EVALUATION OF ROSE GERMPLASM FOR RESISTANCE TO ROSE ROSETTE DISEASE, AND STUDIES OF DISEASE TRANSMISSION AND VECTOR MANAGEMENT

by

Danielle N. Novick

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Sciences

Fall 2017

EVALUATION OF ROSE GERMPLASM FOR RESISTANCE TO ROSE ROSETTE DISEASE, AND STUDIES OF DISEASE TRANSMISSION AND VECTOR MANAGEMENT

by

Danielle N. Novick

Approved: _____
Thomas A. Evans, Ph.D.
Professor in charge of thesis on behalf of Advisory Committee

Approved: _____
Robert E. Lyons, Ph.D.
Interim Chair of the Department of Plant and Soil Sciences

Approved: _____
Mark W. Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my thesis advisor, Dr. Tom Evans, for his constant guidance and encouragement. As a supervisor he is knowledgeable and understanding; as a coworker he is enthusiastic and untiring; as a friend he is generous and engaging. I am also grateful to the additional members of my advisory committee, Dr. David Frey, Dr. Nicole Donofrio, and Dr. Brian Kunkel, for their helpful advice in times of need. This research would not have been possible without the financial support of the United States Department of Agriculture's National Institute of Food and Agriculture or the project director, Dr. David Byrne. Assistance from Michelle Scheiber and Dr. Christina Quinn at Star Roses and Plants was also enormously appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Rose rosette disease (RRD) is a lethal disease of roses (*Rosa* spp.) caused by *Rose rosette emaravirus* (RRV) and vectored by the eriophyid mite *Phyllocoptes fructiphilus*. It is one of the most devastating diseases of landscape roses, producing aesthetically disfiguring symptoms within months and eventually killing the plant. Unfortunately, there is no known resistance among commercial rose cultivars and the few rose species that have demonstrated resistance are not ideal candidates for introgression of resistance genes into commercially viable cultivars. This thesis details three studies relating to the management of RRD. The first is a resistance trial intended to identify rose genotypes that merit consideration as candidates in a breeding scheme for resistance to RRD. A total of 216 genotypes are being screened in an ongoing field trial at the University of Delaware in Newark, DE. In addition to high natural disease pressure, the field has received multiple augmentations since its establishment in May 2015. Augmentation consisted of using twist ties to affix symptomatic shoots of RRD-infected *Rosa multiflora* to actively growing shoot tips of target roses. The vector preferentially feeds on symptomatic tissue, so augmentation artificially enhanced vector populations. Any roses that developed symptoms were subsequently tested by endpoint RT-PCR to confirm the presence of RRV. Thus far 94 genotypes have been verified as susceptible to RRD. The study will continue through 2018 and any roses remaining symptom-free will be assayed to confirm the absence of viral replication. The second and third studies in this thesis investigate (i) mechanical and graft transmission of RRV and (ii) the use of predatory mites as a biological control of the vector, *P. fructiphilus*.

Chapter 1

INTRODUCTION

1.1 Host: *Rosa* spp.

1.1.1 Host Range and Susceptibility

The host range of rose rosette disease is limited to members of the genus *Rosa* in the family Rosaceae, a genus that has been in cultivation for thousands of years (Guoliang 2003; Windham et al. 2014. Apart from roses, this family boasts a number of economically valuable crops such as apples, strawberries, almonds, peaches, and cherries. *Rosa* spp., commonly called roses, include around 120 to 150 species spread across Asia, North America, Europe, and northwest Africa, but the genus is considered to originate in Central Asia where fossils of rose leaflets have been recovered that date to the Eocene Epoch (about 40 million years ago) (Guoliang 2003). Most roses have 7 paired chromosomes but polyploidy is fairly common in the genus with species ranging from diploid to hendecaploid (Zlesac 2009). Polyploidy likely arose as a result of ancestral hybridization, either by natural means or through cultivation, and is believed to have contributed to the rose's broad geographic range and climatic adaptability (Vukosavljev et al. 2013; Zlesac 2009).

Cultivation of roses began thousands of years ago, culminating in more than 20,000 described commercial rose cultivars (Cairns 2003; Guoliang 2003; Krussmann 1981). However, these domesticated ornamental roses derive from only a small subset of wild species, including *R. moschata*, *R. wichurana*, *R. multiflora*, *R. gallica*, *R. chinensis*, *R. gigantea*, *R. foetida*, *R. spinosissima*, *R. cinnamomea*, and *R. rugosa* (Vukosavljev et al. 2013). Only a handful of rose species and no commercial cultivars have proven to be consistently resistant to either rose rosette virus or its eriophyid mite

vector. Resistant genotypes consist mainly of roses native to North America, including *Rosa californica*, *R. carolina*, *R. palustris*, *R. setigera*, *R. spinosissima*, and *R. bracteata* (Amrine 2002). It should be noted that there is little overlap between these two lists. The widespread susceptibility of modern garden roses to RRD is less surprising with the knowledge that resistant species are largely absent from their breeding ancestry.

Other members of Rosaceae and a few members of other economically important families have also been tested for susceptibility to RRV, but none of these plants were successfully infected with RRV either by grafting or by vector transmission (Amrine 2002). Rosaceous plants were also tested for preference and acceptance by *Phyllocoptes fructiphilus* and *P. adalius*, another eriophyid mite commonly found on roses. None of these plants, which included various species from the genera *Prunus*, *Malus*, *Pyrus*, *Rubus*, *Sorbus*, and *Fragaria*, were suitable for egg-laying by *P. fructiphilus* and most could not even sustain survival of the mite for more than a few days (Amrine 2002).

1.1.2 Importance of Roses and Economic Impact

Roses constitute an integral part of the American culture as is evidenced by the fact that in 1986 the 99th Congress designated the rose as the National Floral Emblem of the United States by Senate Joint Resolution 159 (Senate Joint Resolution 159 1986). In his official proclamation to declare this fact, President Reagan invoked the many ways in which roses permeate our society: they're grown in every state, they're given to sweethearts and laid on graves, they were even bred by George Washington himself (Proclamation No. 5574 1986). This botanical reverence extends beyond our borders, with more than ten other countries claiming the rose as the national floral emblem as well (*Floral emblem*).

More importantly, roses are one of the four most cultivated flowers on the planet and are economically the most important ornamental crop worldwide (Guoliang 2003; Whitaker et al. 2009). The Vineland Research and Innovation Centre in Ontario values the Canadian market for landscape roses at Can$149 million and the equivalent U.S. market at Can$928 million (Goodish 2013). The wholesale value alone of container

and bare-root roses in the United States is approximately $194 million (Dobhal et al. 2016). Annual worldwide production estimates include "18 billion cut stems, 60-80 million potted roses and 220 million roses for the landscape with an economic impact in the 10s of billions of dollars" (Debener et al. 2014).

Like any crop, roses are plagued by a multitude of pests and diseases which negatively impact their aesthetic appeal and market value. Black spot (*Diplocarpon rosae*), powdery mildew (*Podosphaera pannosa*), and Japanese beetles (*Popillia japonica*), among others, are frequently encountered by growers, landscapers, retailers, and gardeners, but in the last few decades a newly-characterized virus has been implicated as one of the most serious threats to the garden rose industry (Laney et al. 2011). Rose rosette disease (RRD) is a lethal disease of roses caused by rose rosette Emaravirus (RRV) and transmitted by the eriophyid mite, *P. fructiphilus*.

Rose rosette disease threatens the rose industry in various ways. The lethal and incurable nature of the virus means that it can have an economic impact throughout the supply chain. Producers must shoulder the costs of certified virus-free propagative material, increased scouting and miticide use, removal of local multiflora stands, and rogueing of infected plant material in addition to the possibility of consumers avoiding garden roses as their reliability in the landscape declines. This possibility seems particularly salient considering the increasing demand by consumers for garden roses that are both low-maintenance and disease-resistant (Debener et al. 2014). Rose growers are not the only affected party, though. Both landscape companies as well as public gardens have been tasked with removing large numbers of infected rose plants across the country. For example, in Southlake, Texas, more than 5,400 rosebushes are being replaced in medians and parks, with estimated costs as high as $500,000 (Bahari 2015). Similarly, the Cranford Rose Garden at Brooklyn Botanic Garden first detected rose rosette disease amongst its roses in 2005. Only four years later, the southern beds were so devastated by the disease that the garden was forced to undergo extensive renovations that included destroying at least 20% of the collection (Owens 2011). Unfortunately these are not isolated incidents, and they are only going to become more

common. In September 2017, a rose company called Weeks Roses in Wasco, California, a location previously unaffected by RRD, contacted its customers to disclose the discovery of RRD in its production fields (see Appendix A). If Whitaker and Hokanson were correct in writing that disease susceptibility poses the greatest challenge for producing and maintaining quality roses, then creating RRD-resistant plants should be considered crucial to maintaining the economic viability of the garden rose industry (Whitaker et al. 2009).

1.2 Pathogen: Rose Rosette Virus

1.2.1 Discovery of RRD's Causal Agent

The causal agent of RRD is a relatively recent discovery considering the disease was first described in the 1940 (Conners 1941). The involvement of *P. fructiphilus* was noticed quite early, leading many researchers to believe that the disease was the result of a toxicogenic reaction to the mite's feeding (Doudrick et al. 1986; Slykhuis 1980) However, it was demonstrated as early as 1968 that the mite's feeding was not directly responsible for disease development because RRD could be induced by graft transmission (Allington et al. 1968). At the same time, it was suggested that rose rosette was caused by a virus, but this theory failed to gain traction until a scanning electron microscope study was published in 1983 showing the association of RRD with spherical membrane-bound virus-like particles of about 120-150 nm in size (Allington et al. 1968; Gergerich et al. 1983). Even after this report, it was posited that the causal agent might be a phytoplasma, a phytoplasma-like organism, or mycoplasma-like organism and that root grafting might be responsible for the disease's rapid spread in stands of multiflora rose (Doudrick 1984; Doudrick et al. 1986; Epstein et al. 1993; Gergerich et al. 1983).

Electron microscope studies continued to support the theory of a virus or virus-like organism until a consensus was generally reached in 2011 when rose rosette virus was sequenced and characterized as a member of the genus *Emaravirus* (Ahn et al. 1996; Kim et al. 1995; Laney et al. 2011; Rohozinski et al. 2001; Silvestro et al.

2004). Sequencing revealed that RRV consisted of 4 single-stranded negative-sense RNA segments, which were used to develop a RT-PCR assay to detect the virus in rose tissue (Laney et al. 2011). Only 4 years later, this characterization was modified slightly to reflect the discovery of three additional genome segments as well as the fulfillment of Koch's postulates for the disease (Di Bello 2015; Di Bello et al. 2015b). There are still many unknowns surrounding the pathology of RRD. For example, it's still not clear what effect mixed infections might have on disease development: it's possible that susceptibility to RRV is affected by the presence of additional viruses or other pathogens. Also, the "behavior" of RRV in living rose tissue is not well understood. The virus can be isolated from the roots of infected roses, indicating systemic movement, but there is little work to explain the nature of this movement (Rohozinski et al. 2001; Silvestro et al. 2004).

1.2.2 RRV Morphology and Taxonomy

Rose rosette virus has a multipartite genome consisting of seven single-stranded anti-sense RNAs, each between 1400 and 7100 nucleotides in length (Di Bello et al. 2015b). Transmission electron microscopy of infected rose tissue has revealed the enveloped nature of the virus, with particles ranging in size from 110 to 180 nm (Silvestro et al. 2004). The International Committee on Taxonomy of Viruses (ICTV) considers rose rosette virus to be a member of the relatively new genus *Emaravirus*, a name taken from the type species European mountain ash ringspot associated virus. Other putative members include fig mosaic virus, pigeonpea sterility mosaic virus, raspberry leaf blotch virus, and high plains virus, alternatively called wheat mosaic virus or maize red stripe virus (Mielke-Ehret et al. 2012). The newest proposed *Emaravirus* is blackberry leaf mottle-associated virus, a recently-discovered pathogen of *Rubus* spp. (Hassan et al. 2017).

The *Emaravirus* genus is not categorized into a virus family, but is considered most similar to viruses in the family *Bunyaviridae*, specifically those in the genus *Tenuivirus* (Elbeaino et al. 2013). Unlike the *Bunyaviridae*, emaraviruses have 4 or

more genomic segments (Elbeaino et al. 2013). Several characteristics unite emaraviruses: they possess multipartite genomes of negative sense ssRNA enveloped in double membrane-bound bodies and are transmitted by eriophyid mite vectors (Mielke-Ehret et al. 2012). Some researchers have postulated that the high variability in number of segments present in *Emaravirus* genomes is a sign of the genus's genomic plasticity, with core proteins for replication, assembly, protection, and movement able to re-assort with auxiliary proteins that function in pathogenicity or host range (Di Bello et al. 2015b). This hypothesis is supported by the fact that emaraviruses show detectable sequence homology in RNAs 1, 2, 3, and 4, which code for the RNA-dependent RNA polymerase (RdRp), glycoprotein precursor (GP), nucleocapsid (NC), and putative movement protein respectively, but little to no sequence homology for other segments (Tatineni et al. 2014). When it was still believed that RRV contained only 4 RNA segments, a diversity study of the virus discovered nucleotide identities ranging from 93 to 99% for all regions examined on RNA3 and RNA4, with geographic location having no impact on variability between isolates (Laney et al. 2011). Future studies of this kind might very well find higher genetic diversity on other segments which may be associated with differential pathogenicity depending on the rose species infected.

1.3 Vector: *Phyllocoptes fructiphilus*

1.3.1 General Information and Transmission Details

Eriophyid mites are minute, haplodiploid ectoparasites of plants in the family Eriophyidae (superfamily Eriophyoidea) and are commonly called gall, rust, bud, or blister mites (Keifer et al. 1982). They are soft-bodied and spindle-shaped, consisting of two body regions – the gnathosoma (mouthparts) and idiosoma (body) – and unlike other mites, they possess only two pairs of legs (Keifer et al. 1982). Behind the Tetranychidae, eriophyid mites are the second most economically important family of plant-feeding mites and make particularly good adventive species because of how difficult they are to detect and control (Navia et al. 2010). Worldwide, a total of 19 eriophyoid species from 10 genera have been found on roses, with six of those species

reported on roses in the United States. These include *P. fructiphilus* Keifer, *P. adalius* Keifer, *P. chorites* Keifer, *Epitrimerus linegranulatus* Styer, *Eriophyses eremus* Druciarek & Lewandowski, and *Callyntrotus schlechtendali* Nalepa (Druciarek et al. 2016a; Ochoa et al. 2016). Of these, *P. fructiphilus* and *P. adalius* are the most commonly found on roses but unfortunately the classification of these two species has been fraught with changes due to their independent discovery in different locations and due to morphological differences between female forms of the same species (Druciarek et al. 2016b). Recent corrections to the genus synonymized *P. fructiphilus* with *P. slinkardensis* Keifer and *P. adalius* with *P. rosarum* Liro, but these mites are very similar to each other and mistaken identification is common (Druciarek et al. 2016b; Ochoa et al. 2016).

The eriophyid mite *P. fructiphilus* has been implicated as the vector for the rose rosette disease pathogen since 1968 and it was made clear in 1997 that only a single mite is required for transmision to occur (Allington et al. 1968; Epstein et al. 1997). However, studies conducted over the last five years have greatly improved our understanding of mite transmission. In 2015, positive strand RRV was isolated from eriophyid mites that had fed on infected rose tissue, thereby proving two things: (1) that *P. fructiphilus* transmits rose rosette virus and (2) that the virus replicates within the eriophyid mite (Di Bello 2015; Di Bello et al. 2015a). The latter is inferred from the fact that negative strand RNA viruses like RRV only produce positive strand RNA during replication (Di Bello et al. 2015a). Propagative viruses are generally associated with longer vector feeding durations and latent periods and RRV is no exception to this: *Phyllocoptes fructiphilus* requires a feeding time of 5 days to become viruliferous and can then transmit the virus to a healthy rose in less than an hour (Di Bello 2015). Additional research is required to determine if the virus is transmitted transovarially.

1.3.2 Biology and Life Cycle

Phyllocoptes fructiphilus is a yellowish eriophyid that measures 140-170 microns long and can be identified by a distinctive pattern on its dorsal shield (Keifer et al.

