

MITE-VIRUS-PLANT COMPLEXES OF IMPORTANCE FOR FLORIDA
AGRICULTURE: EARLY DETECTION, CHEMICAL ECOLOGY AND BIOCONTROL
OF *PHYLLOCOPTES FRUCTIPHILUS* AND *BREVIPALPUS CALIFORNICUS*

By
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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
UNIVERSITY OF FLORIDA

2021

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For Liz, Violet, Juniper and Fifes to come

ACKNOWLEDGMENTS

Acknowledgments must be written in complete sentences. Do not use direct address.

For example, instead of Thanks, Mom and Dad!, you should say I thank my parents.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

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2021

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Rose Rosette Virus (genus Emaraviridae) is the most devastating disease of roses. Rose Rosette Virus (RRV) creates witches brooms, rosetting, deforms flowers, increases prickle density, elongates shoots, reddens of plant tissues, causes dieback and ultimately plant death. RRV is spread by a microscopic eriophyid mite known as *Phyllocoptes fructiphilus* Keifer (Trombidiformes: Eriophyidae). Few management options are available: Current mite control is achieved by removing infected roses and frequent pesticide applications. Growers are interested in alternative and less expensive management options to combat *P. fructiphilus* and RRV. Predatory mites have potential to fulfill this need: mites from the family Phytoseiidae are being investigated as biocontrol agents for the management of *P. fructiphilus*. Preliminary data suggest that the phytoseiid mite *Amblyseius swirskii* Athias-Henriot (Mesostigmata: Phytoseiidae) orients itself towards volatiles of RRV-infected roses. This attraction may have synergistic effects for *P. fructiphilus* control. *A. swirskii* and three other commercially-available phytoseiid mites will be tested in olfactometer choice tests to identify specific volatile compounds which may be causing this behavior. Findings will help to develop chemical lures and promote depredation on *P. fructiphilus* in Rose Rosette-infected roses. This research will

contribute a biocontrol option for the management of *P. fructiphilus* in southern Georgia and northern Florida.

CHAPTER 1

LITERATURE REVIEW

1.1 A small introduction to the Acari

Mites and ticks belong to a subclass of small arachnids known as the Acari, an incredibly diverse group of arthropods. Despite their ubiquity, mites and invertebrates in general remain understudied relative to other animal fauna (Grodsby et al. 2015, Rosenthal et al. 2017, Titley et al. 2017). Hoy (2011) speculates that our understanding of mite diversity and abundance may be around 50-100 years behind the taxonomy of the Insecta: The small size and cryptic habits of mites make them easy to overlook, and difficult to observe. In addition, their disputed placement in the Arachnida (Giribet 2018) presents a real challenge for taxonomists (Giangrande 2003). Lastly, many mite species have been missclassified, and cryptic species make taxonomic certainty elusive (Bickford et al. 2007). In spite of these impediments, technological improvements, such as Low-Temperature Scanning Electron Microscopy (Achor et al. 2001, Wergin et al. 2006), Confocal Laser Scanning Microscopy (Chetverikov 2012, Chetverikov et al. 2012), X-ray computed tomography (Dunlop et al. 2011, Facchini et al. 2019), and advances in molecular biology—including high-throughput sequencing—(Dasch et al. 2019), have helped to alleviate the pains of mite identification. The combination of these techniques allows for greater taxonomic certainty (Chetverikov et al. 2012).

1.2 Herbivorous mites

The most well known species of mites have gained scientific recognition primarily due to their pest status (Savory 1964, Jeppson et al. 1975, Hoy 2011): many acari are parasites of plants and animals, causing disease and economic injury (Jeppson et al. 1975, Hoy 2011, Walter and Proctor 2013). Even so, the majority of mite species are of no economic importance, and are harmless, or even beneficial: many show promise as biological control agents for weeds and arthropod pests (Gerson et al. 2003, Carrillo et al. 2015). Although the majority of arachnids are predatory, mites are unique in that there

are species which feed on plants (Savory 1964). Phytophagy arose at least seven times in the Trombidiformes: Parasitengonae, Tetranychidae, Raphignathoidea, Heterostigmata, Eupodoidea, Tydeoidea and Eriophyoidea all have species which feed on plants (Lindquist 1999). Phytophagy is thought to be a facultative development for the majority of mite taxa outside of the Tetranychidae and Eriophyoidea; few species of Eupodoidea and Raphignathoidea are obligate herbivores—only *Halotydeus*, *Penthaleus* (Raphignathoidea: Penthaleidae) and *Eustigmaeus* (Raphignathoidea: Stigmeidae (Gerson 1971))—and the other mite groups have few morphological adaptations associated with plant feeding (Krantz and Lindquist 1979, Lindquist 1999). An important development in the evolution of acarine phytophagy is the reduction of the chelicerae into sharp stylets used for piercing plant tissues (Lillo et al. 2018). These styliform mouthparts are thought to reduce damage to the plant in order to avoid some of the toxic chemistry plants use to defend themselves from arthropod feeding (Brattsten and Ahmad 1986). Herbivorous mite damage is dependent on the specific mite-plant interactions for a given plant spp. or cultivar (Petanović and Kielkiewicz 2010). Mites generally feed on plant epidermal and mesophyll cells (McCoy and Albrigo 1996, Rancic et al. 2006). High mite populations can reduce the amount of chlorophyll available to the plant (Khederi et al. 2018), primarily causing bronzing/russetting/silvering by direct feeding, but this damage often spreads to the surrounding tissues as the plant's immune system responds (Bensoussan et al. 2016). Mite salivary secretions can also cause a condition known as toxemia, which causes plant tissues to become chlorotic or discolored (Oldfield 1996a). Feeding on young tissues often forms distortions and delays plant growth, and some mites—many eriophyoidea and two spp. of Tenuipalpidae—form galls on their host plants (Jeppson et al. 1975, Westphal and Manson 1996, Oldfield 2005). The majority of herbivorous mites do not transmit pathogens (Oldfield and Proeseler 1996), but those which do act as vectors of plant viruses principally from two families from the Prostigmata: Eriophyidae and Tenuipalpidae (Slykhuis 1965). Members of these mite superfamilies are obligate herbivores considered to

be some of the more ancient lineages of phytophagous mites (Lindquist 1999). There have been singular reports of spider mites—tetranychidae—associated with viruses (Slykhuis 1965, Robertson and Carroll 1988), but other studies have failed to reproduce similar results (Granillo and Smith 1974). Plant mites are generally considered secondary pests, but often cause significant losses when conditions are optimal, due to the fast reproductive rate of many pest species (Gerson and Cohen 1989, Dutcher 2007). Although the development of phytophagy can be considered an evolutionary advantage for the Acari, it puts the interest of these minute arachnids in direct conflict with the interests of modern agriculture, and motivates the need for management of mite populations to avoid reaching a crop's economic injury level (Stern et al. 1959).

1.3 In-depth With the Plant Specialists: The Eriophyoidea

The eriophyoidea are the second most economically-important group of herbivorous mites, right behind Tetranychidae. Where eriophyoids lose out in damage output, they pull ahead in diversity: It is estimated that the 2,838 species reported in the “Catalog of the Eriophyoidea of the World” represent only about 10% of the total number of species which exist (Amrine and Stasny 1994). Publications of new species descriptions of eriophyids averaged about 70 per year from 1996 to 2007, and have greatly increased since the publication of *Eriophyoid Mites – their Biology, Natural Enemies and Control* (Lindquist et al. 1996), a landmark publication for the field of eriophyoid studies. Eriophyids range in size from 80-500 μm long (Nuzzaci and Lillo 1996), and seem to have evolved specifically for plant feeding (Krantz and Lindquist 1979, Oldfield and Proeseler 1996, Lindquist 1999, Skoracka and Dabert 2010, Lillo et al. 2018): they have styliform chelicera covered with a protective sheath (Lindquist 1999, Bolton et al. 2018), elongate vermiform bodies, and have a reduced number of legs from the typical eight to four (Lindquist 1996). The stylets of eriophyids are short, $\leq 20 \mu\text{m}$ (Oldfield and Proeseler 1996), which primarily limits their feeding to epidermal cells. Eriophyoid mites also have an abbreviated lifecycle, progressing from egg, to protonymph, followed by deutonymph, then adult, skipping the

tritionymph stage (Manson and Oldfield 1996). Many eriophyoid mites have summer forms (protogynes) which readily proliferate and winter forms (deutogynes), which are able to survive harsher conditions until they can disperse when environmental conditions are moderate (Kassar and Amrine Jr 1990). Eriophyids are not able to disperse very far by walking (Calvet et al. 2020), but are known to disperse aerially (Kuczyński et al. 2020) and passively (Galvão et al. 2012), rarely by phoresy on other animals (Li et al. 2018). The majority of eriophyids studied to date are considered host specific, limited to feeding on a single genus, or one host plant. (Oldfield and Proeseler 1996, Lillo et al. 2018). The few mite species which appear to have a broader host range may be misidentified cryptic species (Navia et al. 2012, Skoracka et al. 2013), a hypothesis strengthened by their limited ability to disperse and close associations with their host plants (Magalhães et al. 2007). These lifestyles and feeding habits of the Eriophyoidea are thought to have shaped both their relationships with their host plants and their ability to transmit pathogens (Mauck et al. 2012, Biere and Bennett 2013): Eriophyid mites represent the majority of mites involved in virus transmission to plants Lillo et al. (2018). In the absence of an infectious agent, most damage from the feeding of vagrant forms of eriophyids is superficial and causes minimal damage to their host plant (Krantz and Lindquist 1979, Oldfield and Proeseler 1996). To date, eriophyids have only been associated with plant viruses (Lillo et al. 2018), which may be explained by the small size of eriophyoid mouths and foreguts, which preclude the acquisition and circulation of large pathogens (Oldfield and Proeseler 1996).

1.3.1 *Phyllocoptes fructiphilus* (Acari: Trombidiformes: Prostigmata: Eriophyoidea: Eriophyidae)

Phyllocoptes fructiphilus Keifer, (Trombidiformes: Eriophyidae) is an eriophyoid mite from the Prostigmata group. *P. fructiphilus* grows from egg to adult in 11 days (Kassar and Amrine Jr 1990).

1.4 The Tetranychoidea

The mite superfamily Tetranychoidea is comprised of 2,000 species divided into 5 families (Krantz 2009), two of which have economic significance, Tetranychidae—the spider mites—and Tenuipalpidae. Tenuipalpidae are known colloquially as the false spider mites, or flat mites due to their flattened character and superficial similarity to tetranychidae. In contrast to tetranychids, tenuipalpids do not spin webs and are considered to be a pest of reduced severity: Krantz (2009) places them as the ‘third most important family of phytophagous mites.’

1.4.1 *Brevipalpus californicus* (Acari: Trombidiformes: Prostigmata: Tetranychoidea: Tenuipalpidae)

Brevipalpus californicus (Banks), (Trombidiformes: Tenuipalpidae) is a tenuipalpid mite from .

1.4.2 1.2 Rose Rosette Virus and Rose Rosette Disease

Like many other eriophyoids, the relationships *P. fructiphilus* has with its host and virus are very specific (Krantz and Lindquist 1979, Oldfield and Proeseler 1996): *P. fructiphilus* only feeds on plants in the genus *Rosa* (roses) and doesn’t create noticeable damage by feeding. An increased interest in *P. fructiphilus* stems from its relationship with a virus known as Rose Rosette Virus (RRV) Emaraviridae (Allington et al. 1968, Tzanetakis et al. 2006, Laney et al. 2011).

P. fructiphilus transmits RRV while feeding on the rose epidermis (Allington et al. 1968). Infection creates the following symptoms: clusters of deformed flowers known as rosettes/witches’ brooms, increased thorniness, elongated shoots, reddened leaves and stems, and increased cane die-back which ultimately kills the rose host (Epstein and Hill 1995). This disease is known as Rose Rosette Disease (RRD) and is the most serious disease of roses, creating millions of dollars of losses for growers (Babu et al. 2014) and threatening the ornamental rose industry Rwahnih et al. (2019).

RRD was first described in North America in 1941 from an outbreak in Manitoba, Canada (Conners 1941). *P. fructiphilus* later became recognized as the vector for RRV (Allington et al. 1968, Doudrick et al. 1986, Jesse et al. 2006) and RRV was eventually confirmed to be the casual agent for the RRD (Doudrick et al. 1987, Tzanetakis et al. 2006, Laney et al. 2011, Bello et al. 2015, Dobhal et al. 2016, Di Bello et al. 2017). The mite and virus were generally associated with invasive multiflora rose, *Rosa multiflora* (Thunb) Amrine Jr (2002) and spread along with the rose throughout the central US (Crowe 1983), and the east (Hindal et al. 1988). Initially the relationship between *R. multiflora* and the pathogen was considered as a type of natural biological control (Epstein and Hill 1999), and some studies even considered artificially spreading RRD to eradicate these pestilent roses (Tipping and Sindermann 2000). The relationship between, *R. multiflora*, mite and virus also has a positive influence on *P. fructiphilus* fecundity: Epstein and Hill (1999) reported a 17-fold increase in the mite population of diseased roses compared to uninfected plants. Like other plant-feeding mites, *P. fructiphilus* reproduce via arrhenotokous parthenogenesis, meaning that unfertilized eggs become male, while fertilized eggs become female. This allows a single female to found a new colony without being fertilized *a priori* to dispersion (Helle and Wysoki 1996).

