally poor health are typical symptoms of herbicide damage and are often confused with RRD symptoms. Weed B Gon symptoms has not been observed in the field before however, the ability of the treated plants to outgrow herbicide damage had been observed previously.

Herbicides such as Preen granular and Fusilade II are labeled for use on roses and did not produce any plant damage throughout the two herbicide experiments. Preen is a pre-emergent herbicide and does not affect plants that have already germinated. Fusilade II is a monocot specific herbicide and does not appear to have a mode of action that affects roses.

Figure 5.1. Diluted herbicide rate symptoms. The picture on the left shows glyphosate damage symptoms that can occur on roses. Herbicide damaged plants displaying large amounts of these rosettes can be mistaken for RRD symptoms. The picture on the right shows thin, distorted leaves that resulted from herbicide damage Weed B Gon, which is a combination of 2,4-D and Dicamba active ingredients.

Future Studies

Screening of cultivars for resistance

While the use of field trials enables a realistic estimation of how species cultivars perform in field settings, the utilization of grafting could enable a more rapid screen for susceptibility or tolerance/resistance. I have shown that infected buds from RRD symptomatic plants graft easily and quickly transmit RRV from bud to rootstock. Utilization of grafting in a greenhouse setting, especially when plants are small, could allow for a quicker screen and reduce the amount of species roses that need to be screened for RRD susceptibility in a field trial.

Determination of suppressor of plant RNA silencing

While a suppressor of silencing has not been determined for RRV, it is very likely that one exists in segments 5-7. Literature has already been published on other Emaraviruses that have suppressors of ssRNA or dsRNA silencing; two suppressors of silencing have been found for *High Plains wheat mosaic virus* (HPWMoV) that are encoded by segments 7 and 8 of that viral genome (Gupta, et al., 2018). Further genomic studies of RRV are needed to discover the functions of segments 5-7 and how these segments and the proteins they encode effect the ability of RRV to cause infection. The documentation of a suppressor of silencing could further the search for an RRD resistant rose.

High temperature driven RNA silencing

Several studies have documented the seasonality of virus and host plant interactions (Honjo, et al., 2019). The optimal temperature range for plant viruses to

replicate in their hosts is believed to be between 15°C and 30°C (Honjo, et al., 2019). The average summer high temperature in Dallas, Texas, where RRD pressure is very high and the disease is endemic to rose landscapes, is 36°C; this location is one that heat masking has been observed both phenotypically and genotypically in field trial roses. Studies that use a temperature gradient and test for presence or absence of RRV could provide better documentation of the occurrence of temperature related RNA silencing.

Asymptomatic, infected roses and their implications

Asymptomatic, infected plants have been observed to remain such for more than 18 months. It is likely that these plants stay undetected, but still contribute for RRV spread. Plants from Tennessee and Texas have been documented to be both infected and asymptomatic through this project. While this could be a sign of RRV tolerance, many of the cultivars are known susceptible plants. It is possible that asymptomatic, infected plants could be a result of RNA silencing. Most of these plant samples come from areas where the high temperature is outside of the optimal virus replication range for much of the year. In addition, research has found that plants infected by viruses at an early growth stage and grown at a higher temperature were less symptomatic than plants grown at lower temperatures (Obrepalska-Steplowska, et al., 2015) Experiments should be completed to determine if temperature plays a role in plants remaining asymptomatic. These could include infecting roses at different ages and maintaining them at various temperatures and making observations on symptom development. There should also be experiments completed that look more into viral genetics to determine if symptom development is related to a satellite or a co-infection with other rose viruses. Previous

studies have found that satellite RNA (satRNA) can enhance the development of symptoms of different viruses (Obrepalska-Steplowska, et al., 2015). Further genomic studies could be completed to determine if there is satellite viruses or RNA associated with RRV that determines symptom development.

Detection of Other Rose Viruses Using Raman Spectroscopy

While the initial PCR detection and Raman Spectroscopy have been completed on the other rose virus samples, data analysis has not been completed. This is due in part by the inability to identify virus free plants of the same cultivar in Florida. Some virus free cultivars have been identified but are located in Texas. One limitation that has been noted with Raman Spectroscopy is that both healthy and infected plants must be collected from the same region to establish a fingerprint. The inability to make inferences about diseases without all types of samples is a potential limitation for the application of Raman Spectroscopy in an industry setting. Therefore, this project is ongoing and there are plans to collect virus infected and virus free material from one location only, then compare those to infected plants from other locations to see if location truly affects the detection ability of this technology.

Effect of Location on Raman Spectroscopy

To determine the impact of location on Raman Spectroscopy, two sets of experiments have been planned, but have not yet been completed. The first is to complete a statewide sampling around and across Texas. In this experiment, 'The Double Knock Out© Rose' cultivar will be utilized. Thirty leaflet samples from healthy (and infected, if available) will be collected by collaborators and sent in for Raman

Spectroscopy to be performed. This experiment will determine if differences in climate and temperature affect the fingerprint developed for this specific rose cultivar. The second experiment is to collect 30 leaflets from healthy (and infected, if available) plants from across the United States. The preferred cultivar is 'The Double Knock Out© Rose'. These nationwide samples would be read through Raman Spectroscopy and further determinations can be made about the effect of location and environment on the ability to detect RRV.

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