1982). It is commonly confused with *P. adalius*, a mite that has become a serious pest in greenhouse rose production because of the feeding damage it causes (Amrine 2002; Druciarek et al. 2016b). The life cycle of *P. fructiphilus* is relatively simple compared to other eriophyids (Keifer et al. 1982). Fertilized adult females (deutogynes) overwinter in protected areas on living rose tissue, spending the cold months under vegetative bud scales, under loose bark, or on overwintering foliage and in early spring the deutogynes move to tender rose shoots to lay eggs, often ovipositing in the leaf axil between the stem and a leaf petiole (Amrine 1996, 2002). This egg placement allows immature mites to start feeding in an ideal location, in the leaf axil, as soon as possible. After the eggs hatch, mites develop through two immature stages (referred to either as the protonymph and deutonymph or larva and nymph) before becoming adults. The average time from egg laying to adulthood is 11 days (Kassar et al. 1990). During the growing season females are called protogynes and live about 30 days, laying around 1 egg per day (Amrine 1996; Manson et al. 1996). Eriophyid sexual reproduction is by indirect insemination with a spermatophore that is deposited on a substrate by males and later picked up by females (Michalska et al. 2010).

Because females are diploid and capable of parthenogenetic reproduction, a population can be established from a single female mite (Manson et al. 1996). Development continues throughout the growing season and populations of the mite on multiflora rose are reported to peak in August or September in Iowa (Epstein et al. 1997). Roses are the only known host of *P. fructiphilus* that are suitable for reproduction and survival beyond a few days (Amrine 2002; Keifer et al. 1982). Mites require tender growth for feeding and reproduction, which means that climatic conditions affecting rose health will also have a strong effect on mite populations. For example extreme low temperatures or daily fluctuation in excess of 17 degrees Celsius during winter or drought during the spring months can significantly reduce the mite population for a growing season because roses produce less tender growth under these harsh conditions (Amrine 2002; Epstein et al. 1999). Since RRD-infected rose tissue shows increased succulence as a primary symptom, it follows that *P. fructiphilus* preferentially feeds on symptomatic

rose shoots and can be found in extremely high numbers on infected roses compared to healthy roses. The average number of mites per symptomatic shoot can easily be in the low hundreds while healthy shoots from the same plots normally carry fewer than ten mites (Amrine 2002). Mite enumeration studies performed by Epstein and Hill showed a normal distribution of eriophyids on shoot tips, with the leaf axil at the fifth node from the tip carrying the highest number (Epstein et al. 1994).

The minuscule size of eriophyid mites affects both their feeding depth and ability to disperse. Eripohyid stylets are about 20 microns in length and are generally only able to puncture the cytoplasm of epidermal cells (Navia et al. 2010). Movement between hosts is by one of four methods: (1) walking between adjacent plants that are touching, (2) coasting on air currents, (3) moving phoretically on birds or other arthropods, and (4) dispersal by rain (Michalska et al. 2010). While dispersal on air currents is likely the most common, it is also the riskiest because an individual mite has no control over where it lands. This strategy of rapid reproduction and risky dispersal possibly reflects an adaptation to low host concentration. Unfortunately, the spread of an invasive host and the increased use of roses in landscaping over the past few decades has allowed for the unprecedented spread of *P. fructiphilus* and RRV.

1.4 Non-Vector Transmission

The primary means of transmission of RRV in nature is through *P. fructiphilus*, although it was at one point suggested that root grafting may contribute to the rapid spread of RRD in large stands of multiflora rose (Di Bello et al. 2015a; Doudrick 1984). Direct evidence of root grafting in the field was never demonstrated (Epstein et al. 1999). Various other forms of transmission have been studied in the lab, but only graft transmission has proven to be consistently reproducible (Allington et al. 1968; Amrine et al. 1988; Di Bello 2015; Epstein et al. 1997). However, articles purporting successful graft transmission should have their methods closely examined because a number of these researchers studied grafting in the context of biological control of invasive multiflora rose (Amrine et al. 1988; Epstein et al. 1997; Hindal et al. 1988).

As such, they did nothing to ensure that grafted plants were kept clean of eriophyid mites, with some experiments even taking place in the field proximal to infected roses (Epstein et al. 1997).

Contamination of grafted plants with *P. fructiphilus* erases the validity of the grafting results, which makes designing a grafting study relatively difficult. *P. fructiphilus* prefers to hide in leaf axils and bud scales of symptomatic tissue, making it difficult to ensure that the scionwood from infected plants used in grafting is mite-free. Graft transmission can be accomplished using bud-free internodal shields of cortical tissue, though, which avoids the risk of hidden eriophyids (Allington et al. 1968). In general, graft transmission studies have used vegetative buds with a surrounding shield of cortical tissue taken from multiflora rose, producing success rates as high as 100% with disease symptoms often appearing in as little as 30 days (Allington et al. 1968; Amrine et al. 1988; Epstein et al. 1994). Transmission is more likely to occur when the scion is attached on the upper portion of current-season canes, when the rootstock is large and vigorous, and when the apical bud and leaves surrounding the graft site are removed (Amrine et al. 1988; Epstein et al. 1994).

Other forms of inoculation have been consistently unsuccessful. Rose to rose transmission by abrasion, vascular puncture, soil, seed, injection, ballistic inoculation, dodder (*Cuscuta campestris*, *C. gronovi*, and *C. pentagona*), and spores of powdery mildew (*Sphaerotheca pannos*) all failed to produce RRD symptoms with one exception: one multiflora rose out of 120 that were stab inoculated with sap extracted in buffer developed symptoms characteristic of RRD (Epstein et al. 1994). It should be noted that at the time of these experiments, diagnosis of RRD was limited to symptom identification. Other emaraviruses can be transmitted by abrasion or vascular puncture, but rose leaf and stem tissues have high levels of tannins, phenolics, and phenolic oxidases that make intact extraction of viral RNA difficult (Rohozinski et al. 2001). In one experiment, though, mechanical transmission of RRV from multiflora rose to two species of tobacco (*Nicotiana benthaniana* and *N. glutinosa*) was allegedly achieved, although there were no serological or molecular means of confirming virus transmission

(Rohozinski et al. 2001).

1.5 Disease: Rose Rosette Disease

1.5.1 Symptoms and Disease Development

Rose rosette disease is most importantly a lethal disease, able to kill roses in a matter of months or years depending on the size of the plant. Symptoms of the disease can vary, but include excessive growth of lateral shoots, increased thorniness and succulence of shoots, abnormal reddening of new growth, leaf, bud, and flower distortion, witches' broom, stunting, and defoliation (Windham et al. 2014). Disease development has been described in three stages, which progress more rapidly in smaller plants than in larger, multi-crowned plants.

Stage 1 of disease development involves red or pink discoloration of leaf veins, leaves, and shoots, vigorous growth and extended succulence of shoots, proliferation of thorns, and distortion of leaves and blossoms (Epstein et al. 1999). These visible symptoms are accompanied by a depletion of stored starch, reduced root growth, and changes in the balance of sugars from predominantly sucrose to predominantly fructose and glucose (Epstein et al. 1994). Sometimes infected roses can revert back to healthy-looking growth, but this is only a temporary improvement (Epstein et al. 1999).

Stage 2, also called the early rosette stage, marks the initial development of RRD's characteristic symptom: the witches' broom. Leaves become elongated and rugose and show mosaic with intense red discoloration. Petioles and internodal distances are shortened as lateral buds break dormancy, producing a rosette. Healthy-looking portions of the plant may become stunted, flower production is extremely reduced, and the plant is extremely susceptible to frost damage (Epstein et al. 1999).

Stage 3 is an advancement in severity of all the symptoms described thus far. Leaves become even more reduced and distorted, apical growth is weak, and root development is extremely reduced (Epstein et al. 1999). Roses at this stage often succumb to winter kill even when conditions are relatively mild. Small plants often die

within a year of infection and mature multiflora may survive up to 5 years (Epstein et al. 1994).

1.5.2 History of Rose Rosette Disease in North America

Rose rosette was originally observed in the early 1940s in California, Wyoming, and Manitoba, Canada (Conners 1941; Thomas et al. 1953). Since then its spread through the midwestern, southern and eastern United States has been intertwined with the dissemination of one of its most susceptible hosts, *Rosa multiflora*. Multiflora rose is native to East Asia, but was introduced to North American gardens as early as the late 1700s (Amrine 2002). Its centuries-long use as a rootstock for ornamental roses is a testament to the species' robust quality and ease of naturalization. The plant truly found its footing, though, when it was marketed in the mid-1900s to combat soil erosion, form cheap and effective living fences, and provide shelter and food for wildlife. From the 1940s to the 1960s more than 34 million multiflora roses were planted in West Virginia and North Carolina alone (Amrine 2002). Multiflora's usefulness for each of the advertised purposes informs its success at establishing itself as an invasive species in much of the United States. It is currently considered a noxious weed in at least ten states and infests approximately 45 million acres in the eastern U.S. (Jesse et al. 2006).

The biological factors that contribute to multiflora's noxious nature can be put into two categories: propagation and vigor. Rosa multiflora reproduces not only by animal dispersal of hypanthia-encased achenes, but also by root suckering and the rooting of branch tips that often droop to make contact with soil (Hindal et al. 1988). Individual mature plants can produce as many as a half million readily germinating seeds in a single year, which persist such that seedlings can continue to sprout for decades after the mother plant is removed (Amrine 2002). In addition to its two asexual means of propagation, R. multiflora's dense and fibrous root system, rapid growth, and intimidating wealth of stiff, curved prickles produce what many have called "impenetrable thickets" that crowd out competing species in habitats ranging from pastures and forest edges to the borders of marshes (Amrine 2002). The geographic

range of *R. multiflora* in the United States is bounded on the North and South largely by winter temperatures: in the far North plants are unable to survive the winter cold while in the deep South seeds do not experience the stratification necessary for germination (Ochoa et al. 2016). Mechanical and chemical means of controlling the species have proven both expensive and ineffective, which led researchers to consider biological methods of reducing multiflora populations without acknowledging the extent of the risk posed to ornamental roses. RRV was intentionally introduced to stands of multiflora rose in many locations by means of grafting or the release of viruliferous mites (Amrine et al. 1988; Brown 1994; Doudrick 1984; Epstein et al. 1997; Hindal et al. 1988).

Throughout the last century, RRD's range has expanded concurrently with the distribution of its susceptible host. Apart from infesting huge tracts of land, multiflora rose functions as the primary repository for rose rosette virus and its eriophyid mite vector. Enumerating the many challenges associated with multiflora rose control may seem tangential when discussing rose rosette disease, but every trait that contributes to the success of the virus' primary weed host also contributes to the success of the virus. In fact, decades of failed attempts to eradicate wild multiflora stands suggest that a focus on resistance breeding is the only reasonable approach to mitigating the impact of the RRD.

While widely distributed multiflora rose facilitated the spread of RRD across the country, another susceptible rose may be largely responsible for bringing the disease into view of the general public. Knock Out® roses were introduced in 2000 and quickly became the most popular series of roses in the U.S. because of their low maintenance, continuous blooming, and disease resistance (Babu et al. 2014). They were planted as groups or in hedges across the country, replacing other popular shrubs in the process. Because they tend to be found in mass plantings, RRD infections can spread quickly in an area and as such are more apparent to passersby. As a result, even though Knock Out® roses are no more susceptible than other garden roses, they are reported as having RRD at a higher rate (Windham et al. 2014).

1.5.3 Geographic Distribution

Rose rosette disease was first described on roses in Manitoba, Canada in 1940, but symptoms were also reported from Wyoming and California in 1941 (Conners 1941; Thomas et al. 1953). By 1961 it had been spotted in Nebraska, by 1976 in Kansas, and by 1978 it was already widespread in Missouri (Crowe 1983; Viehmeyer 1961). Symptomatic roses were observed in Arkansas and Oklahoma by 1982 and since then the disease has been reported across the midwestern, eastern, and southern United States, including Iowa, Wisconsin, Illinois, Indiana, Ohio, Alabama, Pennsylvania, Texas, Maryland, Utah, and Florida (Amrine et al. 1988; Babu et al. 2014; Brown 1994; Crowe 1983; Doudrick 1984; Epstein et al. 1993; Hindal et al. 1988).

Until recently, RRD was only known to infect roses in North America, specifically in Canada and the United States; however, a study published in 2016 reported the isolation of RRV from twenty symptomatic roses in northeast India (Chakraborty et al. 2017). These roses were collected from two ornamental gardens in the Siliguri subdivision of West Bengal. Many details of this report remain unclear, namely how the virus made its way to India, if *P. fructiphilus* was found on these infected roses, and how much of the subcontinent is affected by the disease. It seems likely that RRD will continue to spread both nationally and internationally wherever there are large concentrations of roses and a climate that is suitable to *P. fructiphilus*.

1.5.4 Disease Management

Guidelines for managing rose rosette disease are fairly straightforward considering that the virus is systemic and incurable. Rose plantings should be scouted frequently during the growing season for symptoms and infected roses should immediately be bagged, removed, and disposed of away from other roses (Amrine et al. 1988; Windham et al. 2014). Local stands of multiflora should be controlled to eliminate sources of the virus and vector. Garden roses should be pruned in winter to remove overwintering mites and to avoid excessive tender growth that is attractive to eriophyids (Hoy 2013). For similar reasons, the use of nitrogenous fertilizers should be minimized. It is also

prudent to avoid using leafblowers in the garden to prevent the unnecessary dispersal of *P. fructiphilus*. Some horticulturists have suggested that pruning infected canes can save a rose from RRD if performed when symptoms first appear, but it's possible that the disappearance of symptoms is a manifestation of the temporary "reversion" that can occur after Stage 1 in disease development (Epstein et al. 1999; Windham et al. 2014). There is ongoing research to determine the effectiveness of various acaricides against *P. fructiphilus* and also to measure the impact of barriers in impeding the movement of eriophyid mites (M. Windham, *personal communication*). In general, chemical control measures often fail to significantly reduce eriophyid mite populations under field conditions (Aratchige et al. 2016; Ueckermann 2010).

Diagnosing RRD by its symptoms is highly effective, but our ability to control the disease necessitates the development of reliable early detection techniques. Reverse transcription polymerase-chain reaction assays are currently used to provide molecular confirmation of viral replication, but are expensive, time-consuming, and require symptomatic tissue (Babu et al. 2016; Dobhal et al. 2016). This is of little use when most growers don't have access to a suitable laboratory and when the time between infection and symptom development is long enough for roses to already have been sold, shipped, and planted.

REFERENCES

Ahn, K.K., K.S. Kim, R.C. Gergerich, S.G. Jensen, and E.J. Anderson (1996). "Comparative ultrastructure of double membrane-bound particles and inclusions associated with eriophyid mite-borne plant diseases of unknown etiology: a potentially new group of plant viruses". In: *Journal of Submicroscopic Cytology and Pathology* 28, pp. 345–355.

Allington, W.B., R. Staples, and G. Viehmeyer (1968). "Transmission of rose rosette virus by the eriophyid mite *Phyllcoptes fructiphilus*". In: *Journal of Economic Entomology* 61(5), pp. 1137–1140.

Amrine J.W., Jr. (1996). "*Phyllocoptes fructiphilus* and biological control of multiflora rose". In: *Eriophyid Mites - Their Biology, Natural Enemies and Control*. Ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin. Amsterdam: Elsevier, pp. 741–749.

Amrine J.W., Jr. (2002). "Multiflora rose". In: *Biological Control of Invasive Plants in the Eastern United States*. Ed. by R. Van Driesche, S. Lyon, B. Blossey, M. Hoddle, and R. Reardon. USDA Forest Service Publication FHTET-2002-04, pp. 265–292.

Amrine J.W., Jr., D.F. Hindal, T.A. Stasny, R.L. Williams, and C.C. Coffman (1988). "Transmission of the rose rosette disease agent to *Rosa multiflora* by *Phyllocoptes fructiphilus* (Acari: Eriophyidae)". In: *Entomological News* 99(5), pp. 239–252.

Aratchige, N.S., A.D.N.T. Kumara, and N.I. Suwandharthne (2016). "The coconut mite: Current global scenario". In: *Economic and Ecological Significance of Arthropods in Diversified Ecosystems*. Ed. by A.K. Chakravarthy and S. Sridhara. Springer Nature, pp. 321–342.

Babu, B., H. Dankers, and E. Newberry (2014). "First report of *Rose rosette virus* associated with rose rosette disease infecting knockout roses in Florida". In: *Plant Disease* 98(10), p. 1449.

Babu, B., A. Jeyaprakash, D. Jones, T.S. Schubert, C. Baker, B.K. Washburn, S.H. Miller, K. Poduch, G.W. Knox, F.M. Ochoa-Corona, and M.L. Paret (2016). "Development of a rapid, sensitive TaqMan real-time RT-PCR assay for the detection of *Rose rosette virus* using multiple gene targets". In: *Journal of Virological Methods* 235, pp. 41–50.