Together, these factors likely contributed to *P. fructiphilus*'s ability to disperse. RRD and *P. fructiphilus* can spread through the landscape in various ways: RRV can be spread by grafting (Doudrick et al. 1987) and the mites can crawl from plant to plant or be blown by the wind over long distances (Zhao and Amrine 1997, Zhao and James 1997, Michalska et al. 2009). Unfortunately, *P. fructiphilus* and RRD have the ability to infest commercial rose cultivars as well (Epstein and Hill 1995, Byrne et al. 2018), and can be spread by humans moving infested plants (Navia et al. 2009).

1.4.3 1.3 Disease Management

Rose Rosette Virus (RRV), the casual agent of Rose Rosette Disease (RRD) and *P. fructiphilus* Kiefer invaded the southeastern United States on the multiflora rose, *R.*

multiflora (Thunb) as it spread its range towards the coast (Amrine Jr 2002, Otero-Colina et al. 2018). RRD is present throughout the US, including Decatur County, GA, near the Florida border (*Figure 1*), (EDDMapS 2019). Currently, all roses are known to host *P. fructiphilus*, and few roses show signs of resistance to RRV (Di Bello et al. 2017). Nursery managers tentatively control the disease by removing sick plants and spraying acaracides. Acaricide applications are expensive, and eriophyid mites may develop resistance to acaricides with increased applications (Omoto et al. 1995). In addition, to date there is limited information regarding the toxicity and effectiveness of acaricides used to combat *P. fructiphilus*. Pesticide applications are further complicated by the biology of the mite: *P. fructiphilus* are a refuge seeking species of eriophyoids which prefer to feed on the small plant hairs on the sepals, underneath the petals (Amrine and Stasny 1994, Jesse et al. 2006, Lillo et al. 2018, Otero-Colina et al. 2018). The petals help shield the mites from conventional pesticide treatments. Furthermore, a single mite is potentially enough to transmit the virus (Di Bello et al. 2017), which can infect a rose in less than an hour, yet plants can remain sick and symptomless for months to years (Amrine Jr 1996, Di Bello et al. 2017). This slow onset of disease symptoms creates an additional challenge for management (Di Bello et al. 2017), because by the time the disease is noticed, the mites may have already spread to the whole garden. Disease detection is also difficult: Symptoms can appear similar to natural plant growth or herbicide damage, making it hard to diagnose in the field (Hong et al. 2012). Molecular methods for testing RRV are becoming readily available Di Bello et al. (2017), and newer technologies, such as Raman spectroscopy (Farber et al. 2019) are being developed to test for RRV, but it remains to be seen if these methods are capable of identifying asymptomatic infections or if these tests are suitable for disease monitoring on larger scales. Overall, rose growers need better methods to combat *P. fructiphilus* and RRV.



Figure 2: Typical symptoms of Rose Rosette Disease (RRD), caused by Rose Rosette Virus: clusters of deformed flowers known as rosettes/witches' brooms, increased thorniness, elongated shoots, reddened leaves and stems. RRD ultimately kills the rose host.

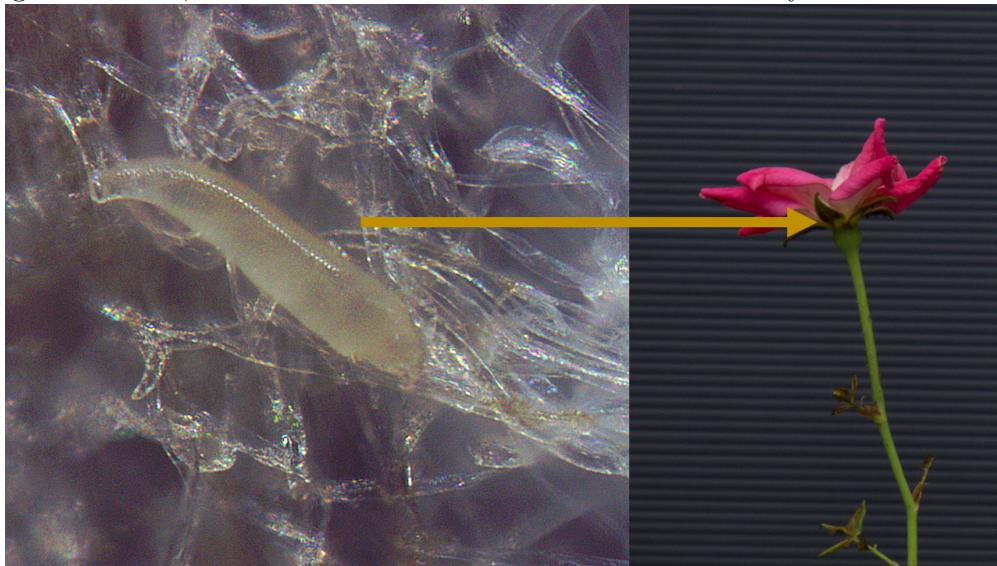


Figure 3: Illustration of the typical location of *Phyllocoptes fructiphilus* on roses. *P. fructiphilus* are difficult to manage with pesticides due to the protection offered by the sepals.

1.5 Integrated Pest Management (IPM)

Integrated Pest Management (IPM) is a philosophy of pest control based on integrating as many different types of control to keep pest populations underneath their economic injury level (EIL) (Stern et al. 1959, Flint and Bosch 1981). The EIL is a breaking point, where the cost of controlling the damage from pests exceeds the costs of crop production (Stern et al. 1959). This EIL is informed by an economic threshold, a point where a specific pest density has been exceeded, and interventions are required to prevent the crop from reaching the EIL (Stern et al. 1959). A useful framework for controlling plant pathogens has been developed in the concept of the disease triangle. For a long time, plant pathologists have recognized the importance of pathogen, environment and host on disease proliferation (Gäumann 1950), and all of these factors must be present in sufficient quantity and quality for disease to occur (McNew 1960, N 1960, Agrios 2004). Extensions of the basic disease triangle have been considered, including adding extra dimensions to account for more variables (Francl 2001), or applying it to different systems (Scholthof 2006), but the basic concept is that removing of one of these main factors of disease gives a point of attack for control efforts: Management may focus on removing a suitable host by crop rotation, or by using cultivars resistant/tolerant to pests and/or pathogens. Pathogens can be excluded from the crop via sanitation measures such as insect screens, tissue culture, seed certification programs, cleaning harvesting equipment, or by growing crops in a greenhouse. Environment can also be manipulated by growing crops in areas where the pest/pathogen isn't present or can't survive under normal conditions. The IPM paradigm encourages the combination of as many of these methods as possible for improved pest control. Although there is some overlap in the technologies and terminologies used, pest management traditionally divides pest interventions into four main categories: chemical, mechanical, cultural (environmental), and biological control methods (Bradley and Moore 2018). Chemical controls are an effective way to quickly control pest

outbreaks, but pesticides have many drawbacks as well: they pose a risk to the applicator, harm beneficial insects/pollinators, leave residues on crops meant for consumption, and can harm the environment through runoff/drift, polluting surface and groundwater (Driesche et al. 2007, Marquina et al. 2010). Pesticides also can create secondary pest outbreaks (Gerson and Cohen 1989) and pest resurgence by killing natural predators in the environment (Driesche et al. 2007), while encouraging pesticide resistance in surviving pest populations (Dutcher 2007, Ciancio and Mukerji 2007). Even so, chemical controls remain useful when used judiciously in an IPM program (Dent 2000, Driesche et al. 2007). Mechanical control is the physical manipulation of plants to prevent pests and pathogens. Mechanical controls include pruning, raking, tilling, removing pests and infected plants (roguing) by hand, creating physical barriers such as raised beds, insect screens, organic or plastic/reflective mulch, solarizing/heating the soil, sticky traps/barriers, and etc. (Ciancio and Mukerji 2007, Bradley and Moore 2018). Cultural control has some overlap with mechanical control methods, but cultural controls tend to refer to controlling the environment of your crops to exclude pests. Some examples include: selecting resistant cultivars, cultivating and planting in healthy soil, managing weeds, interplanting/trap cropping, aerating the soil, choosing appropriate planting dates, rotating crops, letting the field lie fallow, and etc. (Ciancio and Mukerji 2007, Bradley and Moore 2018).

Biological control in the classical sense relies on reintroducing the various biological entities which keep pest populations in check in their natural/native environments (Heimpel and Mills 2017, Hajek and Eilenberg 2019). Biological control agents include parasitoids, predators, herbivores and pathogens which injure pest populations sufficiently to provide control (Heimpel and Mills 2017, Hajek and Eilenberg 2019). One of the primary benefits of biological control is that these natural enemies can become established in the environment, creating long term control while adapting to fluctuations in pest populations over time (Hajek and Eilenberg 2019). Biological control has found success in

a variety of natural and agricultural environments, protecting crops against insect pests and combating invasive weed species (Driesche et al. 2010).

1.6 Predatory mites

One of the first recorded attempts at biological control in the United States was a mite *Tyroglyphus phylloxerae* Riley, intended to control the grape phylloxera, *Daktulosphaira vitifoliae* (Fitch 1855) (Dent 2000, Kirchmair et al. 2009), albeit unsuccessfully. Since then, several mite species have been involved in biological control, both as predator and prey (Bellows Jr et al. 1996). The most well-studied species of predatory mites used for biological is the Phytoseiidae (Gerson et al. 2003, Carrillo et al. 2015). Phytoseiid mites have been commercially mass-reared for their release in greenhouses (Gerson et al. 2003).

Phytoseiids have been noted for their attraction to volatile chemicals from plants (Maeda and Takabayashi 2001, Boom et al. 2002, Boer and Dicke 2004a, Maeda and Liu 2006).

1.6.1 *Amblyseius swirskii*

1.6.2 Plant defenses and Systemic Acquires Resistance (SAR)

Plants are primarily sessile organisms which aren't able to run or hide when an herbivorous mite attacks it. Instead, plants rely heavily on their ability to protect themselves in-situ, via a myriad of different physical and chemical defenses (Walling 2000). These defenses are categorized as either constitutive defenses or induced defenses (Farmer 2016). Constitutive defenses are always 'on,' being produced by the plant constantly, such as tannins and latex, while inducible defenses rely on some sort of signal before the plant will produce them. Physical defenses of herbivory includes spines, prickles, thorns, glandular trichomes, latex, sclereids, epicuticular wax, bark, thick cell walls, and compensatory growth to prevent tissue damage while increasing wear on herbivore mouthparts (Farmer 2016). In addition to these physical barriers to herbivory, plants are also efficient chemical factories which produce a bevy of secondary plant metabolites,

including inhibitory proteins, enzymes, and toxins which reduce palatability of plant tissues, prevent uptake of essential amino acids, or kill the herbivore outright (Farmer 2016). The hypotheses surrounding inducible defenses vary, from considering the costs of resource allocation (Optimal Defense Theory) (Adler and Karban 1994) The majority of chemical defenses are inducible in nature (**citation?**), which permits them to be exploited for laboratory studies and pest management.

There are a number of hypotheses about plant defenses, to be a trade-off between growth and defense

Induction of plant defenses can have negative consequences for the herbivores as well as the predators (Pappas et al. 2017): Ataide et al. (2016) observed that inducing a plant JA pathway reduces phytophagous mite performance, but also negatively affected ovophagy by predatory mites. Also, the predatory mites preferred eggs from herbivorous mites unaffected by the induced plant defenses (Ataide et al. 2016). This reduction in predation allowed the resurgence of the pest mite later (Ataide et al. 2016).

Kant et al. (2007) found examples of interspecific variation of *T. urticae*'s ability to induce—and resist—JA defenses.

The interaction of SA and JA varies by species, and exhibit negative cross-talk in some plant systems *Aculops lycopersici* induce SA defenses while suppressing JA pathways, not via antagonistic cross-talk between the responses, but by suppressing downstream accumulation of JA (Glas et al. 2014). *T. urticae* feeding induces both JA and SA pathways, but when both mites were introduced to the same plant, the JA response plummeted and SA doubled (Glas et al. 2014). When the mites feed on the same plant, *T. urticae* populations benefit from the reduction of JA caused by *A. lycopersici*, but *A. lycopersici* populations suffer (Glas et al. 2014). Meanwhile, the effects of increased SA from eriophyid mite feeding prevented a secondary bacterial infection.