Bahari, S. (2015). "'Rose rosette is an epidemic, and North Texas is the epicenter'". In: *Star-Telegram*. URL: http://www.star-telegram.com/news/local/community/fort-worth/article27534709.html. Fort Worth, TX.

Epstein, A.H. and J.H. Hill, eds. (1994). *United States distribution of rose rosette disease*. Proceedings of the International Symposium: Rose Rosette and Other Eriophyid Mite-Transmitted Plant Disease Agents of Uncertain Etiology, pp. 53–56.

Cairns, T. (2003). "Horticultural classification schemes". In: *Encyclopedia of Rose Science*. Ed. by A.V. Roberts, T. Debener, and S. Gudin. Oxford: Elsevier, pp. 117–124.

Chakraborty, P., S. Das, B. Saha, A. Karmakar, D. Saha, and A. Saha (2017). "textitRose rosette virus: An emerging pathogen of garden roses in India". In: *Australasian Plant Pathology*. DOI: 10.1007/s13313-017-0479-y.

Conners, L. (1941). "Twentieth Annual Report of the Canadian Plant Report Survey 1940". In: p. 98.

Crowe, F.J. (1983). "Witches' broom of rose: A new outbreak in several central states". In: *Plant Disease* 67(5), pp. 544–546.

Debener, T. and D.H. Byrne (2014). "Disease resistance breeding in rose: Current status and potential of biotechnological tools". In: *Plant Science*. DOI: 10.1016/j.plantsci.2014.04.005.

Di Bello, P.L. (2015). "Understanding the causal agent of rose rosette disease". Master's Thesis. University of Arkansas.

Di Bello, P.L., T. Ho, and I.E. Tzanetakis (2015a). "Identification of the rose rosette disease agent". In: *Acta Horticulturae* 1064, pp. 295–298.

Di Bello, P.L., T. Ho, and I.E. Tzanetakis (2015b). "The evolution of emaraviruses is becoming more complex: Seven segments identified in the causal agent of rose rosette disease". In: *Virus Research* 210, pp. 241–244.

Dobhal, S., J.D. Olson, M. Arif, J.A. Garcia Suarez, and F.M. Ochoa-Corona (2016). "A simplified strategy for sensitive detection of *Rose rosette virus* compatible with three RT-PCR chemistries". In: *Journal of Virological Methods* 232, pp. 47–56.

Doudrick, R.L. (1984). "Etiological studies of rose rosette". Master's Thesis. University of Missouri.

Doudrick, R.L., W.R. Enns, M.F. Brown, and D.F. Millikan (1986). "Characteristics and role of the mite, *Phyllocoptes fructiphilus* (Acari: Eriophyidae) in the etiology of Rose rosette". In: *Entomological News* 97(4), pp. 163–168.

Druciarek, T., M. Kozak, M. Maroufpoor, and M. Lewandowski (2016a). "A new species in the genus Phyllocoptes Nalepa (Eriophyidae) from greenhouse roses in Poland". In: *Acarologia* 56(2), pp. 225–235.

Druciarek, T., M. Kozak, M. Maroufpoor, and M. Lewandowski (2016b). "Morphological variability of *Phyllocoptes adalius* female forms (Acari: Eriophyoidea), with a supplementary description of the species". In: *Systematic and Applied Acarology* 21(2), pp. 181–194.

Elbeaino, T., A. Whitfield, M. Sharma, and M. Digiaro (2013). "*Emaravirus*-specific degenerate PCR primers allowed the identification of partial RNA-dependent RNA polymerase sequences of *Maize red stripe virus* and *Pigeonpea sterility mosaic virus*". In: *Journal of Virological Methods* 188, pp. 37–40.

Epstein, A.H. and J.H. Hill (1993). "Rose Rosette disease". In: *Iowa State Univ Ext Publ No PM 1532* Ames, Iowa.

Epstein, A.H. and J.H. Hill (1994). "The biology of rose rosette disease: A mite-associated disease of uncertain aetiology". In: *Journal of Phytopathology* 143, pp. 353–360.

Epstein, A.H., J.H. Hill, and F.W. Nutter Jr. (1997). "Augmentation of rose rosette disease for biocontrol of multiflora rose (*Rosa multiflora*)". In: *Weed Science* 45, pp. 172–178.

Epstein, A.H. and J.H. Hill (1999). "Status of rose rosette disease as a biological control for multiflora rose". In: *Plant Disease* 83(2), pp. 92–101.

Floral emblem. Wikipedia. URL: https://en.wikipedia.org/wiki/Floral_emblem (visited on 02/12/2017).

Gergerich, R.C. and K.S. Kim (1983). "A particle of unique morphology associated with a disease of rose in northwest Arkansas". In: *Phytopathology* 73, pp. 500–501.

Goodish, K. (2013). *The Innovation Report*. Vineland Research and Innovation Centre.

Guoliang, W. (2003). "Ancient Chinese roses". In: *Encyclopedia of Rose Science*. Ed. by A.V. Roberts, T. Debener, and S. Gudin. Oxford: Elsevier, pp. 387–394.

Hassan, M., P.L. Di Bello, K.E. Keller, R.R. Martin, S. Sabanadzovic, and I.E. Tzanetakis (2017). "A new, widespread emaravirus discovered in blackberry". In: *Virus Research* 235, pp. 1–5.

Hindal, D.F. and S.M. Wong (1988). "Potential Biocontrol of multiflora rose, *Rosa multiflora*". In: *Weed Technology* 2(2), pp. 122–131.

Hoy, M. (2013). *Eriophyid mite vector of rose rosette disease (RRD) Phyllocoptes fructiphilus Keifer (Arachnida: Acari: Eriophyidae)*. University of Florida IFAS Extension.

Jesse, L.C., K.A. Moloney, and J.J. Obrycki (2006). "Abundance of arthropods on the branch tips of the invasive plant, *Rosa multiflora* (Rosaceae)". In: *Weed Biology and Management* 6, pp. 204–211.

Kassar, A. and J.W. Amrine Jr. (1990). "Rearing and development of *Phyllocoptes fructiphilus* (Acari: Eriophyidae)". In: *Entomological news*, pp. 276–282.

Keifer, H.H., E.W. Baker, T. Kono, M. Delfinado, and W.E. Styer (1982). *An Illustrated Guide to Plant Abnormalities Caused by Eriophyid Mites in North America*. Agricultural Handbook Number 573. Washington, D.C.: USDA Agricultural Research Service.

Epstein, A.H. and J.H. Hill, eds. (1995). *Double membrane-bound virus-like particles associated with rose rosette and some other diseases transmitted by eriophyid mites*. Proceedings of the International Symposium: Rose Rosette and Other Eriophyid Mite-Transmitted Plant Disease Agents of Uncertain Etiology, pp. 34–41.

Krussmann, G. (1981). *The complete book of roses*. Portland, OR: Timber Press.

Laney, A.G., K.E. Keller, R.R. Martin, and I.E. Tzanetakis (2011). "A discovery 70 years in the making: Characterization of the *Rose rosette virus*". In: *Journal of General Virology* 92, pp. 1727–1732.

Manson, D.C.M. and G.N. Oldfield (1996). "Life forms, deuterogyny, diapause and seasonal development". In: *Eriophyid Mites - Their Biology, Natural Enemies and Control*. Ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin. Amsterdam: Elsevier, pp. 173–183.

Michalska, K., A. Skoracka, D. Navia, and J.W. Amrine (2010). "Behavioural studies on eriophyoid mites: an overview". In: *Experimental Applied Acarology* 51, pp. 31–59.

Mielke-Ehret, N. and H.P. Muhlbach (2012). "*Emaravirus*: A novel genus of multipartite, negative strand RNA plant viruses". In: *Viruses* 4, pp. 1515–1536.

Navia, D., R. Ochoa, C. Welbourn, and F. Ferragut (2010). "Adventive eriophyoid mites: a global review of their impact, pathways, prevention and challenges". In: *Experimental Applied Acarology* 51, pp. 225–255.

Current knowledge of eriophyid mites associated with roses in the United States (2016). Combating Rose Rosette Disease SCRI Project Review.

Owens, S. (2011). *Restoration of the Cranford Rose Garden*. Brooklyn Botanic Garden. URL: http://www.bbg.org/news/restoration_cranford.

Proclamation No. 5574 (1986). *Designation of the Rose as the National Floral Emblem of the United States of America*. Code of Federal Regulations 101.

Rohozinski, J., A.H. Epstein, and J.H. Hill (2001). "Probable mechanical transmission of a virus-like agent from rose rosette disease-infected multiflora rose to *Nicotiana* species". In: *Association of Applied Biologists* 138, pp. 181–186.

Senate Joint Resolution 159 (1986). *A joint resolution to designate the rose as the national floral emblem*. 132 Congressional Record 25390.

Silvestro, S.R. and G.B. Chapman (2004). "A transmission electron microscope study of "New Dawn" climber rose (*Rosa wichuriana x safrano*) exhibiting rose rosette disease". In: *Plant Cell Reports* 23, pp. 345–351.

Slykhuis, J.T. (1980). "Mites". In: *Vectors of Plant Pathogens*. Ed. by K.F. Harris and K. Maramorosch. N.Y.: Academic Press, pp. 325–356.

Tatineni, S., A.J. McMechan, E.N. Wosula, S.N. Wegulo, R.A. Graybosch, R. French, and G.L. Hein (2014). "An eriophyid mite-transmitted plant virus contain eight genomic RNA segments with unusual heterogeneity in the nucleocapsid protein". In: *Journal of Virology* 88(20), pp. 11834–11845.

Thomas, H.E. and C.E. Scott (1953). "Rosette of rose". In: *Phytopathology* 43, pp. 218–219.

Ueckermann, E.A., ed. (2010). *Eriophyoid Mites: Progress and Prognoses*. Springer.

Viehmeyer, G. (1961). "A "new" disease of roses". In: *American Rose Annual*, pp. 98–101.

Vukosavljev, M., J. Zhang, G.D. Esselink, W.P.C. van 't Westende, P. Cox, R.G.F. Visser, P. Arens, and M.J.M. Smulders (2013). "Genetic diversity and differentiation in roses: A garden rose perspective". In: *Scientia Horticulturae* 162, pp. 320–332.

Whitaker, V.W. and S.C. Hokanson (2009). "Breeding roses for disease resistance". In: *Plant Breeding Reviews* 31, pp. 277–324.

Windham, M., A. Windham, F. Hale, and J. Amrine Jr. (2014). *Observations on rose rosette disease*. The University of Tennessee Institute of Agriculture.

Zlesac, D.C. (2009). "Pollen diameter and guard cell length as predictors of ploidy in diverse rose cultivars, species, and breeding lines". In: *Floriculture and Ornamental Biotechnology* 3, pp. 53–70.

Chapter 2

SCREENING OF *ROSA* GERMPLASM FOR RESISTANCE TO ROSE ROSETTE DISEASE UNDER FIELD CONDITIONS

2.1 Introduction

Identifying new sources of resistance to rose rosette disease (RRD) is particularly important because RRD cannot be treated or cured and because the virus can currently only be detected symptomatic tissue, which poses significant challenges to preventing the spread of the disease (Babu et al. 2016; Dobhal et al. 2016. Roses infected by rose rosette Emaravirus (RRV) typically die within three to five years and the only universally recommended management protocol for RRD is to remove and destroy infected plants. Rose rosette disease is also difficult to prevent, especially in areas with high disease pressure, because the eriophyid mite vector's small size and concealed habits allow it to elude most pest control practices (Epstein et al. 1999; Hoy 2013). Additionally, landscape roses are a high-value ornamental with a North American retail market approaching one billion dollars in value, making them a financially worthwhile candidate for breeding strategies such as marker assisted breeding (Goodish 2013). Since marker assisted breeding takes advantage of quantitative trait loci, roses that display some level of RRV tolerance that is less than that of complete resistance could also be used for breeding. This tolerance might take the form of milder symptoms or extended survival despite infection. However, tolerance is difficult to characterize because symptoms of RRD vary significantly among different individuals and developmental stages of a single genotype. Therefore this thesis focuses on identifying sources of resistance rather than tolerance.

Resistance to RRD has been subdivided into two main categories: resistance to rose rosette virus and resistance to the vector, *Phyllocoptes fructiphilus*. The former

implies some mechanism by which the virus cannot replicate inside its host rose, thereby preventing the accrual of a viral load large enough to produce symptoms or pass to new vectors. The latter, resistance to the vector, is not well-understood but may involve morphological characteristics such as bud structure, cuticle thickness, leaf abscission, hormonal responses, and the density and location of glandular hairs (Epstein et al. 1999; Sabelis et al. 1996). Distinguishing between the two types of resistance involves grafting vector-free infected buds onto presumed-resistant roses; if symptoms can be produced through grafting but not in field trials, the rose is likely resistant to *P. fructiphilus*. In Pigeon pea sterility mosaic virus (PPSMV), another *Emaravirus* closely related to RRV and also vectored by an eriophyid mite, it was determined that durable resistance and disease management could be accomplished in genotypes that were resistant to the virus, mite, or both (Jones et al. 2004). This indicates that any form of resistance we can find among roses is worth validating and pursuing through breeding.

Rose rosette disease has been found in every class of hybrid rose, including hybrid teas, floribundas, grandifloras, miniatures, and climbers. It has also been documented in antique roses as well as a number of species roses (Szyndel 2003). The only reported resistance is among a handful of species, mostly native to the United States, including *Rosa californica*, *R. carolina*, *R. palustris*, *R. setigera*, and *R. spinosissima*. Additionally, *R. bracteata* has shown to be resistant to the mite vector (Amrine 2002). For a number of these species, replication trials are still necessary to confirm resistance. Resistance trials that took place prior to the last few years did not benefit from molecular or serological diagnostic assays such as RT-PCR in order to confirm presence of the virus in symptomatic tissue. However, a study published in 2011 showed that symptomatology is a valuable stand-in for molecular assays, with the virus being detected in 100% of 84 roses exhibiting typical disease symptoms (Laney et al. 2011). Regardless, there is a strong need for resistance data that is supported by modern molecular techniques, and the use of a high-quality diagnostic assay in this study sets it apart from those conducted in the past.

Only one study, which is currently awaiting peer-review, provides molecular

confirmation of resistance data. It used both mite transmission and grafting to screen 20 rose genotypes, eventually finding only one cultivar that did not become infected over the course of the analysis (Di Bello 2015). This cultivar, 'Stormy Weather' was included in the resistance screening described in this thesis and can no longer claim to be completely resistant to the virus. Unfortunately, these trials have only identified resistance in rose species that aren't commonly used in breeding. This makes introgression of resistance genes into commercial germplasm a greater challenge.

A small number of the genotypes included in this screening have previously-published resistance data available. Specifically, 20 genotypes (see Table 2.1) of the 151 screened in this study were classified as susceptible, resistant to the virus, or resistant to the vector prior to this trial (Amrine 2002, Di Bello 2015). The classification of 'Bonica' as resistant to *P. fructiphilus* was upheld in a few studies until just recently, when Di Bello was able to produce symptoms in the cultivar using both mite transmission and grafting (Epstein et al. 1997, Di Bello 2015). This raises an important point: resistance data is often not consistent across studies. The genotypes that fail to produce symptoms in this screening require further evaluation to confirm the results and to determine the type of resistance. Many of these will likely prove to be susceptible with more time and more devoted attention to thorough inoculation. It is also possible that genetic diversity in either the virus or the vector creates variability in their virulence, or that co-infection with other rose viruses affects the pathogenicity of RRV.

2.2 Materials and Methods

A total of 151 rose genotypes were included in the screening, with 144 genotypes planted in May 2015, 7 planted in June 2016, and 65 planted in May 2017. Rose material was received from Texas A&M University, UC Davis and nurseries across the United States, including Star Roses and Plants, David Austin Roses, Bailey Nurseries, Greenheart Farms, Antique Rose Emporium, Tyler Francis Roses and any roses received in advance of the planting date were potted and maintained in a greenhouse.

Table 2.1: Genotypes in this study that have existing resistance data

***Rosa* species or Cultivar**	**Location**	**Source**
Susceptible		
American Pillar	Alabama	Amrine 2002
Belinda's Dream	Arkansas	Di Bello 2015
Bonica	Arkansas	Di Bello 2015
Dr. Huey	Tennessee	Amrine 2002
Francis Meilland	Arkansas	Di Bello 2015
Iceberg	Arkansas	Di Bello 2015
Julia Child	Arkansas	Di Bello 2015
The Knock Out Rose	Arkansas	Di Bello 2015
Marmalade Skies	Arkansas	Di Bello 2015
Mermaid	Alabama	Amrine 2002
Mr. Lincoln	Missouri	Amrine 2002
Old Blush	Alabama	Amrine 2002
Queen Elizabeth	Arkansas	Di Bello 2015
Rosa odorata	California	Amrine 2002
Rosa soulieana	Nebraska	Amrine 2002
Rosa woodsii	California	Amrine 2002
Resistant to Vector		
Bonica	Iowa	Amrine 2002
Resistant to Virus		
Stormy Weather	Arkansas	Di Bello 2015
Rosa arkansana	Iowa	Amrine 2002
Rosa palustris	West Virginia	Amrine 2002
Rosa setigera	West Virginia, Iowa	Amrine 2002

The trial was conducted at the Agricultural Experiment Station of the University of Delaware's College of Agriculture and Natural Resources in Newark, Delaware. The field was organized using a randomized block design with three replications. In some cases, only one or two roses were available to plant in the field rather than three and as a result the randomized blocks were not complete. Similarly, some rose genotypes were accessioned twice because they were received from multiple suppliers. These genotypes (e.g. 'Carefree Delight') have six copies present in the field, with 2 copies in each block. Each of the three replicates consisted of 5-6 rows with 8 foot spacing between rows. Within rows, roses were planted with 3 foot spacing between each plant.

This design allowed the canes of adjacent roses to touch each other as the plants matured, producing additional routes for mite movement (Michalska et al. 2010). The field was located next to existing stands of RRD-infected *Rosa multiflora*, and lines of additional multiflora roses were planted along the east and west edges of the field. Supplemental watering was provided to the roses for a few weeks after initial planting, but not thereafter. The location received full sun and good air circulation, which are associated with increased incidence and faster symptom development (Epstein et al. 1997). Roses were planted by hand and kept weeded, mulched, and pruned during the growing season to encourage the growth of soft tissue, which is required by the eriophyid mite vector for feeding and reproduction. Pruned branches were left in the rows.

Augmentation – a term borrowed from biological control that refers to the release of a large population of natural enemies – was used to intensify natural disease pressure in the field by introducing large numbers of viruliferous mites directly to the roses being screened. In this trial, augmentation consisted of twist-tying freshly cut symptomatic shoots from RRD-infected multiflora roses to actively growing shoot tips of a target rose. Rose rosette symptoms are a strong indicator of the presence of viruliferous mites on rose shoot tips, so augmentation is expected to artificially enhance vector populations. This strategy has proven effective at significantly increasing the rate of disease spread compared to nonaugmented plots (Amrine et al. 1988; Epstein et al. 1999). In past studies researchers often augmented roses by grafting symptomatic buds or shoots to the target roses, but this was determined to be unnecessarily time-consuming considering the vector's natural efficiency (Epstein et al. 1997). *Rosa multiflora* material used for augmentation typically ranged from 5 to 15cm in length, showed characteristic symptoms of rose rosette disease, and was collected within the state of Delaware. Throughout the course of the screening thus far (between May 2015 and December 2016), augmentation took place eight times: June 2015, September 2015, June 2016, July 2016, September 2016, June 2017, July 2017, and September 2017. The entirety of the screening field was augmented each time, with at least one

Figure 2.1: Aerial view of field used for resistance screening of rose germplasm with overlay showing organization of field into three replicates

symptomatic shoot being tied to each rose.

To gauge the levels of eriophyid mite populations, mite enumerations were performed in August 2015 and August 2016. The enumerations were conducted as follows: 15 plants were randomly chosen from each of the three blocks to have two shoot tips examined for the presence of eriophyid mites. This produced a total of 90 samples from 45 plants. With each of the 90 samples, the five terminal leaf axils were examined under a dissecting scope and any visible eriophyid mites were counted. Other techniques for counting eriophyid mites were explored which involved submerging rose tissue in a wash solution, which is then run through a set of sieves and rinsed into a counting plate (de Lillo 2001, Jesse et al. 2006). These methods were considered to be unnecessarily time consuming for our purposes.

All roses that displayed symptoms of RRD or any type of abnormal growth were tested for presence of the virus using the two-step endpoint RT-PCR assay described by Dobhal *et al.* using the RRV2 primer pair (Dobhal et al. 2016). Samples of leaf tissue were either processed while fresh or kept frozen at -80°C until processing could occur. Approximately 100mg of symptomatic leaf tissue from each rose was submerged in liquid nitrogen and agitated in a Mini-Beadbeater for 40 seconds. Total RNA was extracted using Qiagen's RNeasy Plant Mini Kit and immediately used in first-strand cDNA synthesis. PCR amplification was performed the same day and products were electrophoresed on 2% agarose. The full diagnostic protocol is detailed in Appendix B.

2.3 Results and Discussion

The roses were evaluated for symptoms nine times over the course of the study with the first symptoms sighted in the field in August 2015, only three months after the plot was established. A total of 94 of the 216 genotypes included in the screening have developed rose rosette disease. This leaves 122 genotypes (see Table 2.2) that have thus far failed to show symptoms of RRD. There are four genotypes - 'American Pillar', 'Mr. Lincoln', 'Old Blush', and *Rosa woodsii* - that are known to be susceptible based on previous resistance screenings but have not yet developed symptoms. This

suggests that additional genotypes will reveal themselves to be susceptible over time. Two other genotypes produced results that contradict previously published resistance data. 'Bonica' was considered to be resistant to *P. fructiphilus* until recently, and this study confirms that 'Bonica' is indeed susceptible to both *P. fructiphilus* and rose rosette virus (Di Bello 2015). 'Stormy Weather' was very recently heralded as a promising source of resistance, but this study contradicts that claim (Di Bello 2015).

Results from the mite enumerations conducted in August 2015 and August 2016 indicate that there was a dramatic increase in the field eriophyid mite population within the first year after planting (see Figure 2.2). In August 2015, 3 months after the rose screening field was established, only 5 of the 90 samples had eriophyid mites present, with mite numbers ranging from 1 to 23 per sample. A total of 55 eriophyid mites were counted on all five samples. A year later in August 2016, 37 of the 90 samples contained eriophyid mites and a total of 503 mites were counted on those 37 samples. In the absence of a control field, these numbers can't be used to draw conclusions about the effectiveness of our inoculation methods. However, the purpose of counting mites was to demonstrate that the field used for screening rose germplasm was supporting a substantial population of the vector.

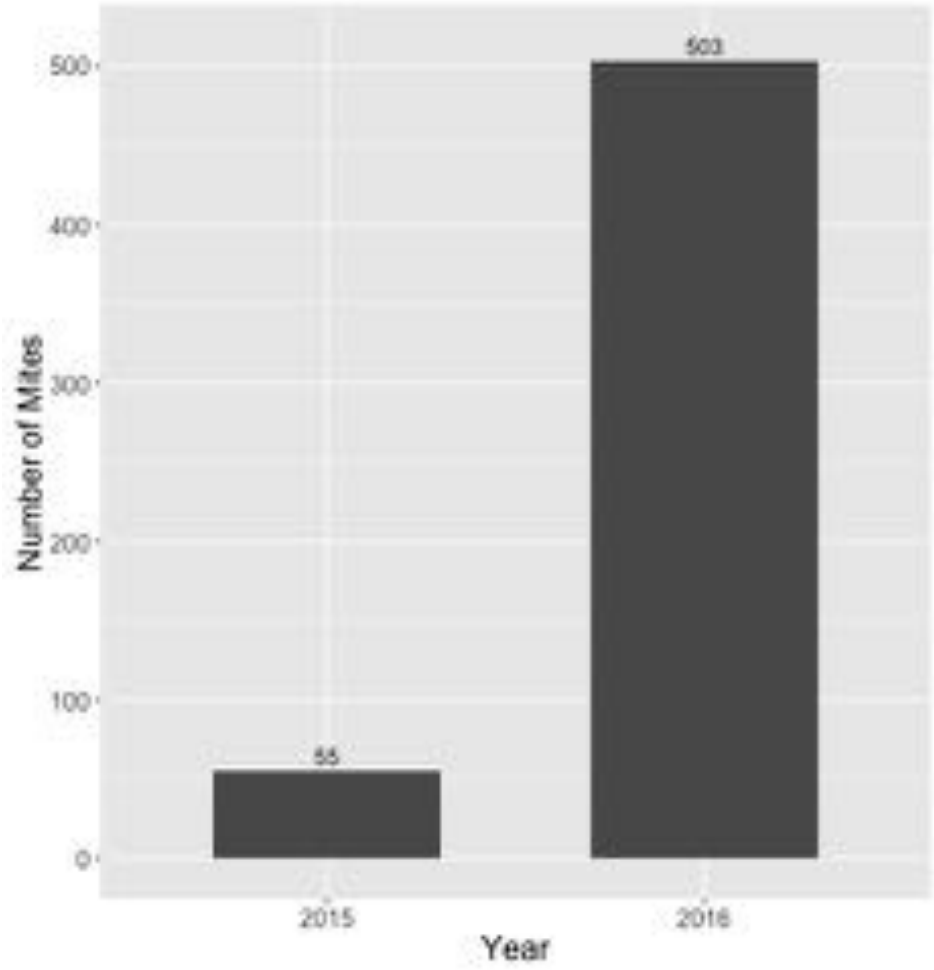


Figure 2.2: Mite enumerations were performed in August 2015 and August 2016 to gauge the level of eriophyid mite populations present in the screening field. Approximately a ten-fold increase was seen between years.

Table 2.2: Results from resistance screening for rose rosette disease, including the number of plants infected and number planted in the field.

Rosa Cultivar or Species	Number Infected	Number Planted
10FA3	0	1
10FA7	0	1
11FA6	0	1
11FA7	0	1
12FA6	0	1
13FA7	0	1
14FA7	0	1
15003-N001	0	1
15003-N002	0	1
15003-N003	0	1
15021-N001	0	1
15045-N002	0	1
15FA3	0	1
15FA7	0	1
16FA3	0	1
195-95	0	3
1FA2	0	1
1FA3	0	1
1FA5	0	1
1FA6	0	1
1FA9	0	1
2-30-07	0	3
201-98-A	0	3
262-97-4	1	3
2FA2	0	1
2FA7	0	1
3FA2	0	1
3FA3	0	1
3FA6	0	1
3FA7	0	1
3FA8	0	1
3FA9	0	1
4-48-07	0	3
4FA2	0	1
4FA3	0	1

Table 2.2: Continued

Rosa Cultivar or Species	Number Infected	Number Planted
4FA5	0	1
4FA7	0	1
5FA5	0	1
5FA7	0	1
6-91-9	0	3
66-84-18	0	3
6FA3	0	1
6FA7	0	1
7FA3	0	1
7FA7	0	1
89-1	1	1
8FA3	0	1
8FA5	0	1
8FA7	0	1
90-1C	0	1
90-82	0	2
9FA3	0	1
9FA6	0	1
Abbaye de Cluny	1	3
Adobe Sunrise	1	3
Amber gem	1	3
American Pillar	3	3
Apricot Nectar	0	3
Basye's Blueberry	0	3
Basye's Purple	0	3
Belinda's Dream	1	3
Betty Prior	0	2
Bonica	2	3
Brite Eyes	0	3
Caldwell Pink	0	3
Carefree Beauty	3	3
Carefree Celebration	2	3
Carefree Delight	4	6
Carefree Sunshine	2	3
Carefree Wonder	1	6

Table 2.2: Continued

Rosa Cultivar or Species	Number Infected	Number Planted
Champlain	4	6
Champneys Pink Noisette	1	3
Charisma	3	6
Cherry Parfait	0	3
Chuckles	0	3
CK25	1	3
Coral Drift	2	3
Day Breaker	0	3
De La Grifferaie	1	3
Dee-lish	1	3
Desmond Tutu	3	3
Doubleloons	0	3
Dr. Huey	2	3
Dream Come True	1	3
Ducher	2	3
E02-15-4	2	2
E02-17-3	2	2
Easy Elegance Calypso	3	3
Easy Elegance Como Park	0	3
Easy Elegance High Voltage	0	3
Easy Elegance Kashmir	3	3
Easy Elegance Kiss Me	0	3
Easy Elegance My Girl	2	3
Easy Elegance Mystic Fairytale	0	3
Easy Elegance Screaming Neon Red	0	3
Easy Elegance Yellow Brick Road	0	3
Easy Elegance Yellow Submarine	0	3
EE1130	0	1
EE1139-N002	0	1
Electron	0	3
Elle	1	3
Eyeconic Melon Lemonade	1	3
Fair Molly	0	3
Fame	0	3
Fire Meidiland	2	3

Table 2.2: Continued

Rosa Cultivar or Species	Number Infected	Number Planted
First Editions Above and Beyond	0	3
Fortuniana	2	3
Francis Meilland	2	3
Frau Dagmar Hartopp	0	3
Fuzzy Wuzzy Red	0	3
G02-2-1	0	3
GNIS	1	3
Golden Fairy Tale	1	3
Gypsy	1	3
Hot Cocoa	2	3
I03-4-5	3	3
Iceberg	3	6
Intrigue	1	6
J. P. Connell	1	3
J06-20-14-3	2	3
John Cabot	2	3
John Davis	0	3
Joseph's Coat	1	3
Julia Child	2	3
Kordes Perfecta	0	3
Korsteimm	3	3
La Marne	3	3
Lady of Shalott	0	3
Laev 17-10	3	3
Lafter	0	3
Limoncello	3	3
Linda Campbell	2	3
Little Buckaroo	0	3
Love	0	3
M4-4	0	3
Manetti	0	1
Marmalade Skies	1	3
Mermaid	2	3
Mevrouw Nathalie Nypels	0	3
Michelangelo	0	3

Table 2.2: Continued

Rosa Cultivar or Species	Number Infected	Number Planted
Miracle on the Hudson	1	3
Moore's Striped Rugosa	0	3
Morden Blush	0	3
Morden Centennial	0	3
Morden Fireglow	0	3
MORsoucrest	2	3
Mr. Lincoln	0	2
Nearly Wild	4	6
Nicole Carol Miller	0	3
Old Blush	0	3
ORA 050.07	3	3
ORA 295.08	2	3
Oso Easy Cherry Pie	1	2
Oso Easy Double Red	1	2
Oso Easy Fragrant Spreader	2	2
Oso Easy Honey Bun	2	2
Oso Easy Italian Ice	2	2
Oso Easy Lemon Zest	2	2
Oso Easy Mango Cream	2	2
Oso Easy Mango Salsa	2	2
Oso Easy Paprika	2	2
Oso Easy Pink Cupcake	1	2
Oso Happy Candy Oh	2	2
Oso Happy Petite Pink	2	2
Oso Happy Smoothie	2	3
Papa Hemeray	0	3
Peter Mayle	0	3
Poseidon	1	3
Purple Pavement	0	3
Queen Elizabeth	1	3
Raspberry Kiss	1	3
Red Drift	1	3
Rosa arkansana - ForestFarm	0	3
Rosa bracteata - RM	0	3
Rosa carolina - ForestFarm	0	3

Table 2.2: Continued

Rosa Cultivar or Species	Number Infected	Number Planted
Rosa folialosa - ARE	0	3
Rosa odorata	3	3
Rosa palustris	0	3
Rosa palustris - EVBLG	0	3
Rosa roxburghii - ARE	3	3
Rosa rugosa	0	3
Rosa rugosa alba	0	3
Rosa rugosa alba - RVR	0	3
Rosa setigera - ForestFarm	0	3
Rosa soulieana	3	3
Rosa virginiana - ForestFarm	0	3
Rosa wichuraiana - thornless	0	3
Rosa wichuraiana poterifolia	1	3
Rosa woodsii	0	3
Rosarium Uetersen	3	3
Sally Holmes	2	3
Sevillana	3	3
Sir Thomas Lipton	0	3
Skylark	0	3
Sophy's Rose	1	3
Sorcerer	0	3
St. Patrick	0	2
Star Delight	0	3
Stormy Weather	1	3
Strawberry Hill	1	3
Tahitian Treasure	3	3
Tamango	0	2
Teasing Georgia	1	3
Tequila	3	3
Tequila Sunrise	0	3
The Endeavor	0	3
The Knock Out Rose	1	3
The Sunny Knock Out	0	3
Therese Bugnet	0	3
Tournament of Roses	1	3

Table 2.2: Continued

Rosa Cultivar or Species	Number Infected	Number Planted
Traviata	0	2
Westerland	2	3
Windermere	2	3
Winner's Circle	2	3
Winnipeg Parks	0	3
Zephirine Drouhin	2	3