When a plant is attacked by an herbivore and a plant pathogen at the same time, it creates pressure on the plant to defend on two fronts, which is further complicated by

the negative cross-talk which sometimes occurs when defenses are induced (Belluire et al. 2010). This struggle has clear benefits for vectors of plant pathogens (Belluire et al. 2010).

T. urticae and *Brevipalpus* suppress plant defenses (Alba et al. 2014, Arena et al. 2018)

Plant cross-talk during the induction of plant defenses is put under pressure when mites and viruses team up? (Belluire et al. 2010)

CHAPTER 2
FIRST REPORT OF *PHYLLOCOPTES FRUCTIPHILUS* IN FLORIDA



First Report of *Phyllocoptes fructiphilus* Keifer (Eriophyidae), the Vector of the Rose Rosette Virus, in Florida, USA

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Source: Florida Entomologist, 103(3) : 411-414

Published By: Florida Entomological Society

URL: <https://doi.org/10.1653/024.103.0317>

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First report of *Phyllocoptes fructiphilus* Keifer (Eriophyidae), the vector of the rose rosette virus, in Florida, USA

Austin Fife¹, Samuel Bolton², Jessica L. Griesheimer¹, Mathews Paret¹, and Xavier Martini^{1,*}

Phyllocoptes fructiphilus Keifer (Acari: Eriophyidae) is a microscopic eriophyid mite. Eriophyid mites are very host specific (Oldfield 1996; Skoracka et al. 2009) and *P. fructiphilus* feeds only on plants in the genus *Rosa* (Amrine 1996). *Phyllocoptes fructiphilus* is associated with the rose rosette emaravirus (rose rosette virus) and acts as the only known vector of rose rosette virus. Infection is associated commonly with the following symptoms: witches' broom, rosetting, deformed flowers, increased thorn density, elongated shoots, reddened leaves and stems, and increased die-back that ultimately kills the rose host (Amrine 1996) (Fig. 1A, B). This disease is known as rose rosette disease and is the most serious illness of roses, affecting the US commercial rose industry which is worth millions of dollars. Rose rosette disease and *P. fructiphilus* have invaded the southeastern US as they followed the range expansion of the non-native *Rosa multiflora* (Thunb.) (Rosaceae) towards the east coast (Amrine 2002; Otero-Colina et al. 2018).

In 2013, a nursery in Quincy, Gadsden County, Florida, USA, detected witches' brooms and other rose rosette disease symptoms on 15 knockout roses that had been imported from out of state. Eight symptomatic plants were tested and found to be positive for rose rosette disease, but *P. fructiphilus* was not detected on the roses at that time (Babu et al. 2014). In 2018, we began a series of surveys along the borders of northern Florida and southern Georgia to determine if this mite was present and acting as a vector for the disease.

Survey efforts initially focused on counties around Leon County, Florida. Rose tissue samples were taken from the periphery of various roses in the landscape; sampling was focused on the flowering tips of roses and included a mixture of flowers, fruits, buds, and short lengths of rose cane. The average sample contained 26.8 ± 1.5 g of undried plant tissue. Samples were trimmed with bypass pruners, dried plant tissue with 70% ethanol between cuts and stored in quart sized plastic bags (Ziploc®, S.C. Johnson & Son, Racine, Wisconsin, USA). Rose cultivars, species, and coordinates were recorded to map out sites that had predatory mites, eriophyid mites, or possible rose rosette disease.

Samples were processed using a washing method derived from Monfreda et al. (2007); cut roses were soaked in a 500 mL beaker with a solution of 1:1 bleach:water with a few drops of concentrated liquid dish washing detergent. The solution was stirred vigorously with a glass rod to dislodge any mites, then poured over a stack of sieves with decreasing screen sizes: 180 µm, 53 µm, and 25 µm. The bea-

ker and rose pieces were further rinsed with tap water over the sieve stack to dislodge any remaining mites. The 53 µm and 25 µm sieves were processed separately; the 53 µm sieve retained larger mites while the 25 µm sieve retained smaller mites, including *P. fructiphilus*. The sieves were then backwashed from the underside of their screen with a water-filled wash bottle, starting from the highest point of a sieve and working to the bottom to flush any trapped debris and mites into a 50 mL centrifuge tube for storage and future observation. Samples were observed under a dissecting microscope. Mites found among the plant debris were siphoned off with a glass pipette and subsequently stored in micro-centrifuge containers with 95% ethanol as a preservative. Some specimens were mounted directly into Hoyer's slide mounting media (Hempstead Halide, Inc., Galveston, Texas, USA), dried at 90 °C, then a ring of nail polish was painted over the edges of the coverslip to seal the slide.

On 14 Feb 2019, we found a total of 42 eriophyid mites from 6 samples obtained from Pink Double Knock Out® roses while surveying roses in the landscape in Tallahassee, Leon County, Florida, USA (Fig. 2A). The mites were sent to the Florida Department of Agriculture and Consumer Services, Division of Plant Industry and were identified as *P. fructiphilus* based, among other characters, on the distinctive pattern of ridges on the prodorsal shield (Bauchan et al. 2019) (Fig. 1C, D). Whereas 2 other eriophyid mites *Eriophyes eremus* Druciarek & Lewandowski and *Phyllocoptes adalius* Keifer (both Acari: Eriophyoidea) are found in roses in the central and eastern US, neither of them were found in the samples analyzed. The roses did not show signs or symptoms of rose rosette disease.

On 16 Jul 2019, we conducted an additional survey of 33 sites with Pink Double Knock Out® roses near the initial site of discovery, including the rose sites where *P. fructiphilus* was detected originally (Fig. 2B). Each sample contained more than 50 eriophyid mites, with some samples containing over 300 mites. We compared the samples collected during Feb and Jul in the same locations with a paired t-test and found a significant increase in the *P. fructiphilus* population between the 2 sampling dates (see Fig. 1C; $P = 0.001$; $\alpha = 0.05$; $df = 4$). Mites that were slide mounted were confirmed subsequently as *P. fructiphilus*.

This is the first record for *P. fructiphilus* in Florida. None of the mite-infested roses showed symptoms of rose rosette disease and none tested positive for rose rosette virus based on detection tools devel-

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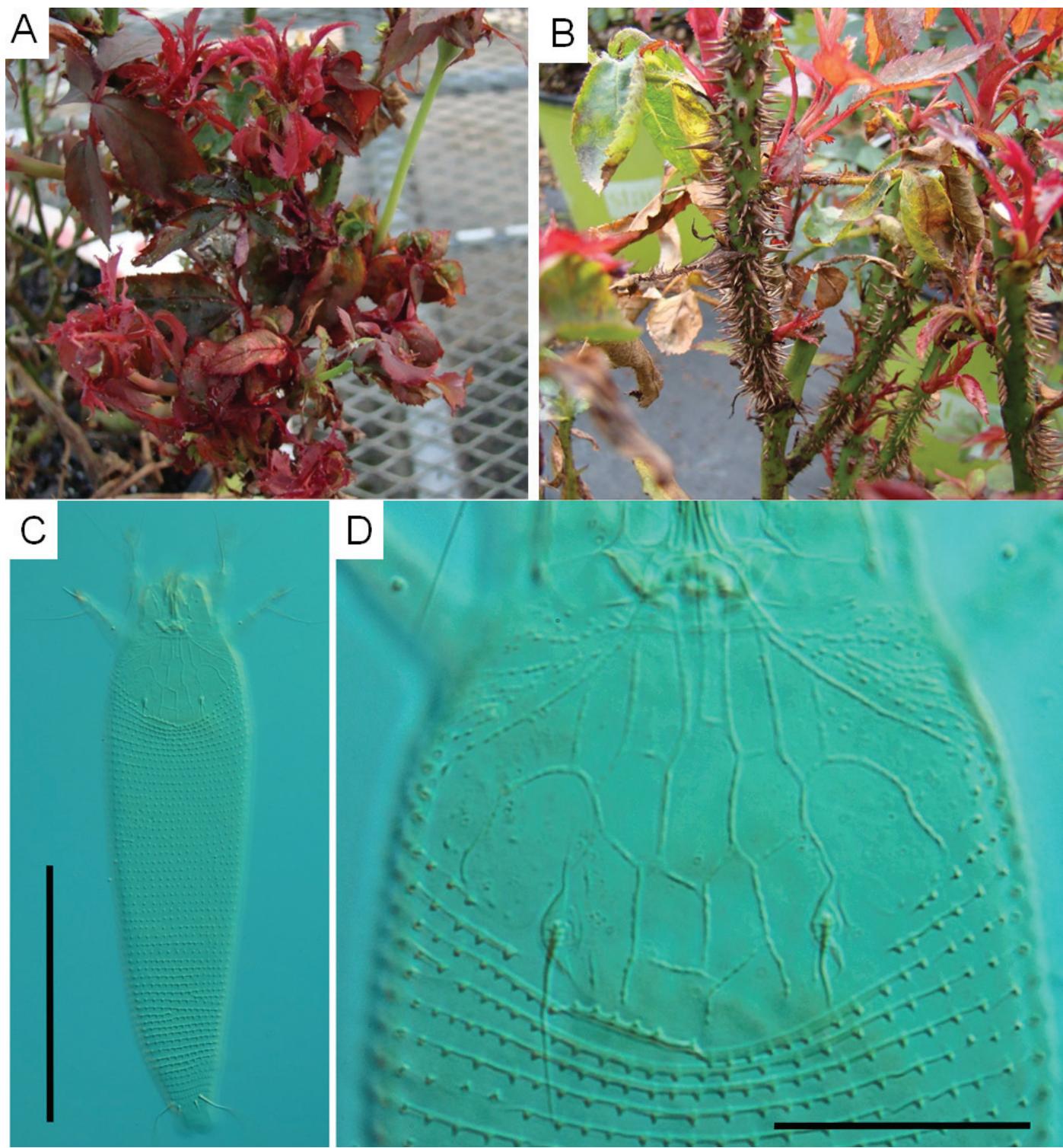


Fig. 1. (A) Symptoms of rose rosette disease: witches' broom, and (B) excessive thorn proliferation; (C) *Phyllocoptes fructiphilus* Keifer (female) from Leon County, Florida, USA: body (scale bar = 100 µm); (D) enlargement of *P. fructiphilus* prodorsal shield to show detail (scale bar = 20 µm).

oped to date. However, the presence of *P. fructiphilus*, along with past detections of rose rosette virus in Florida warrants increased monitoring for the mite and virus in Florida. There is a critical need to develop methods to manage *P. fructiphilus* and rose rosette virus, or homeowners, commercial landscapers, and the US rose industry stands to lose millions of dollars and established plantings in the coming yr.

This research was supported by a grant awarded by the Florida Nursery, Growers and Landscape Association, and USDA-AFRI-CPPM (2017-70006-27268). We thank James Brannin and Fanny Iriarte for assistance during the experiments. We also thank the Florida Department of Agriculture and Consumer Services, Division of Plant Industry for their support for this contribution.