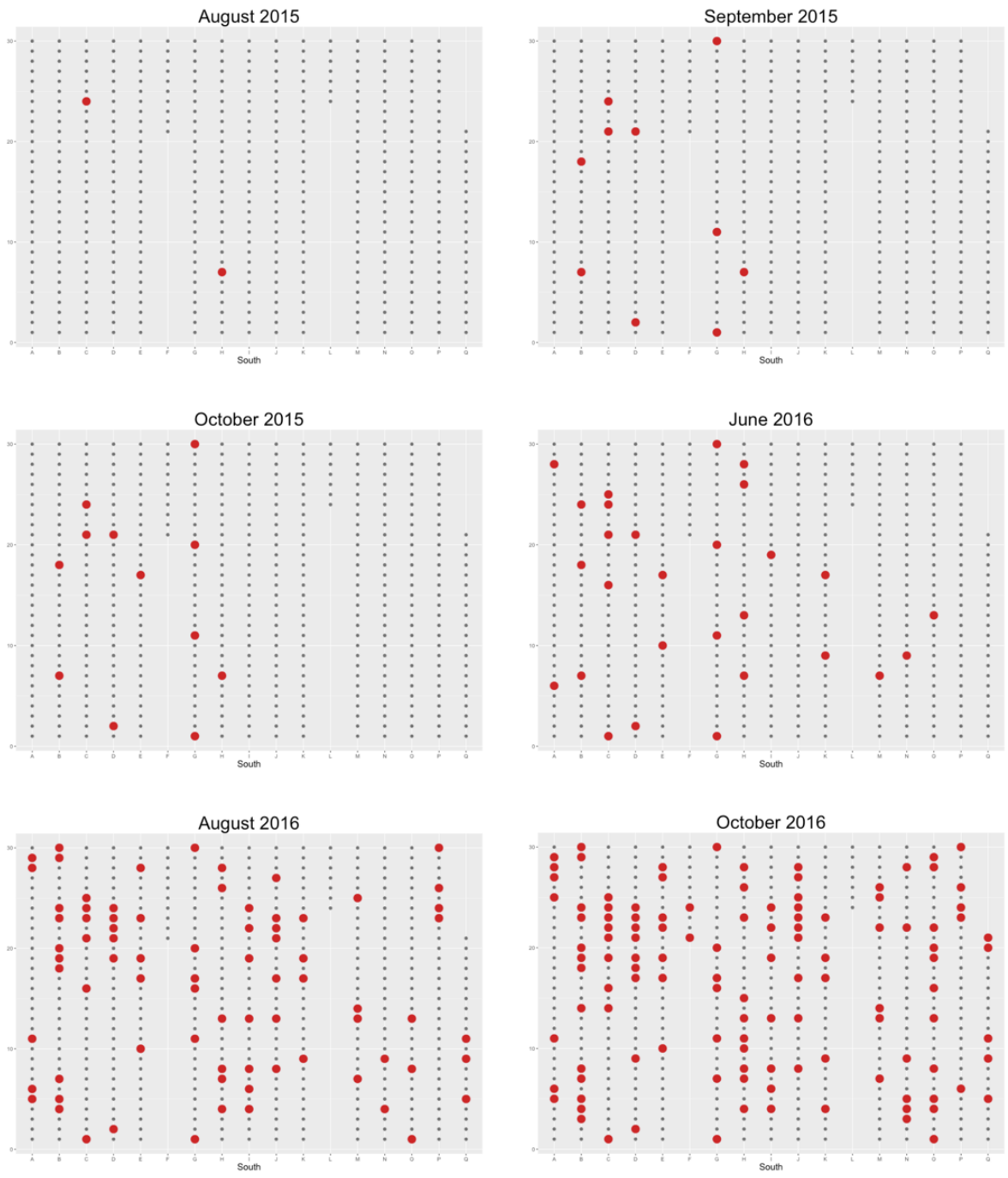


Figure 2.3: Depiction of an overhead view of the resistance trial field, with subplots showing the spread of the spread of disease between August 2015 and October 2016. Red dots represent roses that have tested positive via RT-PCR for the presence of rose rosette virus; grey dots represent roses that have not shown symptoms of RRD and/or have not tested positive for the virus.

2.3.1 Randomness Testing for Spread of Rose Rosette Emaravirus in Field

An ordinary run analysis was conducted to identify the pattern of disease spread in the field (Madden et al. 1982). This type of evaluation can inform the methods used in future resistance screenings by demonstrating whether or not the eriophyid mite vector, *P. fructiphilus*, is spreading from plant to plant by walking. A random pattern of infection would indicate that factors such as augmentation frequency and aerial dispersal of mites are important for disease spread. Aggregations of infection, on the other hand, would indicate that mites are largely moving from plant to plant within rows by walking. The formulas used to derive the values in Table 2.3 are below and additional information on the analysis can be found in the original article (Madden et al. 1982).

$$E(U) = 1 + 2m(N - m)/N$$

$$s_u = (2m(N - m)[2m(N - m) - N]/[N^2(N - 1)])^{1/2}$$

$$Z_u = [U + 0.5 - E(U)]/s_u$$

The rows within field replicates were adjacent, so they were combined for the analysis. Ordinary runs were calculated for each sampling date to show change over time. Sampling dates are defined here as the month and year when tissue was collected from newly (since the previous sampling date) symptomatic field plants, regardless of when the RT-PCR assay was performed on that tissue. The final sampling date in November 2017 therefore includes all resistance data collected over the course of this study. The data in Table 2.3 can be interpreted by examining the p-values in the right-most column. Using a significance threshold of $p = 0.0.5$, the null hypothesis of random RRD infection can only be rejected in the first replicate (Block 1) for the final three sample dates (August, October, and November 2016).

These results have a few different implications. To start, it is apparent that RRD infection in the field is largely random. Random infection may be due to mite movement on air currents, either within the field or from external vector reservoirs, or

Table 2.3: Ordinary run analysis (Madden et al. 1982)

Block 1							
Month	m	N	Observed	Expected	s_u	Z_u	p-value
2015 Aug.	1	149	3	2.99	0.12	4.46	0.999
2015 Sept.	6	149	13	12.52	0.90	1.09	0.862
2015 Oct.	7	149	15	14.34	1.05	1.10	0.864
2016 June	14	160	27	26.55	1.99	0.48	0.684
2016 Aug.	32	160	42	52.20	4.02	-2.41	0.008
2016 Oct.	47	160	52	67.39	5.23	-2.85	0.002
2016 Nov.	56	160	60	73.80	5.73	-2.32	0.010
Block 2							
Month	m	N	Observed	Expected	s_u	Z_u	p-value
2015 Aug.	1	146	3	2.99	0.12	4.42	0.999
2015 Sept.	4	146	8	8.78	0.60	-0.47	0.320
2015 Oct.	5	146	10	10.66	0.76	-0.21	0.417
2016 June	11	157	22	21.46	1.60	0.65	0.742
2016 Aug.	30	157	52	49.54	3.85	0.77	0.780
2016 Oct.	39	157	62	59.62	4.65	0.62	0.732
2016 Nov.	47	157	68	66.86	5.23	0.31	0.622
Block 3							
Month	m	N	Observed	Expected	s_u	Z_u	p-value
2015 Aug.	0	131	1	1.00	0.00	-	-
2015 Sept.	0	131	1	1.00	0.00	-	-
2015 Oct.	0	131	1	1.00	0.00	-	-
2016 June	3	141	7	6.87	0.45	1.39	0.918
2016 Aug.	16	141	29	29.37	2.35	0.06	0.524
2016 Oct.	33	141	48	51.55	4.23	-0.72	0.236
2016 Nov.	39	141	56	57.43	4.73	-0.20	0.421

due to the frequent, repeated augmentations. There is one previously ignored detail that provides evidence that augmentation is more important than independent mite movement. The rows of plants in this field are contiguous with those of another RRV screening field in which augmentation has been incomplete (not all roses were augmented) and inconsistent. That adjacent field has had very little rose rosette disease despite being planted in years earlier. Other factors such as shade and plant spacing may have contributed to this discrepancy, but augmentation frequency is the most glaring difference.

Another interesting implication of this analysis is temporal in nature. It's possible that the non-random, within-row spread of RRD in Block 1 occurred only after the plants had enough time to grow such that canes of adjacent plants could touch. This would explain why the effect isn't seen until the final three sample dates. The contradictory results among the three blocks might be manifestations of this same concept. For example, some type of field effect could have produced more inter-plant contact in Block 1 compared to the other two replicates. Block 1 is the only section of the field that borders a tree line rather than large swaths of grass meaning it receives more shade and consequently might have an entirely different microclimate. Also, this analysis was designed for use with a single cultivar or genotype rather than in a heterogeneous field. The results would be affected by the fact that the roses are different genotypes (with different levels of resistance), sizes, maturities, and had different planting dates. To sum up, the major conclusions of this analysis are as follows: (1) frequent augmentation is important for increasing the rate of infection and (2) more studies are needed to determine the effect of field design on the spread of disease.

2.4 Conclusions and Future Needs

Results from this study are presented with high confidence with regard to susceptible genotypes, but low confidence with regard to any potentially resistant genotypes. The use of a high quality RRV detection protocol (RT-PCR) to confirm presence of the virus in symptomatic tissue sets this study apart from others conducted in the past.

Unfortunately, we have seen that most genotypes will prove to be susceptible to RRD given enough time and this study has thus far only spanned two growing seasons. Not only is there a statistical likelihood that some susceptible roses might escape infection, but we also know that the virus can lie dormant in infected plants for years even after symptoms develop. Variation in RRV isolates and co-infection with other rose viruses might also contribute to inconsistent results from different resistance trials.

The screening field should continue to be monitored for disease as long as possible to continue narrowing down the list of potentially resistant genotypes. Assuming more sensitive detection techniques are developed, roses that do not show symptoms should also be tested for presence of the virus to determine if viral replication is somehow happening without characteristic symptom development. Any genotype that shows promise for resistance should undergo graft transmission studies to determine if this resistance is to the virus or to the vector.

REFERENCES

Amrine J.W., Jr. (2002). "Multiflora rose". In: *Biological Control of Invasive Plants in the Eastern United States*. Ed. by R. Van Driesche, S. Lyon, B. Blossey, M. Hoddle, and R. Reardon. USDA Forest Service Publication FHTET-2002-04, pp. 265–292.

Amrine J.W., Jr., D.F. Hindal, T.A. Stasny, R.L. Williams, and C.C. Coffman (1988). "Transmission of the rose rosette disease agent to *Rosa multiflora* by *Phyllocoptes fructiphilus* (Acari: Eriophyidae)". In: *Entomological News* 99(5), pp. 239–252.

Babu, B., A. Jeyaprakash, D. Jones, T.S. Schubert, C. Baker, B.K. Washburn, S.H. Miller, K. Poduch, G.W. Knox, F.M. Ochoa-Corona, and M.L. Paret (2016). "Development of a rapid, sensitive TaqMan real-time RT-PCR assay for the detection of *Rose rosette virus* using multiple gene targets". In: *Journal of Virological Methods* 235, pp. 41–50.

de Lillo, E. (2001). "A modified method for eriophyoid mite extraction (Acari: Eriophyoidea)". In: *International Journal of Acarology* 27(1), pp. 67–70.

Di Bello, P.L. (2015). "Understanding the causal agent of rose rosette disease". Master's Thesis. University of Arkansas.

Dobhal, S., J.D. Olson, M. Arif, J.A. Garcia Suarez, and F.M. Ochoa-Corona (2016). "A simplified strategy for sensitive detection of *Rose rosette virus* compatible with three RT-PCR chemistries". In: *Journal of Virological Methods* 232, pp. 47–56.

Epstein, A.H., J.H. Hill, and F.W. Nutter Jr. (1997). "Augmentation of rose rosette disease for biocontrol of multiflora rose (*Rosa multiflora*)". In: *Weed Science* 45, pp. 172–178.

Epstein, A.H. and J.H. Hill (1999). "Status of rose rosette disease as a biological control for multiflora rose". In: *Plant Disease* 83(2), pp. 92–101.

Goodish, K. (2013). *The Innovation Report*. Vineland Research and Innovation Centre.

Hoy, M. (2013). *Eriophyid mite vector of rose rosette disease (RRD) Phyllocoptes fructiphilus Keifer (Arachnida: Acari: Eriophyidae)*. University of Florida IFAS Extension.

Jesse, L.C., K.A. Moloney, and J.J. Obrycki (2006). "Abundance of arthropods on the branch tips of the invasive plant, *Rosa multiflora* (Rosaceae)". In: *Weed Biology and Management* 6, pp. 204–211.

Jones, A.T., P. Lava Kumar, K.B. Saxena, N.K. Kulkarni, V. Muniyappa, and F. Waliyar (2004). "Sterility mosaic disease - the "Green Plague" of pigeonpea". In: *Plant Disease* 88, pp. 436–445.

Laney, A.G., K.E. Keller, R.R. Martin, and I.E. Tzanetakis (2011). "A discovery 70 years in the making: Characterization of the *Rose rosette virus*". In: *Journal of General Virology* 92, pp. 1727–1732.

Madden, L.V., R. Louie, J.J. Abt, and J.K. Knoke (1982). "Evaluation of tests for randomness of infected plants". In: *Phytopathology* 72, pp. 195–198.

Michalska, K., A. Skoracka, D. Navia, and J.W. Amrine (2010). "Behavioural studies on eriophyoid mites: an overview". In: *Experimental Applied Acarology* 51, pp. 31–59.

Sabelis, M.W. and J.Bruin (1996). "Evolutionary ecology: Life history patterns, food plant choice and dispersal". In: *Eriophyid Mites - Their Biology, Natural Enemies and Control*. Ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin. Amsterdam: Elsevier, pp. 329–366.

Szyndel, M.S. (2003). "Viruses". In: *Encyclopedia of Rose Science*. Ed. by A.V. Roberts, T. Debener, and S. Gudin. Oxford: Elsevier, pp. 180–189.

Chapter 3

GRAFT AND MECHANICAL TRANSMISSION STUDIES OF ROSE ROSETTE VIRUS

3.1 Introduction

Various mechanical and graft transmission studies have been conducted for rose rosette virus, but in many cases their methods do not inspire confidence in their results. In 1988, a series of graft transmission studies were performed using infected rose material from Missouri, Kentucky, and Indiana as scions (Amrine et al. 1988). Grafts consisted of 3-5 mm long pieces of infected stem tissue, each including at least one vegetative bud, which were inspected and reported to be eriophyid mite-free. However, the rootstock used in this set of grafting studies consisted of rooted cuttings and transplants of field-grown multiflora from Morgantown, WV. These rootstock roses were deemed "healthy" in that they had not developed symptoms of RRD, but they could not be tested for presence of the virus because a suitable diagnostic procedure did not yet exist. Additionally, there is no indication that the rootstock was thoroughly examined for the presence of eriophyid mites before being introduced to the greenhouse where the study took place. There were no valid controls, which could have simply included rooted cuttings or transplants that did not receive grafted buds, described in the study. Each experiment resulted in a high rate of transmission (between 40 and 100%) with symptoms manifesting in as little as 41 days (Amrine et al. 1988). Unfortunately this is not the only oft-cited graft transmission study about rose rosette virus that lacks basic features of standard experimental design.

Epstein and Hill published a paper in 1994 describing a set of experiments that tested for transmission of RRD by graft, sap, soil, seed, dodder (*Cuscuta campestris*, *C. gronovi*, and *C. pentagona*), and spores of powdery mildew (*Sphaerotheca pannosa*)

(Epstein et al. 1994, 1999). The grafting study was carried out in the field and in the greenhouse between April and September using vegetative buds from symptomatic plant as scions and healthy-looking *Rosa multiflora* as rootstock. The symptomatic shoots from which the scion buds were taken had previously been sprayed to run-off with avermectin, an acaricide (Epstein et al. 1994). Some major issues with this study are readily apparent: (1) fields cannot be kept free of eriophyid mites and most greenhouses also aren't completely safe from vector contamination, (2) eriophyid mites hide under bud scales, so scions that contain buds are likely to also contain *Phyllocoptes fructiphilus*, (3) the effectiveness of acaricides on *P. fructiphilus* is only now being tested.

On the other hand, some grafting experiments used methods that should withstand the test of time but lack the confirmation provided by recently-developed diagnostic assays. An experiment performed in 1986 used internodal shields as grafting material, avoiding the risk of eriophyid mites lingering under bud scales (Doudrick et al. 1986). The rootstock consisted of healthy multiflora rose maintained in a separate greenhouse, monitored for the presence of mites, and kept on a rotation of acaricides. Additionally, three of the ten roses that had received grafts were thoroughly dissected and examined for evidence of the vector mite. Of all the studies on graft transmission of RRV published to date, this is the most convincing. All ten roses produced symptoms in 6-14 weeks post-graft and subsequent examinations showed no eggs, shed skins, or live individuals of *P. fructiphilus* (Allington et al. 1968).

Less attention has been paid to mechanical transmission of RRV. Mechanical transmission of the virus has not been known to occur in nature and the very structure of RRV suggests that it would also be difficult to produce in vitro. As a multipartite virus with seven components, the likelihood of inoculating healthy plants with a complete set of viral particles is low. The probability goes down when you consider the fact that single-stranded RNA particles are very unstable. Epstein and Hill attempted sap transmission from symptomatic multiflora to healthy multiflora using

various inoculation techniques including razor cuts, stab wounds, injections, and "ballistic inoculation" with an air pistol (Epstein et al. 1994). Of these methods, only stab inoculation was successful: two out of 120 plants subjected to multiple stab wounds through drops of sap inoculum developed symptoms. One plant showed characteristic symptoms of RRD. The second plant showed "slight reddening of veins of apical leaflets followed by die-back of the apical node" (Epstein et al. 1994). It is unclear if the symptoms displayed in this second plant were actually caused by RRD.

Other researchers have tried to mechanically inoculate standard indicator plants with the RRV and other emaraviruses (Mielke-Ehret et al. 2012; Rohozinski et al. 2001. Unlike previous studies, Rohozinski *et al.* used an inoculum made from the root tissue of multiflora rose to rub inoculate two species of tobacco, *Nicotiana glutinosa* and *N. benthaniana* (Rohozinski et al. 2001). It was believed that root tissue would provide a better source of intact nucleic acids than leaf or stem tissue because it has a lower content of tannins and polyphenols. Inoculum was prepared using root tips from both symptomatic and non-symptomatic roses. Some of the test plants inoculated with sap from symptomatic roses developed symptoms that included the following: pale green regions next to leaf veins, necrotic spots, and splitting along leaf veins. Light microscopy of inoculated *Nicotiana* showed abnormal development at the cellular level even in leaves showing no symptoms as well as what appeared to be membrane-bound virus-like particles (Rohozinski et al. 2001). It bears repeating, though, that this study was conducted before the development of a reliable RT-PCR protocol or serological test that could confirm replication of RRV in affected tissues.

This critique of past transmission studies is not to say that rose rosette virus cannot be transmitted by graft or mechanical means. Rather, it's a call for replications to confirm what we already suspect to be true, that RRV can effectively be transmitted by graft, and that mechanical transmission can be achieved under strict conditions in the lab but is not likely to occur in nature. The experiments described below are an attempt to replicate the methods used in past publications that are frequently cited as concrete evidence of graft and mechanical transmission.

3.2 Graft Transmission

3.2.1 Materials and Methods

Grafting took place in spring of 2016, a time of year when mite populations tend to be low. Two weeks prior to treatments, 35 certified virus-free multiflora roses were pruned to encourage new vegetative growth. On May 2nd, symptomatic shoots were harvested from local infected multiflora roses to serve as scions. Leaves from 10 randomly selected scions were frozen and later tested to confirm presence of the virus and all scions were kept in a refrigerator until the following day when grafting could take place. On May 3rd, the 35 rootstock multiflora roses were randomly divided into three experimental groups. Twenty-five of the roses would receive grafts consisting of a vegetative bud with a small supporting shield of cortical tissue. Each of these twenty-five roses received two buds, which were held in place with small strips of grafting tape. Another five roses received no grafts, but each had two buds affixed to their stems with twist-ties. The final five roses received no treatment. All 35 plants were sprayed and soil-drenched with a systemic acaricide, Kontos SC, after treatments and kept in a greenhouse for the duration of the study. Roses were monitored for symptom development on a weekly basis for the first three months. Thereafter, they were checked occasionally for any changes.

3.2.2 Results and Discussion

Within 48 hours of grafting, the grafted buds and shields were shrunken and wilted. None of the shields formed successful grafts with the rootstock. This is likely due to the fact that increased succulence is a symptom of rose rosette disease, and the symptomatic shoots harvested for scions were very soft. Additionally, the grafted roses were not kept under mist because many of the grafting studies that this experiment tried to replicate were not conducted in mist rooms. By August 2016, three months after grafting, none of the roses displayed any symptoms of rose rosette disease. By November, 9 of the 35 plants displayed characteristic symptoms of RRD. However, these 9 symptomatic roses included 2 members of the control groups that received

no grafts. Upon inspection under a microscope, eriophyid mites were found on the symptomatic tissue from multiple roses. These results indicate that the precautions taken to minimize the risk of contamination by *P. fructiphilus* (e.g. harvesting in early spring, drenching with an acaricide, keeping roses in a greenhouse) were insufficient and that any study using field-collected rose tissue for buds may contain vectors which cannot be detected or treated effectively by miticides.

3.2.3 Conclusions and Future Needs

The outcome of this experiment demonstrates one of the major difficulties of working with eriophyid mites, they are extremely small. This allows them to pass through insect screens, hide on very small pieces of plant tissue, and avoid most means of control. Future studies of this nature might benefit from being conducted in a growth chamber rather than a greenhouse, particularly in locations where RRD is well-established. Better handling of scions would also improve results. Removing all of the leaves from scions and washing stems in horticultural oil would help remove lingering eriophyid mites and eggs. Also, it might be sufficient to graft a small shield of cortical tissue instead of a vegetative bud with supporting shield (Allington et al. 1968). This would avoid the issue of *P. fructiphilus* hiding underneath bud scales. Multiple rounds of grafting accompanied by regular dissections of the tissue under a microscope to check for evidence of eriophyid mites would provide additional assurance that the plants are not infested with the vector.

A major problem with this study was that none of the grafts were actually successful, probably resulting from the extreme succulence of the infected scions. In the time since this experiment took place, it has been demonstrated that RRV is systemic and can be isolated even from the root tissue of infected plants. It is likely unnecessary to use the symptomatic tissue from infected plants as scions. Buds or cortical shields cut from hardier stems would be significantly more likely to form successful graft unions with the rootstock.

3.3 Mechanical Transmission

3.3.1 Materials and Methods

This mechanical transmission experiment was carried out twice, the first time on September 12th, 2016 and the second time on October 19th. Each time, three RT-PCR-confirmed RRV-infected roses were selected from the resistance trial field. The plants were uprooted using a digging fork and the smallest roots were harvested. The samples were rinsed with water and a small portion of each sample was frozen and later tested to confirm presence of the viral genome in the root tissue. Inoculum was prepared by combining samples from all three plants and grinding the roots with a buffer in a blender. Three buffers were used: (a) distilled water; (b) 0.01 M sodium phosphate buffer, pH 7.35 containing 5% 2-mercaptoethanol, 0.01 M (final concentration) ethylenediaminetetraacetic acid EDTA, and 200 micrograms/ml calcium bentonite (Louie et al. 2006); (c) 0.01 M sodium phosphate buffer, pH 7.35 containing 2 mg/ml ascorbic acid and 20 mM (final concentration) 2-mercaptoethanol (Epstein et al. 1994). The mixture was lightly rubbed with a sponge onto Nicotiana leaves dusted with 600 grit Carborundum. Each extract was applied to a total of 12 plants, constituting three each of four different *Nicotiana* species: *N. glutinosa*, *N. clevelandii*, *N. tabacum*, and *N. benthaniana*. Another set of 12 *Nicotiana* received sham inoculations using only the buffer. This control group was treated before the true inoculations took place in order to prevent contamination.

All plants were kept in a greenhouse and monitored for changes for three weeks. Leaf samples were taken from each experimental group to be tested for RRV. Samples were taken selectively from regions of abnormal growth. The second round of this experiment took place with the knowledge that our greenhouse was experiencing an outbreak of blue mold of tobacco, caused by *Peronospora hyoscyami f.sp. tabacina*. On October 24th, after it was apparent that the second set of Nicotiana were also showing symptoms of blue mold, they were sprayed with Ridomil Gold SL.

3.3.2 Results and Discussion

Eleven days post first inoculation (September 23rd), nearly all of the inoculated plants showed leaf mosaic and/or raised green islands. However, the raised green islands were later discovered to be accompanied by sporulation on the bottom of the leaf. Examination of the spores under a microscope revealed that the plants were infected with blue mold of tobacco, a type of downy mildew caused by *Peronospora hyoscyami f.sp. tabacina*. This discovery cut the experiment short because the plants had to be immediately disposed of, but before their disposal I was able to take samples from fully expanded leaves of seven plants to test the tissue for RRV. These seven plants showed the least severe symptoms of blue mold and included representatives of all three buffers and three of the four *Nicotiana* species and one of the seven plants was from the group that received the control inoculation. Electrophoresis of the RT-PCR products produced non-specific DNA smears for all samples, including the sample from the control group.

Plants from the second round of this experiment also developed blue mold, but the disease was largely controlled after the application of Ridomil Gold SL. On November 4th, samples were taken from all experimental groups (each buffer with each species of *Nicotiana*) as well as from an untreated *Nicotiana tabacum* to be tested for the presence of RRV. Results were the same as they were during the first iteration of the experiment. These non-specific DNA smears might be attributed to a variety of factors. The most likely is that the PCR protocol is optimized for rose tissue so the primers may be producing non-target amplification in the tobacco genome. Also, the end point RT-PCR assay may not be sensitive enough to detect the virus at a low titre.

The root tissue used for inoculum was also tested using RT-PCR. Between the two replications, roots from six roses were used. All three root samples from the first round tested positive for RRV. Only two of the three samples tested positive in the second round. This could have been a false negative result or a side effect of attempting to grind and process root tissue in the same manner as leaf tissue. Regardless, this shouldn't have affected the inoculations since all the root samples were combined before

being used in inoculum preparation.

3.3.3 Conclusions and Future Needs

While the graft transmission study described here was not particularly successful, it seems unnecessary to replicate it with improved methods because it has since become well established that RRV is graft transmitted (M. Paret, *personal communication*). On the other hand, mechanical transmission of RRV to either rose or tobacco is not well-studied. In fact, there has been very little research into mechanical transmission of most emaraviruses, possibly because they are all closely associated with eriophyid mite vectors (Mielke-Ehret et al. 2012). Mechanical transmission of RRV between roses does not seem to happen in nature and attempts to inoculate roses with viral extractions has not produced consistent successful results (Epstein et al. 1999. This study was unable to confirm the published research of Rohozinski *et al.*, in which they concluded that the presence of viral particles in inoculated *Nicotiana* were evidence of mechanical transmission of RRV (Rohozinski et al. 2001). It seems more likely that the symptoms they saw on inoculated plants were a result of physical damage. The particles seen under an electron microscope cannot conclusively be identified as RRV. However, diagnostic assays with greater sensitivity than the endpoint RT-PCR used in this study are currently being developed and they might be able to demonstrate for certain if mechanical transmission of RRV is possible. Additionally, future studies might benefit from methods to improve the stability of RRV in buffer. For example, the roots used for inoculum could be ground in liquid nitrogen before addition of the buffer to help reduce the effect of ribonucleases. In our particular location, it would benefit to conduct the study in a growth chamber or another location where the risk of contamination with blue mold is reduced.

REFERENCES

Allington, W.B., R. Staples, and G. Viehmeyer (1968). "Transmission of rose rosette virus by the eriophyid mite *Phyllcoptes fructiphilus*". In: *Journal of Economic Entomology* 61(5), pp. 1137–1140.

Amrine J.W., Jr., D.F. Hindal, T.A. Stasny, R.L. Williams, and C.C. Coffman (1988). "Transmission of the rose rosette disease agent to *Rosa multiflora* by *Phyllocoptes fructiphilus* (Acari: Eriophyidae)". In: *Entomological News* 99(5), pp. 239–252.

Doudrick, R.L., W.R. Enns, M.F. Brown, and D.F. Millikan (1986). "Characteristics and role of the mite, *Phyllocoptes fructiphilus* (Acari: Eriophyidae) in the etiology of Rose rosette". In: *Entomological News* 97(4), pp. 163–168.

Epstein, A.H. and J.H. Hill (1994). "The biology of rose rosette disease: A mite-associated disease of uncertain aetiology". In: *Journal of Phytopathology* 143, pp. 353–360.

Epstein, A.H. and J.H. Hill (1999). "Status of rose rosette disease as a biological control for multiflora rose". In: *Plant Disease* 83(2), pp. 92–101.

Louie, R., D.L. Seifers, and O.E. Bradfute (2006). "Isolation, transmission and purification of the *High plains virus*". In: *Journal of Virological Methods* 135, pp. 214–222.

Mielke-Ehret, N. and H.P. Muhlbach (2012). "*Emaravirus*: A novel genus of multipartite, negative strand RNA plant viruses". In: *Viruses* 4, pp. 1515–1536.

Rohozinski, J., A.H. Epstein, and J.H. Hill (2001). "Probable mechanical transmission of a virus-like agent from rose rosette disease-infected multiflora rose to *Nicotiana* species". In: *Association of Applied Biologists* 138, pp. 181–186.

Chapter 4

BIOLOGICAL CONTROL OF *PHYLLOCOPTES FRUCTIPHILUS* (ACARI: ERIOPHYIDAE) USING PREDATORY MITES FROM THE FAMILY PHYTOSEIIDAE (ACARINA: MESOSTIGMATA)

4.1 Introduction

Commercialization of roses that are resistant to RRD is a long-term goal for the rose industry. However, in the meantime, additional means of managing the disease should be investigated. One approach involves targeting the eriophyid mite vector associated with rose rosette virus. Currently, control of *Phyllocoptes fructiphilus* is achieved by eliminating disease reservoirs — local stands of *Rosa multiflora* — and by application of acaricides, although both of these methods have their drawbacks. Removal of nearby multiflora roses can be very effective, but in many cases it is nearly impossible to accomplish. Multiflora rose is an invasive species in much of the United States and has proven extremely resistant to eradication efforts (Amrine 2002). Acaricides, on the other hand, are relatively easy to use but vary widely in their effectiveness against eriophyids (Aratchige et al. 2016; Ueckermann 2010). Chemical control of eriophyid mites is often unsuccessful as these mites are found in well-protected refuges in plant tissue. For example, *P. fructiphilus* prefers to hide in the leaf axils and flower buds of roses. More importantly, there are no comprehensive resources available that offer recommendations on which acaricides to use and how to use them for *P. fructiphilus* on roses.

This paucity of information extends to biological control practices to prevent RRD. Eriophyid mites in general have been studied to a lesser extent when compared to other arthropod pests. However, there is a strong body of evidence showing that both inoculative (releasing small numbers of natural enemies that are expected to reproduce

and provide more long-term control) and inundative (releasing large numbers of natural enemies for immediate reduction of the pest population) approaches to biocontrol have been used to successfully reduce the populations of a number of different eriophyid mite species under field conditions (Aratchige et al. 2016; da Silva et al. 2016; McCoy 1996; McMurtry et al. 1997; Sabelis 1996; Thistlewood et al. 1996. The natural enemies used to control eriophyids are generally acaropathogenic fungi or predatory mites from the families Stigmaeidae and Phytoseiidae (Sabelis 1996).

In the absence of literature directly related to the biological control of *P. fructiphilus* it is useful to examine a different eriophyid mite species as a reference. The coconut mite, *Aceria guerreronis* is the subject of extensive research due to its economically devastating impact on coconut production (Aratchige et al. 2016; da Silva et al. 2016; Ueckermann 2010. Also, it's considered a good candidate for biological control because (a) many commercially-grown cultivars of coconut palm are too tall for chemical control to be feasible or safe, (b) very few chemical controls have proven effective at reducing populations of *A. guerreronis*, and (c) the use of acaricides, especially those with systemic properties, is further limited because residues are observed in fruits (Aratchige et al. 2016).

The coconut mite is characteristic of many eriophyid mites in that its natural enemies include both predaceous mites and acaropathogenic fungi. Of these natural enemies, two species stand out as promising biological control agents. First is the fungus *Hirsutella thompsonii*. The genus *Hirsutella* is possibly the most well-known and widely studied fungus to parasitize eirophyid mites (Aratchige et al. 2016; McCoy 1996; Van Leeuwen et al. 2010). Dozens of *Hirsutella* species parasitize a wide range of insects and nematodes (McCoy 1996). Commercial mycoacaricides produced from *Hirsutella thompsonii* have shown promise for helping control the coconut mite, *Aceria guerreronis* (Aratchige et al. 2016; Moore et al. 2008). Temperate species and varieties of the fungus might be an interesting option to explore for *P. fructiphilus*.