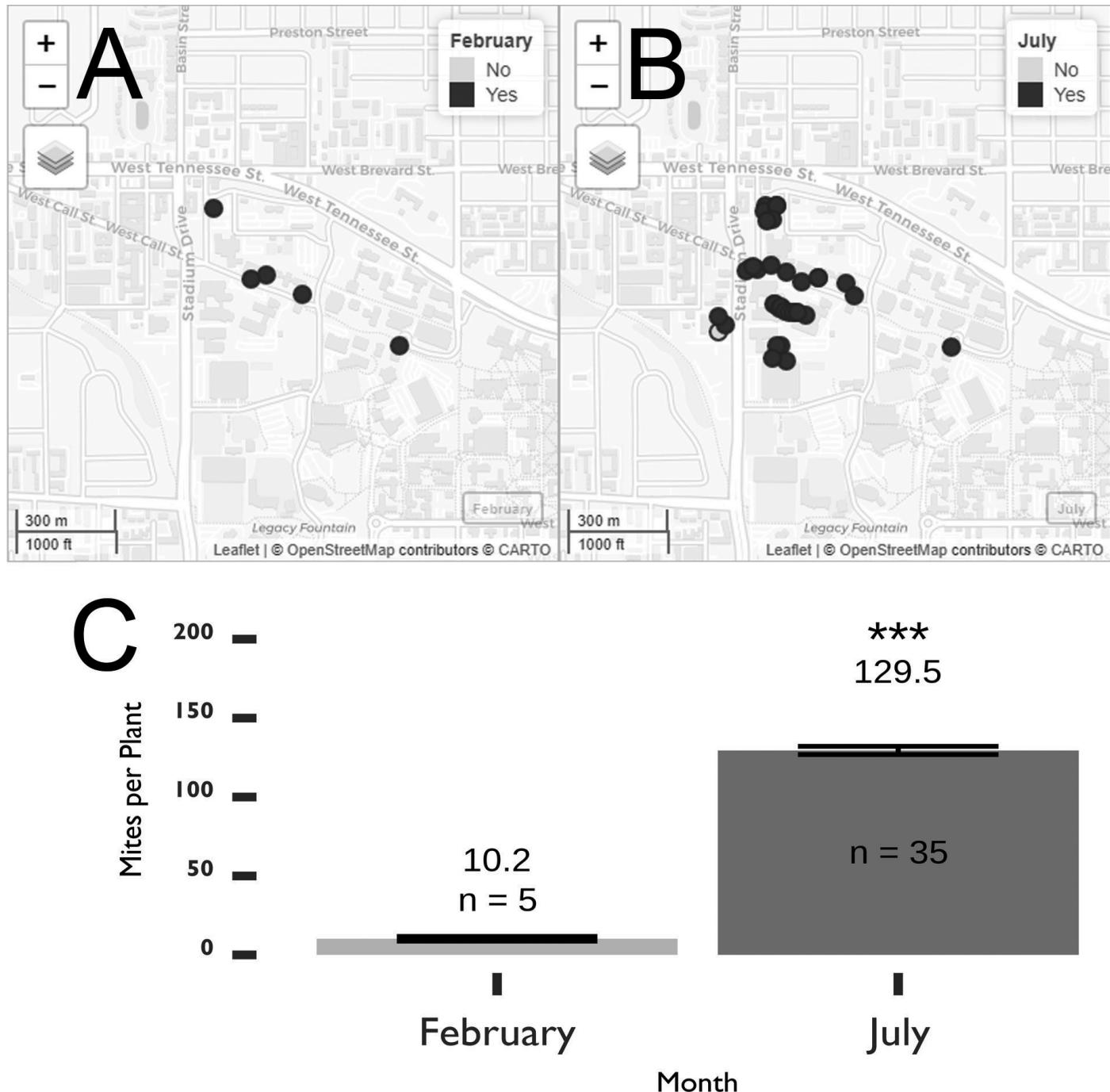


Fig. 2. Presence of *Phyllocoptes fructiphilus* in Leon County, Florida, USA, in (A) Feb 2019 and (B) Jul 2019. Orange dots indicate sites sampled that had *P. fructiphilus*. Gray dots indicate surveyed areas where no *P. fructiphilus* were found. (C) Average number of *P. fructiphilus* per rose sample. Samples were taken from sites in Leon County, Florida, on 14 Feb and 16 Jul 2019. Asterisks represent significant differences as calculated by pairwise t-tests of the 5 sites tested for *P. fructiphilus* during both mo. P -value < 0.001.

Summary

The invasive mite *Phyllocoptes fructiphilus* Keifer (Acari: Eriophyidae) feeds on plants in the genus *Rosa*. *Phyllocoptes fructiphilus* is associated with the rose rosette emaravirus (rose rosette virus) and acts as the only known vector of rose rosette virus, the causal agent of rose rosette disease (Emaravirus).

The mite *P. fructiphilus* is reported for the first time in the state of Florida, USA. No roses showed signs or symptoms of viral infection, and current molecular methods were unable to detect the virus. *Phyllocoptes fructiphilus* represents a potential threat to the Florida rose industry if rose rosette disease becomes established.

Key Words: rose rosette disease; rose rosette virus

Sumario

El ácaro invasivo *Phyllocoptes fructiphilus* Keifer (Acari: Eriophyidae) se alimenta sobre plantas del género *Rosa*. *Phyllocoptes fructiphilus* se asocia con rose rosette emaravirus (virus del arrosetamiento de la rosa), es reconocido principalmente como vector de la virus del arrosetamiento de la rosa, el agente causal de la enfermedad del arrosetamiento de la rosa (Emaraviridae). El ácaro *P. fructiphilus* se reporta por primera vez para el estado de la Florida, USA. Ninguna rosa mostró señales o síntomas de una infección viral, y ningún virus fue detectado con el uso de métodos moleculares de hoy en día. *Phyllocoptes fructiphilus* representa una amenaza potencial para la industria de la rosa en la Florida si Emaraviridae se llega a establecer.

Palabras Clave: virus del arrosetamiento de la rosa; enfermedad del arrosetamiento de la rosa; emaravirus

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CHAPTER 3

2. OBJECTIVE 1: SURVEY FOR THE INVASIVE MITE *PHYLLOCOPTES FRUCTIPHILUS*, ROSE ROSETTE VIRUS (RRV), AND WILD PREDATORY MITES IN NORTHERN FLORIDA

3.1 2.1 *P. fructiphilus* detection in Northern Florida

3.1.0.1 2.1.1 Problem Statement

Rose Rosette Virus (RRV), the casual agent of Rose Rosette Disease (RRD) and *P. fructiphilus* Kiefer invaded the southeastern United States on the multiflora rose, *R. multiflora* (Thunb) as it spread its range towards the coast (Amrine Jr 2002, Otero-Colina et al. 2018). RRD is present throughout the US, including Decatur County, GA, near the Florida border (*Figure 1*), (EDDMapS 2019).



Figure 1: Map of the distribution of Rose Rosette Disease (RRD) in 2019 as reported from the Early Detection & Distribution Mapping System maintained by the University of Georgia's Center for Invasive Species and Ecosystem Health. Areas in green are counties where RRD has been reported.

In 2018, a group of researchers conducted a series of surveys for *P. fructiphilus* and RRD in the southeastern United States (Solo 2018). They encountered *P. fructiphilus* in

Thomas County and Lowndes County, (*Figure 4*), (Solo 2018), less than 20 miles from the northern border of Florida.



Figure 4: Distribution of *Phyllocoptes fructiphilus* in the United States from Solo 2018. The black line circles represent areas where *P. fructiphilus* have been found.

Unfortunately, these surveys did not include Florida (see *Figure 4*), (Solo 2018).

3.1.0.2 2.1.2 First report of *Phyllocoptes fructiphilus* in Florida

Phyllocoptes fructiphilus is a microscopic plant-feeding eriophyid mite. Eriophyoid mites are very host specific (Oldfield 1996b, Skoracka et al. 2009) and *P. fructiphilus* only feeds on plants in the genus *Rosa* (Amrine Jr 1996). *P. fructiphilus* is the vector of Rose Rosette Virus (RRV). RRV infection is commonly associated with the following symptoms: witches' brooms/rosetting, deformed flowers, increased prickle density, elongated shoots, reddened leaves and stems, and increased die-back which ultimately kills the rose host (Amrine Jr 1996). This disease is known as Rose Rosette Disease (RRD). and is the most serious disease of roses, creating millions of dollars of losses for growers. Rose Rosette Disease and the mite have invaded the southeastern united states as they followed the range expansion of the non-native *Rosa multiflora* (Thunb) towards the coast (Amrine Jr 2002, Otero-Colina et al. 2018).

RRD has been detected in Florida in 2014 on 15 plants; however, the plants were destroyed and *P. fructiphilus* were not detected on the roses at that time (Babu et al. 2014). In 2018 we began a series of surveys along the borders of northern Florida and southern Georgia

Cities with populations over 1,000 will be visited along this route and cuttings will be taken from various roses in each city. Rose species, symptoms and coordinates will be recorded to map out sites which have *P. fructiphilus* or possibly Rose Rosette Disease.

Rose tissue samples were taken from the periphery of various roses throughout Leon and Gadsden counties as well as surrounding regions. Rose tissues sampled included a mixture of flowers, fruits, buds and short lengths of rose cane, trimmed with bypass pruners and stored in quart sized plastic baggies. Pruners were sanitized with 70% ethanol between cuts. Rose species and coordinates were recorded to map out sites which had predatory mites, *P. fructiphilus*, or possibly RRD.

Rose samples were processed using a washing method derived from Monfreda et al. (2007): cut roses were soaked in a 500 mL beaker with a solution of 1:1 bleach:water with few drops of dishwasher detergent. The solution was stirred vigorously with a glass rod to dislodge any mites. This solution was then poured over a stack of sieves with decreasing screen sizes: 180 µm, 53 µm and 25 µm. The beaker and rose pieces were further rinsed with tap water over the sieve stack to knock off any remaining mites. The 25 µm sieve screen traps mites which are the size of *P. fructiphilus*. This sieve was then backwashed from the underside of the screen with a water-filled wash bottle, starting from the highest point of the sieve and working to the bottom of the sieve to flush the trapped debris into a 50 ml centrifuge tube for storage and future observation. Samples were observed under a dissecting microscope. Mites found among the plant debris were siphoned off with a glass pipette and subsequently stored in micro-centrifuge containers filled with 95% ethanol as a preservative. Select specimens were mounted directly into Hoyer's slide mountant (Hempstead Halide, Inc. Galveston, TX), dried at 90°C, then ringed with nail polish.

On February 14, 2019, we found a total of 42 eriophyid mites from six samples obtained while surveying roses in Leon County, Florida. (see *Figure 5A*) The mites were sent to the Florida Department of Agriculture and Consumer Services - Department of Plant Industry (FDACS-DPI) and were all identified as *P. fructiphilus* using the keys provided in (Baker et al. 1996). The roses did not show signs or symptoms of RDD. These roses were tested for RRV with RT-qPCR and Reverse Transcription Recombinase Polymerase Amplification (RT-RPA) (Babu et al. 2016, 2017a). However, none of the plants infested with *P. fructiphilus* were positive for RRV.

On July 16th we conducted an additional survey of 33 roses near the initial site of discovery, including the rose sites where *P. fructiphilus* were originally detected. (see *Figure 5B*), Each sample contained more than 50 eriophyid mites, with some samples containing over 300 mites. We compared the samples collected during February and July with a paired t-test and we found a significant increase in *P. fructiphilus* population between the two sampling dates: p-value = 0.001, $\alpha = 0.05$, df = 4. A subsample of these mites were slide mounted and subsequently confirmed as *P. fructiphilus*. Additional rose samples were tested for RRV by RT-qPCR, but no virus was detected.

This is the first record for *P. fructiphilus* in Florida. More importantly, RRV is currently not established in Florida. None of the mite-infested roses had symptoms of RRD and none were positive for RRV. However, the presence of *P. fructiphilus*, along with past detections of RRV in Florida warrants increased monitoring for the mite and virus in Florida. There is a critical need to develop methods to manage *P. fructiphilus* and RRV, or the US rose industry stands to lose millions on mite control.



Figure 5: Presence of *Phyllocoptes fructiphilus* in Leon County, Florida in (A) February 2019 and (B) July 2019. Orange dots indicate sites sampled which had *P. fructiphilus*. Gray areas indicate previously surveyed areas where no *P. fructiphilus* were found.



Figure 6: Log number of *Phyllocoptes fructiphilus* per rose sample. Samples were taken from sites in Leon County, Florida on February 14 and July 16th, 2019. Asterisks

represent significant differences as calculated by pairwise t-tests of the 5 sites tested for *P. fructiphilus* during both months. $\alpha = 0.05$, p-value = 0.001.

3.1.0.3 2.1.3 Proposal: a series of surveys for *P. fructiphilus* and Rose Rosette Disease in northern Florida

A key part of controlling the spread of *P. fructiphilus* is mite and disease monitoring. We propose an expanded survey of mites on roses in Florida to estimate the populations and distribution of *P. fructiphilus*, as well as to detect if Rose Rosette Disease is present. Doing so will provide us with insight into the patterns of how *P. fructiphilus* and RRD spread.

An additional value of rose surveys will be detecting other mites present on roses: there are many species of mites present in Florida with potential to control agricultural pests such as *P. fructiphilus*. Native predatory mites sometimes have an advantage for bio-control because native mites have adapted to the environment where they will be released. Besides predatory mites, we also may encounter other vectors of RRD or different mite species of concern which have not yet been reported in Florida.

3.1.0.4 2.1.4 Materials & Methods

A survey of roses in the landscape was conducted following a transect of northern Florida from west to east, Pensacola to Jacksonville. Cities with populations over 1,000 were visited along this route and cuttings were taken from various roses in each city. Rose cultivar/species, sun exposure and GPS coordinates were recorded to map out sites which had predatory mites, eriophyid mites, or possibly symptoms of Rose Rosette Disease. Rose tissue samples were taken from the periphery of various roses in the landscape; sampling was focused on the flowering tips of roses and included a mixture of flowers, fruits, buds, and short lengths of rose cane. Samples were trimmed with bypass pruners which were routinely sanitized with 70% ethanol between cuts. Samples were stored in 500 mL Nalgene™ Wide-Mouth Polypropylene Copolymer bottles (ThermoFisher Scientific, Waltham, MA, USA) with ~10 mL of 95% ethanol. The rose samples then were

gently shaken to coat the rose tissues sampled with ethanol. Doing so made sure that the sampled mites were killed and acted to preserve both mites and rose tissues until samples could be processed further and checked for mites.