The predatory mite most commonly used to control coconut mites is *Neoseiulus baraki*, a member of the family Phytoseiidae. Even single releases of this predator have

produced significant reductions in eriophyid populations when compared to control plantations of coconut palm (Aratchige et al. 2016). *N. baraki* is well-suited to the job for many reasons. It can be used alongside various acaricides; it preferentially feeds on *A. guerreronis* over other prey options; it can develop to maturity and reproduce on *A. guerreronis*. *N. baraki* also possesses morphological characteristics — a long body and short legs — that enable it to reach its prey in their hidden location under the perianth of coconut fruits (Aratchige et al. 2016). In fact, a recent study demonstrated that the key factor to determine success of a biological control agent in the coconut mite system was predator size relative to the opening of the prey refuge (da Silva et al. 2016). This phenomenon is not limited to coconut mites. Research on the eriophyid tulip bulb mite, *Aceria tulipae*, found that biological control using *N. cucumeris* was only effective alongside repeated ethylene applications because the ethylene increased the distance between inner bulb scales enough to allow entry of the predator (Van Leeuwen et al. 2010).

The two predatory mites chosen for my study are also members of the family Phytoseiidae. *Amblyseius andersoni* and *Neoseiulus californicus* are both polyphagous predators that can tolerate a wide range of temperatures and are known to feed, develop, and reproduce on eriophyid species such as the apple rust mite, *Aculus schlechtendali* (Lesna et al. 1996; McMurtry et al. 1997). *A. andersoni* was even shown to have higher reproductive potential and shorter development times on a diet of eriophyid mites compared to tetranychid mites (Dicke et al. 1990; Kropczynska-Linkiewicz 1971). Both species are commercially available and already widely used by growers across the United States to control pests such as tetranychid mites and thrips.

4.2 Greenhouse Study

4.2.1 Materials and Methods

This experiment was designed to evaluate the effectiveness of two different predatory mite species at reducing populations of *P. fructiphilus* on roses in a greenhouse environment. The overall approach was to establish a population of eriophyid mites

on potted roses in a greenhouse and then randomly divide the infested roses into three treatment groups. One group would be treated with *Neoseiulus californicus*, one with *Amblyseius andersoni*, and one would serve as a control and receive no treatment.

Sixty small drift roses in 15-cell trays were obtained from Star® Roses and Plants in September 2015. These roses were potted into 2-quart pots and kept in a greenhouse for the duration of the study. On October 13th, 2015, the 60 roses were placed in 30 screened insect rearing cages, measuring 24" tall with a 13.5" square footprint, with two roses per cage (see Figure 4.1). Each rose was then augmented with symptomatic shoot tips from local infected mutliflora roses. These shoot tips were affixed with twist ties to an actively growing shoot of each drift rose. The success of this method of augmentation is well documented in studies on the biological control of multiflora rose (Epstein et al. 1997; Hindal et al. 1988). Eriophyid mites are able to walk from one one plant to another when canes are touching, so it is expected that the augmented mites will voluntarily move from the wilting multiflora shoot to the adjoining drift rose (Michalska et al. 2010). From October 2015 to May 2016 the roses were monitored for symptom development and periodically checked for evidence of eriophyid mites by examining cuttings under a dissecting microscope. From May 2016 to August 2016 the roses were augmented monthly and samples were harvested monthly before each augmentation to be examined for eriophyid mites. Aphids were widespread in the greenhouse and were controlled with Imidacloprid, which is not known to negatively impact eriophyid mites.

Unfortunately, this experiment could not be completed because the drift roses didn't develop a suitable population of eriophyid mites within a reasonable amount of time to apply treatments. For posterity, the methods would have been as follows. When the drift roses had been sufficiently infested with eriophyid mites (approximately 50% of samples showing at least five eriophyid mites in any life stage), roses would be randomly divided into three experimental groups and sampled to establish an approximate baseline population of *P. fructiphilus*. Tissue sampling and mite counting would follow the procedure outlined by Jesse *et al.*, which involves washing the arthropods

off samples and screening the wash solution through a series of mesh sieves (Jesse et al. 2006).

Each group of 20 plants in 10 cages would be subjected to one of three treatments: (1) one sachet of *Neoseiulus californicus* per cage, (2) one sachet of *Amblyseius andersoni* per cage, or (3) no treatment. Sachets of predatory mites would be purchased from Biobest USA, Inc. Each sachet of *A. andersoni* is purported to contain a minimum of 250 mites, which breed in the sachet and produce approximately 2000 offspring over the course of 6 weeks. Sachets of *N. californicus* contain a minimum of 100 mites and produce approximately 2000 offspring over 6 weeks (*Biological Control* 2017). The screening of the insect cages is fine enough to contain both species of predatory mites, so contamination between treatments should not be an issue. Over the following six weeks, roses from each treatment would be sampled and the number of all recovered predatory and eriophyid mites would be recorded. At the end of six weeks, all roses would be thoroughly dissected and processed to get a final count of all predatory and eriophyid mites.

4.2.2 Results and Discussion

No evidence of eriophyids was found on any tissue sampled from the drift roses until December 2016. Unfortunately, this was too late to apply treatments and mites were only found a small percentage of the drift roses, which would not have been suitable for data analysis. It's not entirely clear why the drift roses used in this experiment failed to develop a detectable population of eriophyid mites within a reasonable amount of time. Drift roses are known to be susceptible to RRD and a substantial number of eriophyids were introduced to the plants over the course of several months. A variety of environmental conditions likely proved inhospitable to our vector and prevented *P. fructiphilus* from establishing on the plants. For example, the roses produced a negligible amount of new growth during their time in the greenhouse. This could have resulted from a nutrient imbalance or the constant assault of aphids. Regardless, the

Figure 4.1: Two drift roses in an insect cage, ready for inoculation with the RRV vector.

tender growth required by eriophyid mites for feeding and reproduction was largely absent.

Additionally, the greenhouse in which the drift roses were housed had strong fans for proper ventilation. Considering that eriophyid mites tend to disperse by drifting in air currents, it's possible that they were simply blown out of the greenhouse. It should be noted, though, that multiflora roses in a neighboring greenhouse with the same ventilation system became unintentionally infested with *P. fructiphilus*. There is evidence that rearing *P. fructiphilus* in a greenhouse is difficult compared to the rearing of other eriophyid mites because the environment is not ideal for feeding and breeding and because spider mites omnipresent in many greenhouses and must be controlled (Kassar et al. 1990).

Multiflora roses may have been a better choice for this experiment because they are extremely reliable hosts of the vector and easily push new growth, even though they are larger and would require larger insect cages. Finally, it's possible that keeping the drift roses in insect cages may have negatively impacted their development. Insect cages are known to locally increase the temperature and humidity levels, which likely stressed the drift roses and prevented them from pushing tender new growth. As a result, the eriophyid mites that were introduced had no suitable habitat and either dispersed or died.

4.3 Microscope Interaction Study

4.3.1 Materials and Methods

After it was clear that the greenhouse study was no longer viable, it was decided to conduct a significantly smaller experiment to determine if *A. andersoni* and *N. californicus* would even consume *P. fructiphilus* if given the chance. This took the form of observing the interaction of each predatory mite with individuals of *P. fructiphilus* on rose tissue under a dissecting microscope. On October 17th, 2016, predatory mites were received from Biobest USA, Inc. *A. andersoni* was shipped in a container of bran and *N. californicus* was shipped in vermiculite. Over the following two days

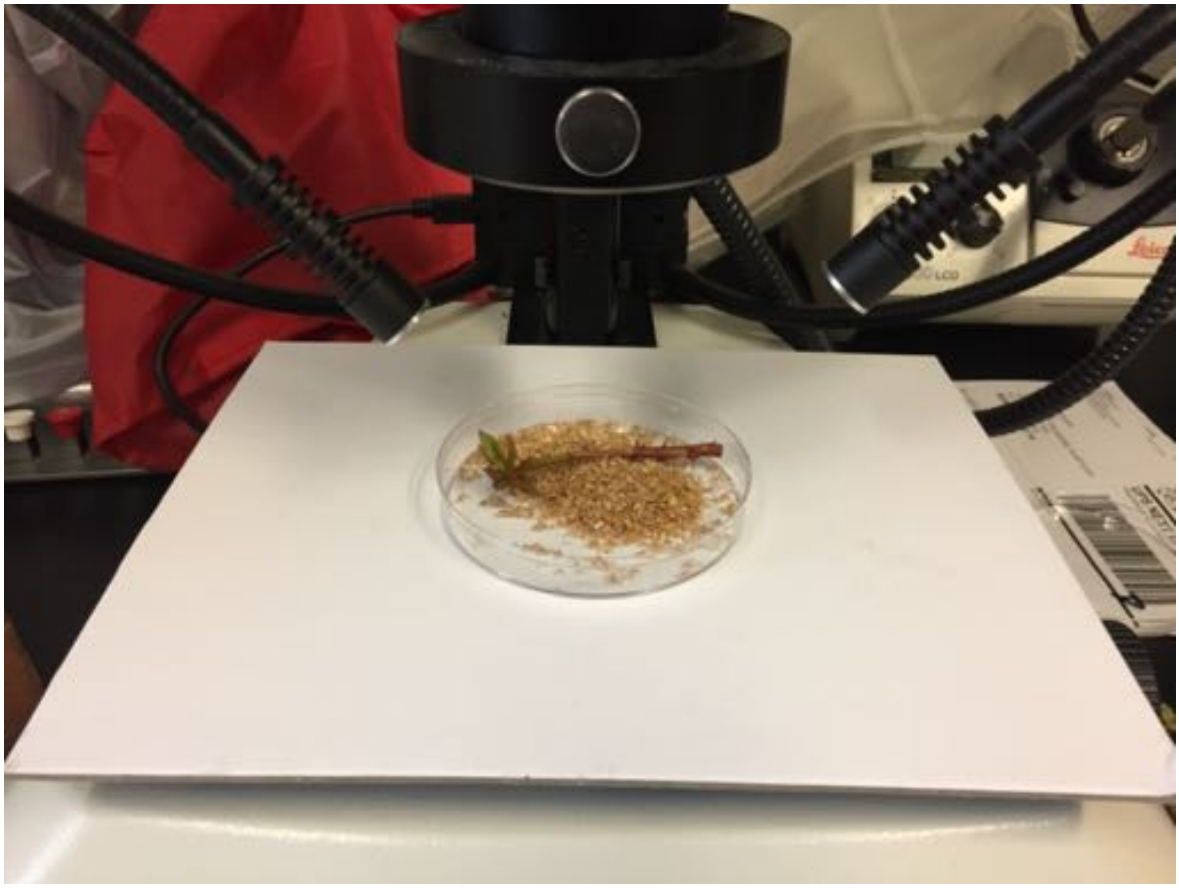


Figure 4.2: Microscope setup of biological control study. Petri dish contains symptomatic shoot of *Rosa multiflora* with leaves removed, surrounded by *Amblyseius andersoni* in bran-flake carrier.

they were both evaluated in association with *P. fructiphilus*. To accomplish this, 3-inch sections of symptomatic multiflora rose shoots were harvested from local infected plants. Shoots were stripped of leaves and examined under a microscope. When a cluster of eriophyid mites was found, generally around a vegetative bud, the multiflora shoot was placed in an empty petri dish and surrounded with one species of predatory mite in its associated carrier material (see Figure 4.2). The cluster of eriophyids was observed until a predatory mite appeared in the field of view. Photographs were taken of the interaction between each predator species and *P. fructiphilus*.

4.3.2 Results and Discussion

Both predatory mite species readily consumed *P. fructiphilus* (see Figures 4.3, 4.4, 4.5). In multiple instances a single predator mite would consume an entire cluster of eriophyids in a matter of minutes. There are many factors that may have positively contributed to the predators' voraciousness. First, they were shipped without access to food and probably arrived hungry. Second, they did not have access to a wide array of food choices on the rose tissue and so it cannot be determined if they would consume the eriophyids if presented with other options. Third, the application rate of the predators was extremely high in the petri dish so they did not have to exhibit much searching behavior. Finally, the leaves of the multiflora had been removed, granting the predators access to the eriophyids' refuge regardless of predator size or persistence.

4.4 Conclusions and Future Needs

The results of this study only indicate that two species of commercially available predatory mites will consume *P. fructiphilus* under the most ideal conditions. This leaves a wide opening for future research, but the first experiment I would conduct is of similar scale to the microscope study described here. Instead of merely observing the interaction of predator an prey, it would be beneficial to quantify the effectiveness of each phytoseiid. This could be accomplished by collecting a number of infected multiflora shoots of equal size and dividing them into two groups. Samples from one group would be left in an empty arena for a given amount of time, say 2 hours, after which they would be dissected and examined for the number of eriophyid mites present. Samples from the other group would be put in an arena for the same amount of time with a known number of predator mites before dissection and examination. Comparison of the two groups would allow me to determine by statistical analysis if the predator substantially reduced the number of eriophyid mites present, which would also demonstrate if the predator was able to enter the refuges of *P. fructiphilus*.

Before substantial resources are allocated to additional greenhouse and field studies, it would be prudent to conduct an economic feasibility study to determine if

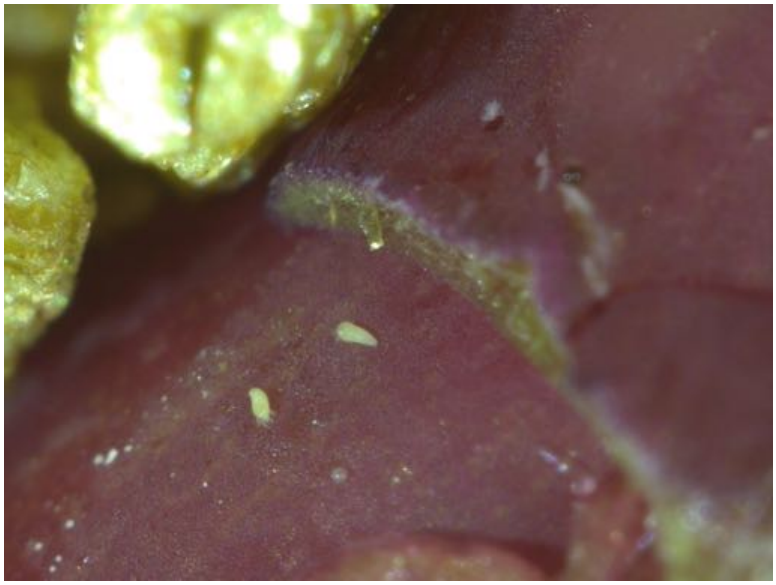


Figure 4.3: Before: Two individuals of *Phyllocoptes fructiphilus* at 25x magnification on a RRD-infected rose stem with the leaves removed. Gold flakes are the vermiculite carrier in which *Neoseiulus californicus* was shipped.