Samples were processed using a washing method derived from Monfreda et al. (2007) used to detect eriphyoid mites such as *P. fructiphilus*: The sampling bottles with ethanol and rose tissues were vigorously shaken to dislodge any mites, then the ethanol in the container was poured over a stack of sieves with decreasing screen sizes: 180 µm, 53 µm, and 25 µm. The bottle and rose pieces were then further rinsed with 95% ethanol over the sieve stack to dislodge any remaining mites. The 53 µm and 25 µm sieves were processed separately; the 53 µm sieve retained larger mites while the 25 µm sieve retained smaller mites, including *P. fructiphilus*. The sieves were then backwashed from the underside of their screen with a 95% ethanol-filled wash bottle, starting from the highest point of a sieve and working to the bottom to flush any trapped debris and mites into a 50 mL centrifuge tube for storage and future observations.

The ethanol solutions of mites and plant debris were allowed to settle until excess ethanol could be siphoned off, allowing us to then pour this concentrated plant-mite mixture into a thin, small petri dishes to be observed under a dissecting microscope. Mites found among the plant debris were counted, then siphoned off with a glass pipette and subsequently stored in micro-centrifuge containers with 95% ethanol as a preservative. 5-10 specimens from each sample were made into prepared microscope slides: Mites were cleared and mounted using the methods of Faraji and Bakker (2008): mites were simultaneously cleared and stained with Faraji and Bakker's modified clearing solution and heated on a hot plate until the specimens were clear. Subsequently these mites were moved with an eyelash tool into an iodine-modified Hoyer's slide mounting media (Hempstead Halide®, Inc., Galveston, Texas, USA), underneath a 12 mm glass coverslip. The prepared slide was then dried at 90 °C before sealing the slide by painting a ring of alkyd insulating enamel (Red Glyptal® 1201, Chelsea, MA, USA) over the edges of

the coverslip to seal the slide, to protect it from damage by air incursion and moisture. These slides could then be observed under a compound microscope with phase-contrast objectives to identify the mite families and species if necessary.

A subset of up to 10 samples per site will be selected for molecular testing using the methods described in Druciarek et al. (2019) for determining mite species identity and RRV presence within those mites.

After mite quantities and species were recorded, a representative sample of eriophyoids putatively identified as *Phyllocoptes fructiphilus* had their identity verified with the acarologist, Dr. Sam Bolton of the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI) to ensure accuracy.

Roses which appeared to show symptoms of Rose Rosette Disease, or which had populations of *P. fructiphilus* present were tested by the Plant Disease Diagnostic Clinic at the NFREC. Plant tissues were tested for Rose rosette virus by Dr. Fanny Iriarte using the currently accepted molecular methods described in Babu et al. (2016), Babu et al. (2017a), and/or Babu et al. (2017b).

3.1.0.5 2.1.5 Potential benefits

Florida is the largest producer of roses with a total value exceeding \$30 million, and stands to lose millions of dollars if RRD and *P. fructiphilus* become established. At this point, it is critical to develop action plans to control eriophyid mites on roses. By conducting this survey and finding *P. fructiphilus* and RRD in the Florida landscape, we can determine the severity of the situation and inform growers who are in areas of greater risk. Informed nurseries can then take actions to protect their ornamentals from mite infestation, and can be vigilant for RRD symptoms.

3.1.0.6 2.1.6 Expected outcomes

Potential outcomes of the mite surveys are fourfold:

- Detect the range of *Phyllocoptes fructiphilus* and/or Rose Rosette Disease in northern Florida

- Identify native predatory mites with bio-control potential
- Identify other possible vectors of RRD
- Detect other mite species of concern on Florida roses

Our results will help identify areas with greater disease risk for *P. fructiphilus* and/or RRV.

Overall, we believe that the various objectives of this proposal will answer fundamental questions regarding the potential spread of *Phyllocoptes fructiphilus* and Rose Rosette Disease (RRD) in Florida. RRD has been found without the mite, and now we may have found the mite without the pathogen at different parts of the year. This suggests that *P. fructiphilus* and RRD have potential to become established in Florida. This research would be a first step in the development of preventative measures against RRD in Florida and will have direct practical outcomes that could be implemented in the short term to protect Florida production of roses.

CHAPTER 4

3. OBJECTIVE 2: POTENTIAL OF THE PREDATORY MITE *AMBLYSEIUS SWIRSKII* TO CONTROL *PHYLLOCOPTES FRUCTIPHILUS*

4.1 3.1 *A. swirskii* attraction to RRV-infected roses

4.1.0.1 3.1.1 Problem Statement

Some pest species can be successfully managed by introducing their natural enemies to control them. Phytoseiid mites such as *Amblyseius swirskii* Athias-Henriot belong to a group of predatory mites which are used in agriculture for pest control (Smith and Papacek 1991, Onzo et al. 2012, Buitenhuis et al. 2015). Phytoseiid mites integrate well into pest management programs and are compatible with certain pesticides (Trumble and Morse 1993, Nicetic et al. 2001, Fernández et al. 2017) and other bio-control agents (Midthassel et al. 2016), which together help control pests when conventional methods are undesirable or unavailable.

A. swirskii are generalist predators which feed on other common agricultural pests such as whiteflies (Bolckmans et al. 2005), spider mites (McMurtry et al. 1970), and thrips (Wimmer et al. 2008). *A. swirskii* can persist on pollen (Loughner et al. 2011, Delisle et al. 2015) and other arthropods even when the pest of concern is absent (Janssen and Sabelis 2015). This allows *A. swirskii* to be released as a preventative measure instead of reacting to an outbreak (Kutuk and Yigit 2011). Predatory mites are able to locate their prey in tight areas when other predators might be too big. These features make *A. swirskii* an interesting option for managing *P. fructiphilus*, but their potential has not yet been tested.

Predators like *A. swirskii* learn to associate their prey with chemical cues (Boer and Dicke 2004b, 2004a, 2005), and become attracted to the Volatile Organic Compounds (VOCs) released when plants are injured by pests or infected with pathogens (Boer and Dicke 2004a).

Salicylic Acid plays a role in activating a plant's immune system and helps protect the plant from pests, bacteria, fungus or viruses (Gozzo and Faoro 2013). We are collaborating

with the University of Georgia to test how activating plant defensive compounds might protect roses from *P. fructiphilus* and/or RRD. We applied Acibenzolar-S-methyl (ASM), a chemical which works like Salicylic Acid to activate plant defenses (Ziadi et al. 2001, Tripathi et al. 2010) at a high and a low rate, as well as a miticide and water as controls. These data suggest that activated plant defenses may work to control *P. fructiphilus*.

4.1.0.2 3.1.2 Preliminary Data

Predatory mites do not feed on the plants they live on, so they shouldn't be harmed by activated plant defensive compounds. In natural systems, predatory mites learn to follow plant defensive chemicals to find their prey (Boer and Dicke 2004b). This suggests that combining ASM-activated plant defenses with predatory mites may enhance the ability of predatory mites to find their prey, but this combination hasn't been tested yet.

Preliminary tests with *A. swirskii* show attraction towards compounds from RRV-infected roses. The lab has tested some of these compounds with Y-tube choice tests to determine their role in attracting *A. swirskii* to the infected roses. Other chemicals remain to be tested. Understanding which chemicals are responsible for attracting *A. swirskii* to RRV-infected roses is a preliminary part of determining if *A. swirskii* will search for *P. fructiphilus* when on a rose.



Figure 7: *Amblyseius swirskii* attraction to healthy and Rose Rosette Virus-infected Pink Double Knock Out® roses. Asterisks represent significant differences as calculated by χ^2 -squared contingency table tests for given probabilities. N.S. = not significant. RRV-infected vs Healthy Rose: χ^2 -squared = 9.33, df = 1, a = 0.05, p-value = 0.002. Filtered Air vs Healthy Rose: χ^2 -squared = 0.47, df = 1, a = 0.05, p-value = 0.4913.

4.2 3.2 Volatile changes in Rose Rosette Virus-infected Plants Treated with Acibenzolar-S-Methyl, a functional analog of Salicylic Acid

4.2.0.1 3.2.1 Problem Statement

Some mites orient themselves towards volatiles of their prey or the host plant of their prey. We intend to investigate differences between RRV-infected and uninfected Pink Double Knock Out® roses and their volatiles, as well as the effects of SAR-induction on rose volatiles. The results will help inform future assays involving predatory mites and their *P. fructiphilus*-seeking behaviors in relation to rose RRV-infection status and the use of SAR-inducers for biological control.

4.2.0.2 3.2.2 Materials & Methods

We plan on collecting plant volatiles from Double Knock Out® roses and comparing the differences between RRV-infected and uninfected roses, using a push-pull volatile collection system as illustrated in *Figure 8*. Filtered air fills an oven bag sealed over rose canes to trap leaf volatiles. Volatiles are then drawn through a clean collection filter by a vacuum. The chemicals on the filter are eluted with 150 ul of dichloromethane, then 5 ul of nonyl acetate are added as an internal standard to the extraction. The extractions then are injected into a GC-MS and their spectra are analyzed. Compounds are identified by comparison to mass spectra databases as well as synthetic standards for confirmation.

Treatments will be the following:

1. Uninfected Pink Double Knock Out® roses
2. RRD infected Pink Double Knock Out® roses
3. SAR-induced uninfected Pink Double Knock Out® roses
4. SAR-induced RRD-infected Pink Double Knock Out® roses

A group of 20 plants will be isolated. Volatiles from the 20 plants will be collected as a baseline (already collected). Then half of the plants will be SAR induced by bi-weekly spray applications of Acibenzolar-S-Methyl. Subsequently half of the remaining plants (5 control and 5 SAR-induced plants) will be grafted with RRV-infected buds to inoculate them with the virus (Doudrick et al. 1987). Volatiles will be collected bi-monthly following SAR-induction and RRV application.

Warning in tiff::readTIFF("figure/vol-extract-trap-filter.tif"):

TIFFFetchNormalTag: Incompatible type for "RichTIFFIPTC"; tag ignored



Figure 9: Volatile collection system for rose headspace sampling. An inert nylon bag is placed around the canes of interest, an air inlet is inserted and sealed at the base with a zip-tie to form a relatively air-tight seal around the base of the rose canes. Once the bag begins to inflate, a small hole is cut in the corner of the bag and a filter inserted and sealed with a zip-tie to form a second seal. The exterior end of the filter is attached to a vacuum airline set to allow for constant static pressure on the bag from inflation. The rose is then left for 24 hours, the filter is eluted with Dichloromethane into a gas chromatography vial, 1 ul of Nonyl Acetate is added as an internal standard, and then the sample is processed using a coupled Gas Chromatography - Mass Spectrometer (GC-MS) for chemical identification.

4.2.0.3 3.2.3 Potential benefits

By determining which chemicals change during infection or with SAR-induction, we can gain insight into the physiological changes occurring during RRV-infection and SAR-induction in roses. Testing headspace volatiles can also give us insight into what VOCs predators like *A. swirskii* may encounter on infected or SAR-induced roses.

4.2.0.4 3.2.4 Expected outcomes

- Determine which headspace VOCs are different between RRV-infected and healthy roses
- Determine what effects SAR-induction has on RRV-infected and healthy roses
- Creates a starting point for volatile testing for future olfactometer studies with *A. swirskii*

Knowing which VOCs affect the host-seeking abilities of a predatory mite like *A. swirskii* is part of preliminary work for determining the compatibility of *A. swirskii* as a predator of *P. fructiphilus*.

4.2.0.5 3.3.1 Proposal: a series of behavioral tests to determine which compounds from RRV-infected Pink Double Knock Out® roses are attractive to *A. swirskii*

We propose observing the behavior of *A. swirskii* with Y-tube choice tests to test *A. swirskii*'s potential to control *P. fructiphilus*.

4.2.0.6 3.3.2 Materials & Methods

Amblyseius swirskii Athias-Henriot will be reared according to procedures adapted from Sarwar (2017): *A. swirskii* mites will be reared in growth chambers set at 25 °C with 70% RH and 16:8 hours light:dark. Colonies will be kept in vermiculite-filled plastic containers, which will be suspended on plastic pylons in a moat of water with a surfactant to break the surface tension, preventing mite escape. Colonies will be fed every 2 days with bee pollen.

1. We will analyze differences in headspace VOCs extracted from both RRV-infected and uninfected roses using paired Gas Chromatography-Mass Spectrometry and perform a Principal Component Analysis to determine which compounds are good candidates for testing *A. swirskii* attraction.
2. We will record the responses of *A. swirskii* mites with two-arm olfactometer assays to determine the attractiveness to the selected compounds and analyze the results using χ^2 -squared tests.

3. If there are significant differences in *A. swirskii* attraction towards single compounds or blends with Y-tube assays, we will perform a series of tests with four-arm olfactometer assays to compare *A. swirskii* choices between different attractive compounds.

4.2.0.7 3.3.3 Potential benefits

A. swirskii are compatible with some pesticides and existing bio-control methods. *A. swirskii* feed on other pests as well, so they can integrate with existing pest management programs. Developing a bio-control option for RRD can increase profitability for rose growers, reduces losses from RRV-infection, and reduce the number of pesticide applications required. Reduced reliance on pesticides promotes the health and safety of growers as well as protecting the environment. Reducing pesticide usage can also prevent *P. fructiphilus* from developing pesticide resistance and decrease risks for pollinators which frequent roses. Predatory mites are low-maintenance to rear and deploy. Mites subsist in the environment without decreasing sustainability in other areas. Overall, predatory mites represent great value for sustainable agriculture.



Figure 8: *Amblyseius swirskii* attraction to Methyl Salicylate (MeSA) and D-L Limonene vs Filtered Air at concentrations of 1 g/mL. 100 m of chemical was applied to 3 cm dental wicks inside of erlenmeyer flasks which were part of the air line attached to the olfactometer. Asterisks represent significant differences as calculated by χ^2 -squared contingency table tests for given probabilities. N.S. = not significant. MeSA vs Air: χ^2 -squared = 0.48649, df = 1, a = 0.05, p-value = 0.4855. D-L Limonene vs Air: χ^2 -squared = 0.94737, df = 1, a = 0.05, p-value = 0.3304.

4.2.0.8 3.3.4 Expected outcomes

Potential outcomes of observing predatory mite behaviors are:

- Identify which RRV-infected rose compounds are attracting *A. swirskii*
- Determine rate of *A. swirskii* predation on *P. fructiphilus*

Nursery managers are interested in alternative and less expensive management options to combat *P. fructiphilus* and Rose Rosette Virus. Predatory mites have potential to fulfill this need: Mites from the family Phytoseiidae are being investigated as biocontrol agents for the management of *P. fructiphilus*. Preliminary data suggest that the phytoseiid mite *A. swirskii* is attracted to volatiles of Rose Rosette-infected roses. This attraction may have synergistic effects for *P. fructiphilus* control. *A. swirskii* This research will contribute testing the suitability of *A. swirskii* as a biocontrol option for the management of *P. fructiphilus*.

CHAPTER 5

INTEGRATED PEST MANAGEMENT OF *PHYLLOCOPTES FRUCTIPHILUS*

5.0.1 Introduction

Rose Rosette Virus (RRV) is a lethal emaravirus (Emaraviridae) vectored by *Phyllocoptes fructiphilus* Kiefer as it feeds (Allington et al. 1968, Laney et al. 2011). RRV creates a condition known as Rose Rosette Disease (RRD), with the following symptoms: witches' brooms/rosetting, deformed flowers, increased prickle density, elongated shoots, reddened leaves and stems, increased die-back and ultimately rose death. RRV and *P. fructiphilus* are widely distributed in the US, and both virus and mite are present in the southeastern United States (Solo 2018), and *P. fructiphilus* has been recently detected in Florida (Fife et al. 2020). The presence of *P. fructiphilus* in Florida emphasizes the need to monitor and manage both mite and virus, to prevent establishment of RRV in Florida. Currently, no roses are known to resist *P. fructiphilus* and few roses show signs of resistance to RRV (Di Bello et al. 2017). Horticulturists manage the disease by removing sick plants and spraying pesticides in an attempt to kill the mite vector. Pesticides are under increased public scrutiny due to concerns about health, the environment, and harm to pollinators. Although eriophyid mites are often controlled via chemical means (Messing and Croft 1996, Leeuwen et al. 2009), a handful of species have developed resistance to some acaricides, including *Phyllocoptura oleivora* (Ashmead) and *Acalitus vaccinii* (Keifer) becoming resistant to dicofol (Omoto et al. 1994, 1995) while *Aculus cornutus* (Banks) and *Aculops lycopersici* (Tryon) have developed resistance to various organophosphates (Baker 1979, Abou-Awad and El-Banhawy 1985). The lack of management options for mites, as well as the increased cost of rose production due to RRV make it difficult for growers to compete with an increasingly competitive international market. Rose growers need better methods to combat *P. fructiphilus* and RRV.

Integrated pest management (IPM) techniques may have the potential to address some of the shortcomings of current control methods (**citation?**). All viable combinations

of chemical and biological control are used in IPM to reduce pest populations to below the economic injury level of the crop in question (**citation?**). By not relying on a single product or method for pest control, IPM is thought to delay or prevent the development of pesticide resistance (**citation?**).

A major aspect of integrated pest management in crops relies on the plant's natural defenses [].

innate immune system (Nurnberger et al. 2004). Plants naturally have resistance genes which protect plants against certain pathogens (Grennan 2006), as well as a suite of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) which are able to recognize parts of invasive pathogens which are not present in plants, such as flagellar proteins (Nurnberger et al. 2004). Plants respond to pathogen detection with the activation of signalling pathways (Biere and Bennett 2013, Gozzo and Faoro 2013). The type of biochemical response is multivariate, based on the pathways involved and the species of plant in question (**citation?**), but activation of an immune response ultimately results in a variety of biophysical/biochemical changes in plant physiology, such as the thickening of cell walls or production of antimicrobial agents, proteins, reactive oxygen species and other defensive compounds (Chisholm et al. 2006, Jones and Dangl 2006, Kachroo and Robin 2013).

The most well-studied hormones known for triggering plant immunity are Jasmonic Acid, Ethylene, and Salicylic Acid (Thomma et al. 2001).

These signalling pathways respond to particular types of pest and pathogen attacks, and many of the pathways overlap or interact with one another in both complementary and antagonistic ways.

Activation of the Salicylic Acid pathway causes a plant defense response known as known as Systemic Acquired Resistance (SAR) (Gozzo and Faoro 2013). SAR can protect a plant from becoming infected, but SAR can also be activated before infection to increase a plant's resistance to future microbial attack Kalaivani et al. (2016). This is done by

exposing plants to chemicals similar to Salicylic Acid (SA), a defensive phytohormone which initiates the signalling cascade for SAR (Gaffney et al. 1993).

5.1 Inducing Systemic Acquired Resistance with Acibenzolar-S-Methyl to reduce populations of *Phyllocoptes fructiphilus*

Acibenzolar-S-methyl (ASM) is a functional analog of SA, which is able to induce SAR (Ziadi et al. 2001, Tripathi et al. 2010). ASM is currently used by growers to protect plants from fungal infection (Ziadi et al. 2001, Tripathi et al. 2010). ASM application has shown chitinase activity in roses (Suo and Leung 2001) and preliminary studies have shown that ASM might prevent RRV progression in roses (Babu et al., submitted). Mites have an exoskeleton comprised of chitin (Nuzzaci and Alberti 1996), suggesting that rose chitinases affect *P. fructiphilus*'s ability to feed or grow on SAR-induced plants. We intend to test how ASM affects RRV progression and *P. fructiphilus* survival by testing two rates of an ASM-based SAR activator on mite populations in areas with high pest pressure in Georgia. Our hypothesis is that there will be fewer *P. fructiphilus* on plants treated with Actigard when compared to the water treated control group.

5.1.0.1 4.1.4 Materials & Methods

Roses This will be a 12-week experiment conducted from August to October simultaneously in Griffin, GA and Athens, GA. Each site will be given 48 Pink Double Knock Out® Roses (Star Roses and Plants, West Grove, PA, USA) which will be planted in 1 gallon buckets filled with potting soil and mixed with granular slow-release fertilizer. Plants will be placed on black plastic mulch and be watered weekly with overhead impact sprinklers.

Mites *Phyllocoptes fructiphilus* are present in the landscape of Georgia. Tissue from RRD-infected roses will be placed onto roses during the first week and 5th week

Spray rates We will be applying the Acibenzolar-S-methyl (ASM) based Actigard50WG® (Syngenta AG, Basel, Switzerland), at two different rates: 50 mg/L (Half

rate) and 100 mg/L (High rate) to observe the effects of inducing Systemic Acquired Resistance (SAR) on *Phyllocoptes fructiphilus* Kiefer. We will have two field sites in Georgia with local populations of *P. fructiphilus*: Griffin GA and Athens, GA. The tests in Griffin will have two controls for chemical applications in this experiment: The first will be the miticide Kontos (Bayer CropScience LP, Cary, NC, USA), at the label rate as a positive control and the second will be water as a negative control. The tests in Athens will have a control of untreated roses as well as water as a negative control.

Data Collection Rose/rosebud cuttings ~10 cm will be taken from each plant before the first treatment to determine the initial populations of *P. fructiphilus* on the roses. We will then take a subset of samples from each rose treatment weekly, rotating samples until each rose plant has been sampled three times. We will also collect samples from all roses at the end of the trial. This experiment will be repeated for two seasons. Rose samples will be placed in 50 mL centrifuge tubes and refrigerated or frozen until floral samples can be processed. Samples will be processed using the washing methods of Monfreda et al. (2007), eriophyoid mites will be counted and identified as previously described.

Plot Design - 2018



Figure 10: Field design for testing the potential of Acibenzolar-S-Methyl to reduce populations of *P. fructiphilus* by inducing Systemic Acquired Resistance in Pink Double Knock Out® roses. Trials were conducted for three months from August to October 2018 in Griffin, GA. Four treatments were applied weekly for 12 weeks: Blue = Water Red = Actigard50WG 100 mg/L (High rate), Pink = Actigard50WG 100 mg/L (Half rate) Turquoise = Kontos (Label rate). Flower cuttings were taken weekly to record *P. fructiphilus* numbers.

Plot Design - 2018

Infected Rose Patch													
Treatments:	Water				Actigard 100 mg/L				Actigard 50 mg/L				N→
	Kontos (Acaricide)												
Row 1	A1	B1	C1	D1	C4	D4	B4	A4	D7	A7	B7	C7	D10 A10 B10 C10
Row 2	A2	B2	C2	D2	C5	D5	B5	A5	D8	A8	B8	C8	D11 A11 B11 C11
Row 3	A3	B3	C3	D3	C6	D6	B6	A6	D9	A9	B9	C9	D12 A12 B12 C12

Figure 10: Field design for testing the potential of Acibenzolar-S-Methyl to reduce populations of *P. fructiphilus* by inducing Systemic Acquired Resistance in Pink Double Knock Out® roses. Trials were conducted for three months from August to October 2018 in Griffin, GA. Four treatments were applied weekly for 12 weeks: Blue = Water Red = Actigard50WG 100 mg/L (High rate), Pink = Actigard50WG 100 mg/L (Half rate) Turquoise = Kontos (Label rate). Flower cuttings were taken weekly to record *P. fructiphilus* numbers.

Plot Design - 2019

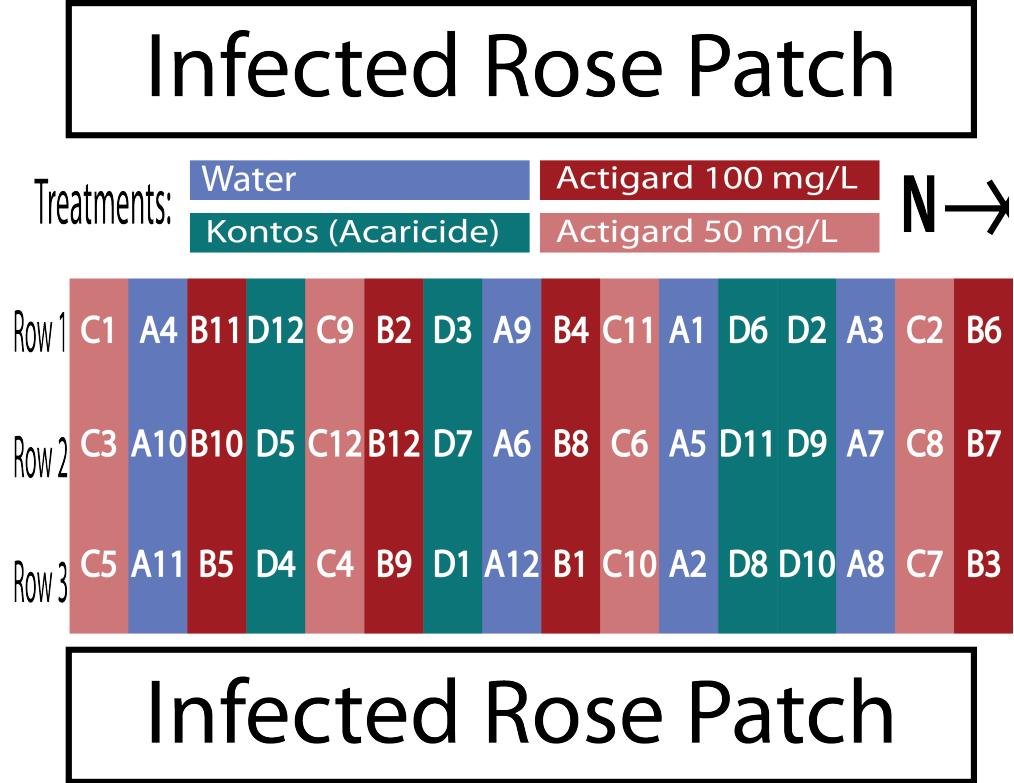


Figure 11: Field design for testing the potential of Acibenzolar-S-Methyl to reduce populations of *P. fructiphilus* by inducing Systemic Acquired Resistance in Pink Double Knock Out® roses. Trials were conducted for three months from September to December 2019 in Griffin, GA. Four treatments were applied weekly for 12 weeks: Blue = Water Red = Actigard50WG 100 mg/L (High rate), Pink = Actigard50WG 100 mg/L (Half rate) Turquoise = Kontos (Label rate). Flower cuttings were taken weekly to record *P. fructiphilus* numbers.