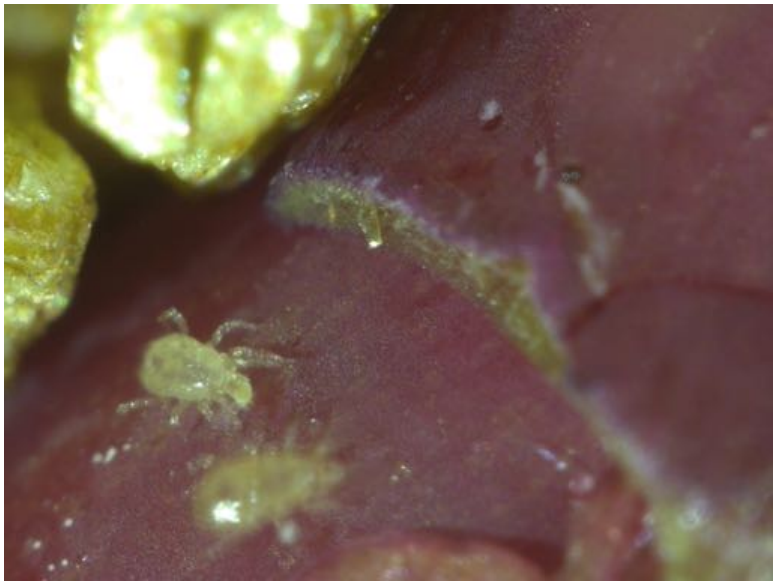
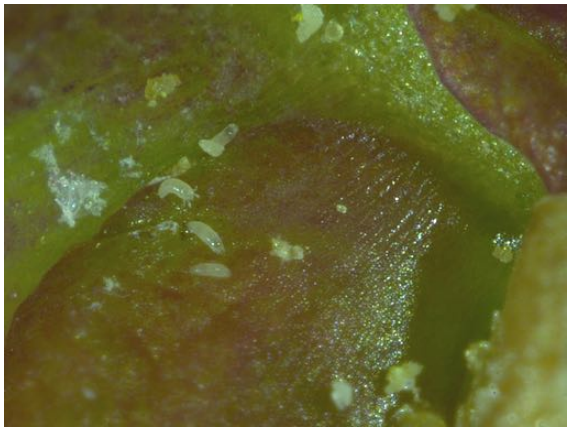
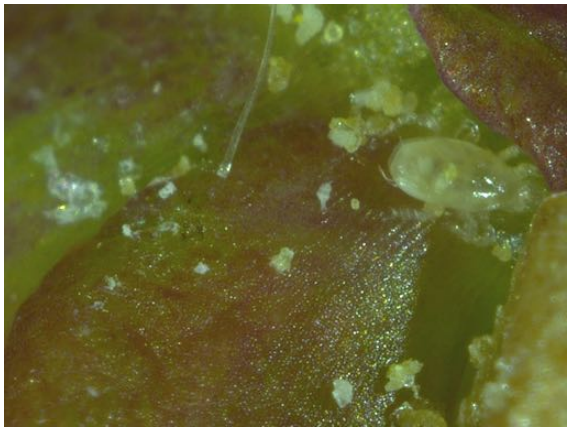


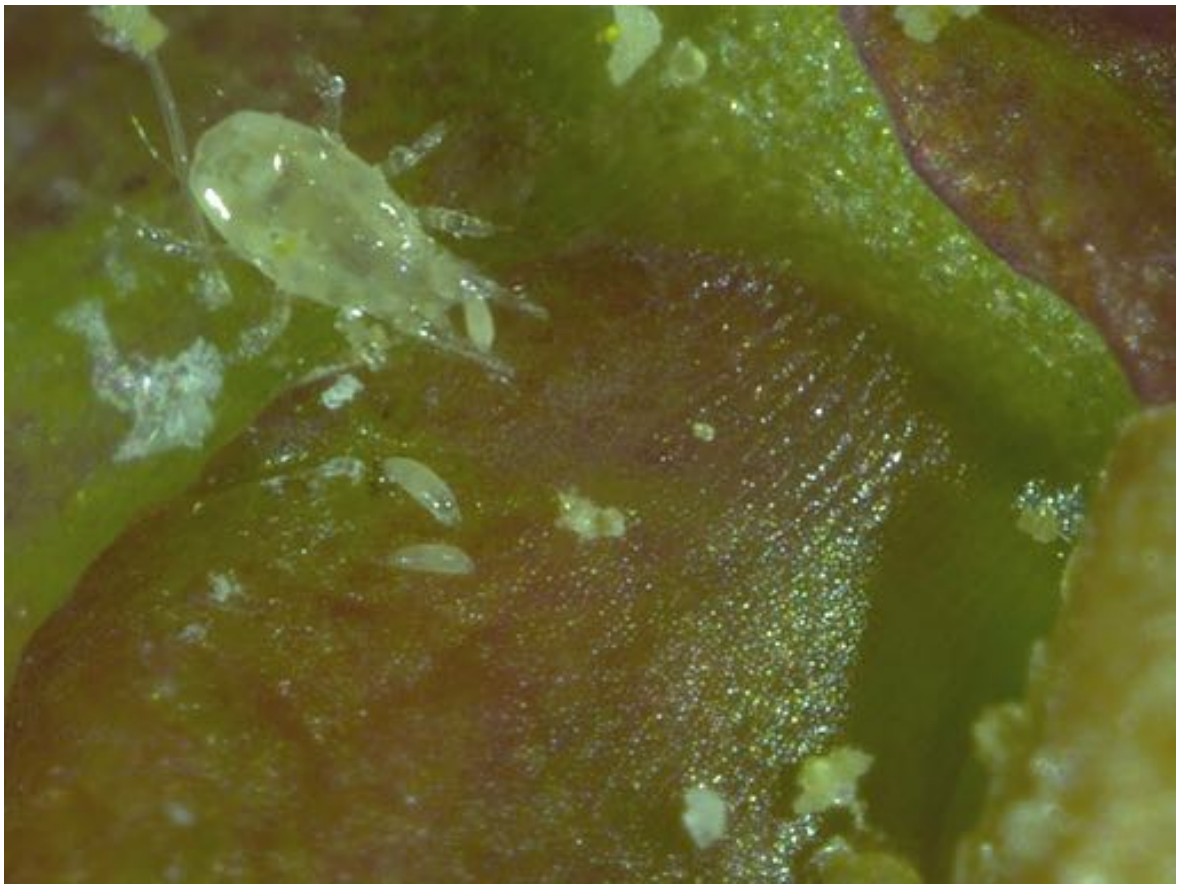
Figure 4.4: After: Two individuals of *Neoseiulus californicus* at 25x magnification in the process of consuming two *P. fructiphilus*. The predatory in mite in focus has an eriophyid in its clutches.



(a) Before



(b) After



(c) While eating

Figure 4.5: A single *Amblyseius andersoni* consumes a cluster of *Phyllocoptes fructiphilus* at 25x magnification. Figure a shows the cluster of eriophyids before arrival of the predatory mite. Figure b shows the predator leaving the scene after finishing its meal. Figure c shows the predator clutching one of the eriophyids from the cluster.

biological control even makes sense from a financial perspective. The coconut mite was discussed in the introduction to this chapter because it has been the subject of a large number of biocontrol studies. However, there are two key differences between the coconut and rose systems. First, the coconut mite causes damage to coconut crops simply by feeding whereas eriophyid-associated damage to roses results from virus transmission. Second, the product of a coconut plantation is the coconut fruits while the product of a rose field is the rose plant. Since RRV can be transmitted by a single viruliferous eriophyid and since a rose is not marketable after infection, the economic injury level for a rose field is by nature extremely low. However, the aim of biological control is not to achieve complete eradication of a pest species, but rather to maintain a balance of predator and prey populations. Because of this, it's possible that biological control is not an economically feasible approach to reducing incidence of RRD. However, the ecology of *P. fructiphilus* bodes well for a successful biocontrol program. Dispersal of the mite is population-dependent rather than associated with a specific season or life stage (Michalska et al. 2010). If the population can be kept relatively low using predatory mites, then RRD outbreaks might remain fairly localized.

If it is determined that biological control is worth pursuing there are a multitude of questions to answer in order to assess the suitability of *N. californicus* and *A. andersoni* for controlling *P. fructiphilus*. For instance, are the predators minute enough to reach the eriophyids in their refuges? Will the predators eat the eriophyids preferentially over alternative prey? Can the predators develop solely on *P. fructiphilus* as a food source? These and other questions must be addressed for any biological control program.

REFERENCES

Amrine J.W., Jr. (2002). "Multiflora rose". In: *Biological Control of Invasive Plants in the Eastern United States*. Ed. by R. Van Driesche, S. Lyon, B. Blossey, M. Hoddle, and R. Reardon. USDA Forest Service Publication FHTET-2002-04, pp. 265–292.

Aratchige, N.S., A.D.N.T. Kumara, and N.I. Suwandharthne (2016). "The coconut mite: Current global scenario". In: *Economic and Ecological Significance of Arthropods in Diversified Ecosystems*. Ed. by A.K. Chakravarthy and S. Sridhara. Springer Nature, pp. 321–342.

Biological Control (2017). BioBest USA, Inc. URL: http://www.biobestgroup.com/en/biobest/products/biological-control-4463/.

da Silva, F.R., G.J. de Moraes, I. Lesna, Y. Sato, C. Vasquez, R¿ Hanna, M.W. Sabelis, and A. Janssen (2016). "Size of predatory mites and refuge entrance determine success of biological control of the coconut mite". In: *BioControl* 61, pp. 681–689.

Dicke, M., M.W. Sabelis, and M. De Jong (1990). "Do phytoseiids select the best prey species in terms of reproductive success?" In: *Experimental Applied Acarology* 8, pp. 161–173.

Epstein, A.H., J.H. Hill, and F.W. Nutter Jr. (1997). "Augmentation of rose rosette disease for biocontrol of multiflora rose (*Rosa multiflora*)". In: *Weed Science* 45, pp. 172–178.

Hindal, D.F. and S.M. Wong (1988). "Potential Biocontrol of multiflora rose, *Rosa multiflora*". In: *Weed Technology* 2(2), pp. 122–131.

Jesse, L.C., K.A. Moloney, and J.J. Obrycki (2006). "Abundance of arthropods on the branch tips of the invasive plant, *Rosa multiflora* (Rosaceae)". In: *Weed Biology and Management* 6, pp. 204–211.

Kassar, A. and J.W. Amrine Jr. (1990). "Rearing and development of *Phyllocoptes fructiphilus* (Acari: Eriophyidae)". In: *Entomological news*, pp. 276–282.

Daniel, M. and B. Rosicky, eds. (1971). *Studies on the feeding of four species of phytoseiid mites (Acarina: Phytoseiidae)*. The 3rd International Congress of Acarology, pp. 225–227.

Lesna, I., C.G.M Conijn, P. Cohen, M.W. Sabelis, and H.R. Bolland (1996). "Candidate natural enemies for control of *Aceria tulipae* (Keifer) in tulip bulbs: exploration in the strage and pre-selection in the laboratory". In: *Experimental Applied Acarology*.

McCoy, C.W. (1996). "Pathogens of eriophyid mites". In: *Eriophyid Mites - Their Biology, Natural Enemies and Control*. Ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin. Amsterdam: Elsevier, pp. 481–490.

McMurtry, J.A. and B.A. Croft (1997). "Life-styles of Phytoseiid mites and their roles in biological control". In: *Annual Review of Entomology* 42, pp. 291–321.

Michalska, K., A. Skoracka, D. Navia, and J.W. Amrine (2010). "Behavioural studies on eriophyoid mites: an overview". In: *Experimental Applied Acarology* 51, pp. 31–59.

Moore, D., L. Alexander, and R.A. hall (2008). "The coconut mite, Eriophyes guerreronis Keifer in St. Lucia: Yield losses and attempts to control it with acaricide, polybutene and Hirsutella fungus". In: *Tropical Pest Management* 35, pp. 83–89.

Sabelis, M.W. (1996). "Phytoseiidae". In: *Eriophyid Mites - Their Biology, Natural Enemies and Control*. Ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin. Amsterdam: Elsevier, pp. 427–456.

Thistlewood, H.M.A., D.R. Clements, and R. Harmsen (1996). "Stigmaeidae". In: *Eriophyid Mites - Their Biology, Natural Enemies and Control*. Ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin. Amsterdam: Elsevier, pp. 457–470.

Ueckermann, E.A., ed. (2010). *Eriophyoid Mites: Progress and Prognoses*. Springer.

Van Leeuwen, T., J. Witters, R. Nauen, C. Duso, and L. Tirry (2010). "The control of eriophyoid mites: state of the art and future challenges". In: *Eriophyoid Mites: Progress and Prognoses*. Ed. by E.A. Ueckermann. Springer, pp. 205–224.

Appendix A

WEEKS ROSES LETTER TO CUSTOMERS

Weeks Roses
30135 McCombs Road ■ Wasco, California 93280
Phone: 661-758-5821 • *Toll-free:* 800-992-4409
Customer Service Hotline: 661-758-5172 • *Fax:* 661-758-8013
eMail: info@weeksroses.com • *Web:* www.weeksroses.com

September 12, 2017

Dear Valued Customer:

We have discovered a small number of Rose Rosette Disease (RRD) infected rose plants in one of our field production areas. Because of this discovery, we have initiated an aggressive program to remove, and destroy, any affected rose plants. We have also been cooperating with scientists from the University of California who have made eradication of RRD one of their top priorities. Since RRD is spread by a tiny mite, we have started spraying a continuous rotation of miticides targeting that mite. Week's grows roses in two production locations. These two areas are separated by one mile. So far, we have only found infected plants in one of the two locations. The other area, has remained free of RRD.

RRD was first reported in California in 1941. It is believed to exist in native roses growing in the high foothills. It has not previously been reported in garden roses in the state. We are committed to preventing the spread of this disease and want you to know the steps we are taking to prevent the introduction and spread of RRD in these states: California, Oregon, Washington, Arizona, New Mexico, Nevada, and Hawaii.

1. All orders for shrub roses, hybrid teas, climber, miniature, floribunda, and grandiflora roses will ONLY be shipped from our fields where we are confident that RRD does not exist.
2. Tree roses will be shipped from a field where only one, early-stage infected rose plant has been found to have RRD. This plant was, of course, immediately bagged in the field and destroyed. With help from the University of California/Davis, we have done randomized tests of 160 additional plants in the tree rose production area. All samples, other than the one early-stage rose, were negative for RRD.
3. Week's trained scouts are continuously walking through all fields to be sure that any symptomatic plants are found, and destroyed. We remove plants for several feet in both directions to ensure that any mites that might have moved from the symptomatic plant to its neighbors are destroyed.
4. Of all the millions of rose plants in Week's production fields only 147 plants with any suspect symptoms have been removed. Some thrips and herbicide injury symptoms look similar to RRD, so many of the plants that we have removed have NOT had RRD. Nevertheless, we are destroying all suspect plants showing any symptoms.

While we are confident that we are finding, and removing all infected plants, Weeks is committed to making an extra effort to prevent movement of RRD. In our efforts to eradicate RRD in our fields, we are taking the extra step of not shipping from fields where multiple infected plants were found to any of the states listed above. What this means for you, is that in your area, some varieties will not be available. If the substitutions we have available are not acceptable to you, we will be happy to work with you to adjust your order. Weeks Sales team and Customer Service are working on substitutions now and will be contacting you in the next few weeks. Please feel free to contact Customer Service if you have any questions at 800-992-4409.

While many may feel that we are being overly cautious, we want all our customers to know that the high standards that Weeks has developed and lived by, for the last 80 years, are still being followed. Weeks will continue to strive to sell only the highest quality roses.

One last note, the mite that spreads RRD and the virus itself is very specific to roses, so it will have no effect on any other plant species grown and sold by Weeks.

Regards,

Niles Kinerk
Owner

Stu Chamberlin
General Manager

Appendix B

ENDPOINT RT-PCR PROTOCOL TO DETECT ROSE ROSETTE EMARAVIRUS

Samples of leaf tissue were either processed while fresh or kept frozen at -80°C until processing could occur. Approximately 100mg of symptomatic leaf tissue from each rose placed in a cryo-safe bead-beater vial with 2 glass beads, which was submerged in liquid nitrogen and agitated in a Mini-Beadbeater for 40 seconds. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and immediately used in first-strand cDNA synthesis. Negative controls included "no enzyme", "no template", asymptomatic. Samples from a known infected multiflora rose were used as a positive control. A second primer set (see Table B.1) that amplifies a highly conserved mitochondrial mRNA in plants was used as an additional internal control.

For cDNA synthesis, the following reagents were combined for each reaction on ice and then incubated at 70°C +/- 3°C for 5 minutes to denature the RNA: 9.5 μl DEPC water, 1 μl 10 mM dNTPs, 0.2 μl random hexamer primers (0.5 μg/μl), and 4 μl RNA template. The RNA template was replaced with nuclease free water for the "no template" control". After incubation, all reactions were placed on ice for 1 minute. The following reagents were then added to each reaction before incuabtion at 37°C +/- 2.5°C for 1 hour: 0.5 μl RNAsin Plus (Promega), 4 μl 5X Buffer M-MLV (Invitrogen), and 0.8 μl M-MLV 200U/μl Reverse Transcriptase (Invitrogen). The reverse transcriptase was replaced with nuclease free water for the "no enzyme" control.

Endpoint RT-PCR was performed in 20 μl reaction volumes consisting of 10 μl GoTaq Green Master Mix (Promega), 1 μl of each RRV2F and RRV2R (5 M) primers, 3 μl of cDNA template, 1.6 μl of BSA (50mg/ml), 2 μl of 10% PVP and 1.4

μl nuclease free water. The RRV2 primers were replaced with Nad5 primers for the internal control. The cycling parameters for amplification were: initial denaturation of 94°C for 3 minutes followed by 38 cycles of denaturation at 94°C for 20 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 3 minutes. PCR products were electrophoresed on 2% agarose alongside a 25 bp DNA ladder.

Table B.1: Primers used in two-step endpoint RT-PCR protocol

Primer name	Sequence (5'-3')	Fragment size	Target	Reference
Nad5-F	GATGCTTCTTGGGGCTTCTTGTT	181 bp	Plant	Menzel et al. 2002
Nad5-R	CTCCAGTCACCAACATTGGCATAA			
RRV2-F	TGCTATAAGTCTCATTGGAAGAGAAA	104 bp	RRV	Dobhal et al. 2016
RRV2-R	CCTATAGCTTCATCATTCCTCTTTG			