5.2 4.2 Integrating Pest Management Methods to control *Phyllocoptes fructiphilus*

5.2.0.1 4.2.1 Problem Statement

5.2.0.2 4.2.2 Proposal: We propose testing various pest management treatments including predatory mites to reduce populations of *Phyllocoptes fructiphilus*

We propose testing two different SAR-inducers as well as predatory mites for their ability to reduce populations of *P. fructiphilus*. We also intend to combine the effects of

predatory mites with a SAR-inducer to determine if these treatments are compatible. All testing will be done in areas with high pest pressure in Georgia. Our hypothesis is that there will be fewer *P. fructiphilus* on plants treated with the SAR-inducers when compared to the water treated control group, and even fewer mites found on plants treated with the combination of a SAR-inducer and predatory mites.

5.2.0.3 4.2.3 Materials & Methods

Our studies are designed to investigate if predatory phytoseiid mites such as *A. swirskii* can be combined with roses' natural systemic activated resistance (SAR) to manage populations of the plant-parasitic mite, *P. fructiphilus*, the vector of Rose Rosette Virus (RRV). Our findings will be used to develop Integrated Pest Management (IPM) programs for *P. fructiphilus* management.

Roses This will be a 12-week experiment conducted from August to October simultaneously in Griffin, GA and Athens, GA. The Athens site will be given 96 Pink Double Knock Out® Roses (Star Roses and Plants, West Grove, PA, USA), while Griffin will use 54 roses due to the smaller plot area available. Bare root roses will be planted 2 months before the trials begin to allow new flush to form. Rose planting media and environmental conditions will be the same as previously described.

Mite Infestation *Phyllocoptes fructiphilus* are present in the landscape of Georgia. Rose cuttings ~10 cm will be taken from roses showing symptoms of Rose Rosette Disease in the landscape and placed in each rose pot on the 1st, 5th and 9th week of the experiment.

Predatory mites

Amblyseius swirskii mites will be applied on the 1st, 5th and 9th week of the experiment. These mites are deployed from polyethylene fiber sachets containing live colonies of *A. swirskii* and a mite which they consume for food. There is a small hole at the bottom of these sachets which allows the mites to be slowly released into the environment.

Field Treatments

1. Water - Control
2. Actigard - 100 mg/L
3. Ninja - label rate
4. Kontos - label rate
5. *A. swirskii* (one sachet per rose treated)
6. *A. swirskii* + Ninja (one sachet per rose treated, label rate)

Data Collection

Georgia collaborators will be collecting flower samples from all roses once before beginning the treatments on week 1 and once at the end of the experiment on week 12. For weeks 2 through 11, Georgia collaborators will collect flower samples starting from the top rows of each block every week, until each row has been sampled three times (see *Figure 12* and *Figure 13*). Georgia collaborators rate disease severity for each rose every week before they spray, rating roses according to the Horsfall-Barratt Scale (Horsfall 1945). Roses displaying symptoms of RRD will have tissues sent to the Plant Disease Diagnostic Clinic at the North Florida Research and Extension Center(PDC) for virus confirmation.

Sample Processing

- A flower cutting of about ~12 cm will be taken and placed the flower petal side down into 50 ml centrifuge tubes filled with 15 ml of 95% ethanol so the entire flower is submerged over the sepals. Once the lid is secure, the tube will be shaken vigorously for a few seconds to help dislodge any mites. Samples will be processed using the washing methods of Monfreda et al. (2007), eriophyoid mites will be counted and identified as previously described.

Plot Design - Athens

The site at Athens, GA has space for five blocks: A, B, C, D and E. Each block is a 3 × 6 plot with 18 plants, with three plants in each treatment. The experiments will be run for 12 weeks. We will be sampling flower cuttings from two rows each week, starting with

the top rows (1-15 and 16-30 for week one) of each block and rotating to the next row each week (31-45 and 46-60 on week 2) continuing until all rows have been sampled three times. In order to avoid confusion, each rose pot will be labeled with a stake that has the plant number and treatment abbreviation: (W, A, K, M, N, +) written on it. Applications will be done on the same day each week, weather permitting, preferably at the beginning of the week.

Water			Actigard			Kontos		
Mites			Ninja			Ninja + Mites		
Week 1	1	2	3	4	5	6	7	8
	16	17	18	19	20	21	22	23
	31	32	33	34	35	36	37	38
	46	47	48	49	50	51	52	53
	61	62	63	64	65	66	67	68
	76	77	78	79	80	81	82	83
Week 2	10	11	12	13	14	15		
	25	26	27	28	29	30		
	40	41	42	43	44	45		
	55	56	57	58	59	60		
	70	71	72	73	74	75		
	85	86	87	88	89	90		

Figure 12: Field design for Integrated Pest Management trials on Pink Double Knock Out® roses to control *P. fructiphilus* in Athens, GA with five treatments. W = Water A = Actigard50WG, K = Kontos, M = *A. swirkii* predatory mite sachets, N = SP2700 (Trade name: Ninja, SePro), + = *A. swirkii* + Ninja combined treatments. All products were applied at their label rates for 12 weeks. Flower cuttings were taken weekly to record *P. fructiphilus* numbers.

Plot Design - Griffin

The site at Griffin, GA has space for three blocks: X, Y, and Z. Each block is a 3×6 plot with 18 plants, with three plants in each treatment. This experiment was run for 12 weeks as well. We will be sampling flower cuttings from two rows each week, starting with

the top rows (1-9 and 10-18 for week one) of each block and rotating to the next row each week (19-27 and 28-36 on week 2) continuing until all rows have been sampled three times.

Labels and applications were conducted in the same manner as previously described.

Water	Actigard	Kontos
Mites	Ninja	Ninja + Mites
1	2	3
10	11	12
19	20	21
28	29	30
37	38	39
46	47	48
4	5	6
13	14	15
22	23	24
31	32	33
40	41	42
49	50	51
7	8	9
16	17	18
25	26	27
34	35	36
43	44	45
52	53	54

Figure 13: Field design for Integrated Pest Management trials on Pink Double Knock Out® roses to control *P. fructiphilus* in Griffin, GA with five treatments. W = Water A = Actigard50WG, K = Kontos, M = *A. swirkii* predatory mite sachets, N = SP2700 (Trade name: Ninja, SePro), + = *A. swirkii* + Ninja combined treatments. All products were applied at their label rates for 12 weeks. Flower cuttings were taken weekly to record *P. fructiphilus* numbers.

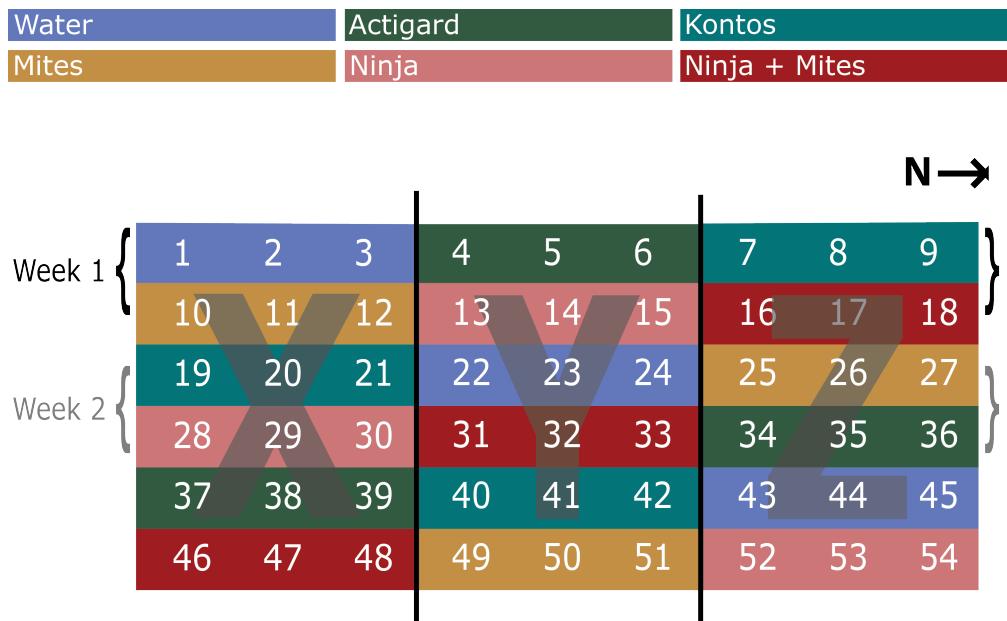


Figure 14: Field design for Integrated Pest Management trials on Pink Double Knock Out® roses to control *P. fructiphilus* in Tallahassee, FL with five treatments: Water, Actigard50WG, Kontos, *Amblyseius swirkii* predatory mite sachets, and *A. swirskii* + Actigard combined treatments. All products were applied at their label rates for 12 weeks. Flower cuttings were taken weekly to record *P. fructiphilus* numbers.



Figure 15: Number of *Phyllocoptes fructiphilus* found in rose samples with five treatments. Statistical significance was determined using Tukey contrasts for multiple Comparisons of means. Groups which share letters are not statistically different from one another. a = 0.05

5.2.1 *Brevipalpus*-transmitted orchid fleck virus infecting three new ornamental hosts in Florida

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6617

5.2.2 Abstract

We describe the first detection of orchid fleck virus (OFV) infecting three unreported hosts: *Liriope muscari*, cv. ‘Gigantea’ (Decaisne) Bailey, *Ophiopogon intermedius* Don and *Aspidistra elatior* Blume (Asparagaceae: Nolinoidaea) in Leon and Alachua Counties, FL. The orchid-infecting subgroup (Orc) of OFV infects over 50 plant species belonging to the plant families Orchidaceae, Asparagaceae (Nolinoidaea), and causes citrus leprosis disease in *Citrus* (Rutaceae). The only known vectors of OFV-Orc are the flat mites, *Brevipalpus californicus* (Banks) *sensu lato* (Trombidiformes: Tenuipalpidae). Florida has various plants in the landscape which *Brevipalpus* spp. feed on, which are susceptible to infection by OFV-Orc. Chlorotic ringspots and flecking were seen affecting Liriopogons (*Liriope* and *Ophiopogon* spp.) in Leon County, FL. Nearby *A. elatior* also appeared chlorotic. Local diagnostics returned negative for common plant pathogens, therefore new samples were sent to the Florida Department of Agriculture and Consumer Services (FDACS) and USDA-ARS for identification. Two orchid-infecting strains of OFV were detected via combinations of conventional RT-PCR, RT-qPCR, Sanger sequencing and High Throughput Sequencing (HTS). Amplicons shared 98% nucleotide identity with OFV-Orc1 and OFV-Orc2 RNA2 genome sequences available in NCBI GenBank. Coinfections were detected in each county, but single strains of OFV-Orc were detected in *L. muscari* (Alachua, OFV-Orc2) and *A. elatior* (Leon, OFV-Orc1). Three potential mite vectors were identified via cryo-scanning electron microscopy (Cryo-SEM): *Brevipalpus californicus* (Banks) *sensu lato*, *B. obovatus* Donnadiieu, and *B. confusus* Baker. In conclusion, OFV orchid strains are present in northern Florida, representing a risk for susceptible plants in the southeastern US.

5.2.3 Resumen

Se describe la primera detección del virus de orchid fleck virus (OFV), infectando a tres huéspedes no reportados: *Liriope muscari*, cv. ‘Gigantea’ (Decaisne) Bailey, *Ophiopogon intermedius* Don and *Aspidistra elatior* Blume (Asparagaceae: Nolinoidaea)

para los condados de Leon y Alachua, FL. Los subgrupos de OFV que infectan a las orquídeas (Orc) puedan infectar más de 50 especies de plantas pertenecientes a las familias Orchidaceae, Asparagaceae (Nolinoidaea), e infecta *Citrus* (Rutaceae) como la enfermedad de la leprrosis de los cítricos. Los únicos vectores de OFV-Orc son los ácaros planos *Brevipalpus californicus* (Banks) *sensu lato* (Trombidiformes: Tenuipalpidae). La Florida tiene varias plantas en el campo ornamental, por lo cuáles las especies de *Brevipalpus* se puedan alimentarse, y esas plantas son susceptibles a las infecciones de OFV-Orc. Se observaron manchas anulares cloróticas y salpicaduras en las hojas de las Serpentinás (*Liriope* y *Ophiopogon* spp.) y también se vieron hojas cloróticas en el *A. elatior* adyacente, situado en el condado de Leon, FL. Los diagnósticos del laboratorio local fueron negativos para los patógenos comunes, por lo tanto, se enviaron nuevos ejemplares al Florida Department of Agriculture and Consumer Services (FDACS) y el USDA-ARS para su identificación. Se detectaron dos cepas del OFV mediante la combinación de RT-PCR convencional, RT-qPCR, secuenciación de Sanger y secuenciación de alto rendimiento. Las ampliaciones compartían una identidad de nucleótidos del 98% con las secuencias del genoma de ARN2 de OFV-Orc1 y OFV-Orc2 disponibles en el NCBI GenBank. Se detectaron coinfecciones del virus en cada condado, pero se detectaron cepas únicas de OFV-Orc en *L. muscari* (Alachua , OFV-Orc2) y *A. elatior* (Leon , OFV-Orc1). Se identificaron tres ácaros mediante microscopía electrónica de barrido criogénico (Cryo-SEM) con potencial de ser vectores: *Brevipalpus californicus* (Banks) *sensu lato*, *B. obovatus* Donnadiue y *B. confusus* Baker. En conclusión, OFV está presente en el norte de Florida, lo que representa un riesgo para las plantas susceptibles en el sureste de Estados Unidos.

5.2.4 Keywords:

False spider mite, flat mite, *Brevipalpus*-transmitted viruses, *Liriope*, Nolinoidaea, *Ophiopogon*, *Aspidistra*, Ruscaceae, Rutaceae, Asparagaceae, orchid, Orchidaceae, pests, ornamental plants, orchid fleck virus.

Orchid fleck virus (OFV), is the type member for the genus *Dichorhavirus*, family *Rhabdoviridae*. The virus is a bacilliform, nuclear rhabdovirus composed of two segments of single-stranded, negative-sense RNA which infects plants (Dietzgen et al. 2014, Walker et al. 2018, Amarasinghe et al. 2019). Only Flat mites (Trombidiformes: Tenuipalpidae) from the genus *Brevipalpus* are known to transmit dichorhaviruses (Maeda 1998). Plants infected with OFV exhibit chlorotic and necrotic flecks on their leaves(Kubo et al. 2009b, Kubo et al. 2009a, Dietzgen et al. 2018b). The virus was first described as infecting *Cymbidium* orchids in Japan (Doi et al. 1977). There have been reports of OFV and OFV-like rhabdoviruses infecting orchids in Asia, Africa, North America, South America, Europe, and Oceania. The prevalence of OFV and its mite vector is thought to be associated with the movement of infected orchids (Dietzgen et al. 2018a). More than fifty species of Orchidaceae (Kitajima et al. 2010, Peng et al. 2013) can naturally become infected with OFV, as well as some Asparagaceae (Nolinoidaea) (Mei et al. 2016, Dietzgen et al. 2018b), and Rutaceae, where infection causes citrus leprosis-like symptoms (Roy et al. 2015, 2020, Cook et al. 2019, Olmedo-Velarde et al. 2021). Mechanical transmission of OFV is possible under laboratory conditions to the plant families Chenopodiaceae, Aizoaceae, Fabaceae, and Solanaceae (Chang et al. 1976, Kondo et al. 2003, Peng et al. 2013).

5.2.4.1 Virus Detection

During June 2020, chlorotic flecks and ringspot patterns of unknown etiology were observed on Giant Liliy turf *Liriope* spp., cv. ‘Gigantea’ in a landscape of Leon County, Florida (Figure 16). *Liriope* belong to a group of plants in the family Asparagaceae, subfamily Nolinoidaea, comprised of grass-like monocotyledonous liliod plants native to southeastern Asia (Chase et al. 2009, Meng et al. 2021). *Liriope* and the closely related *Ophiopogon* (Asparagaceae: Nolinoidaea) are considered the most important ground cover plant in the southeastern United States (Mcharo et al. 2003). Viral infections of suspected leaf samples were initially tested at the Plant Disease Diagnostic Clinic at the North

Florida Research and Education Center (NFREC) in Quincy, FL. All the samples were tested with one step conventional RT-PCR, and were found negative for begomovirus, carlavirus, potyvirus, tospovirus, cucumber mosaic virus and tobacco mosaic virus. As initial diagnostics were inconclusive, samples were taken of putatively infected plants with ringspot symptoms during July and August of 2020. Leaves were taken from *Liriope* spp. and *Ophiopogon* spp., as well as the *Aspidistra elatior* Blume (Asparagaceae: Nolinoidae), nearby, which appeared sickly and chlorotic (Figure 17). Plant materials were sent to the Florida Department of Agriculture and Consumer Services (FDACS) for identification. The FDACS determined that the pathogen was OFV using previously published primers and methods to conduct RT-PCR and Sanger sequencing (Kubo et al. 2009b, Kubo et al. 2009a, Ramos-González et al. 2015). The identity of the virus was verified as OFV Orchid strain 1, (OFV-Orc1), following the methods described in Kondo et al. (2017). Nucleotide sequencing shared 98% nucleotide identity with the OFV-isolates So (Accession No. AB244418) and Br (Accession No. MK522807), which belong to orchid subgroup I (Kondo et al. 2006, 2017). These samples from FDACS were subsequently retested by the USDA-APHIS-PPQ S&T Beltsville laboratory, in conjunction with tests of fresh samples from both Alachua and Leon counties. The USDA used RT-PCR, RT-qPCR, and High Throughput Sequencing (HTS) to reconfirm the presence of OFV. Conventional RT-PCR with Generic R2-Dicho-GF and R2-Dicho-GR primers amplified ~800 nt amplicons of the L-gene (RNA2) (Roy et al. 2020), to detect both OFV-Orc1 and OFV-Orc2 in *O. intermedius* and *A. elatior* from Leon County (Kondo et al. 2017) (GenBank Accession Numbers: MZ852004, MZ852005 MZ852006, and MZ852007). 99% nucleotide sequence identity is shared between OFV-Orc1 and OFV-Orc2 for the RNA2 genome, whereas 90% sequence identity was found between these two reassortment strains. The presence of OFV-Orc1 and OFV-Orc2 in Leon and Alachua counties was reaffirmed with HTS data (Table 1): Analysis of HTS data from Leon County found that the symptomatic *L. muscari* were coinfecte^d with both OFV-Orc1 and OFV-Orc2, while the symptomatic *A.*

elatior were solely infected with OFV-Orc1. Sequence data of symptomatic *L. muscari* from Alachua County revealed infections with OFV-Orc2 (GenBank Accession MZ852006). After the initial identification by FDACS of OFV-Orc, mite samples were collected from symptomatic Asparagaceae in Leon County. Most mites collected were Tenuipalpid mites (flat mites or false spider mites), a pest of ornamental plants, some of which are known to act as vectors for plant viruses (Childers et al. 2003, Childers and Rodrigues 2011).

5.2.4.2 Mite Description

Mite taxonomy is complicated by cryptic species complexes which occur in many plant-feeding groups of the Acari (Umina and Hoffmann 1999, Skoracka and Dabert 2010, Arthur et al. 2011, Skoracka et al. 2013), including tenuipalpid mites from the genus *Brevipalpus* (Navia et al. 2013). The commonly used phase-contrast microscopy is insufficient to detect some diagnostic characters for separation of cryptic species, instead best practices recommend the combination of Differential Interference Contrast (DIC) Microscopy and Scanning Electron Microscopy along with molecular methods to separate cryptic species (Beard et al. 2015). The flat mites collected were initially suspected to belong to *B. californicus* after inspection with phase contrast microscopy. Subsequent observation via DIC microscopy at FDACS agreed with this tentative identification. Unfortunately, the *B. californicus* s.l. species group, *sensu* Baker and Tuttle (1987) is suspected to contain cryptic species (Childers and Rodrigues 2011, Rodrigues and Childers 2013). New mite samples were collected from symptomatic liriopogons and *A. elatior* in Leon County and sent to USDA-ARS's Electron and Confocal Microscopy Unit for analysis. Three mite species were recovered and examined under cryo-scanning electron microscopy (Cryo-SEM): *B. californicus* s.l. (Figure 18), *B. obovatus* Donnadieu and *B. confusus* Baker. The recent report of OFV in the US is thought to be Ko et al. (1985) which describes nuclear inclusions caused by an undescribed bacilliform rhabdovirus in *Brassia* orchids. The significance of this report is their description of the spoke-wheel configurations of the viral particles (Ko et al. 1985), a sign typically associated with

OFV infection (Chang et al. 1976). Unfortunately, this article made no mention of mites or further investigations of the virus. The first report of OFV in the continental US was Bratsch et al. (2015), who confirmed the presence of OFV in *Phalaenopsis* hybrids using Transmission Electron Microscopy of ultrathin sections of plant tissue as well as molecular sequence analysis. They also discuss the association of OFV with *Brevipalpus* mites, but the authors did not make a conclusive species identification beyond suggesting that the mite vector belonged to the *B. californicus* group, referring to Kondo et al. (2003)'s publication (Bratsch et al. 2015). Later reports of OFV described OFV infecting a previously undescribed Nolinoidaea hosts in Australia (Mei et al. 2016, Dietzgen et al. 2018b), including *Liriope spicata* (Thunb.) Lour, a different species of liriopogon than those identified from the Florida sites. We are not aware of any reports of OFV infecting liriopogons, *A. elatior* nor other Nolinoidaea in the US. Although Zheng et al. (2013) had mentioned an association between *B. californicus* and *A. elatior*, they never reported symptoms of OFV-Orc in this plant. We believe that our findings indicate the first report of OFV-Orc infecting ornamental Nolinoidaea in Florida, and possibly the US. This publication also marks the first reports of *A. elatior* and *Ophiopogon* spp. as natural hosts of OFV-Orc. There are two orchid strains of OFV (OFV-Orc1 and OFV-Orc2), and two citrus strains (OFV-Cit1 and OFV-Cit2) (Beltran-Beltran et al. 2020, Roy et al. 2020). The OFV strains detected in Florida are identical in genome sequence to the orchid strains of OFV infecting citrus in Hawaii, Mexico, Colombia, and South Africa (Beltran-Beltran et al. 2020, Roy et al. 2020). Both OFV-Orc1 and OFV-Orc2 infect citrus (Roy et al. 2020), but none of the citrus strains have been reported from any orchid species. The *Brevipalpus* mites collected from liriopogons and *A. elatior* in Leon County were abundant on OFV-infected plants very near to citrus trees, some plants even surrounding the trunk. *B. californicus* s. l. has been reported as a pest of citrus (Childers et al. 2003) and are often collected from citrus fruits (Baker 1949, Baker and Tuttle 1987, Vacante 2010, 2016). The proximity of these mite vectors to citrus raises the question: why these

trees are not currently infected with OFV-Orc? It is important to note the uncertainty surrounding the vector for OFV-Orc. There are three mite species which have been recovered from OFV-Orc infected plants: *B. obovatus*, and *B. confusus* and *B. californicus* s.l., but only *B. californicus* has been described as a vector of OFV. Even so, the *B. californicus* which we find on liriopogons and *A. elatior* may not be the same cryptic species as those found on citrus. Transmission of OFV from populations of *B. californicus* liriopogon/*A. elatior* to citrus may be limited by host preferences, vectorial capacity, viral propagation/circulation in the vector, viral acquisition times, or feeding times required for transmission to citrus. Even so, these types of questions require future study to determine the potential of nolinoidaea to citrus transmission. Best practices for integrated pest management have not been created for controlling *Brevipalpus* mites on these ornamentals, but methods designed to control *Brevipalpus* in other systems may be applicable. The most common method used to control *Brevipalpus* are synthetic acaricides (Andrade et al. 2010, 2019). Unfortunately, some acaricides and their residues can harm beneficial predatory mites as well (Fernández et al. 2017), even at low doses (Havasi et al. 2021), and mixing different chemistries can be detrimental for mite control (Vechia et al. 2018). In addition, pesticide resistance has been reported in various *Brevipalpus* populations (Alves et al. 2000, Omoto et al. 2000, Campos and Omoto 2002, Rocha et al. 2021), due to exposure to pesticides used to control other arthropod pests (Vechia et al. 2021). In addition, predatory mites (Chen et al. 2006, Argolo et al. 2020), entomopathogenic fungi (Magalhães et al. 2005, Rossi-Zalaf et al. 2008, Peña et al. 2015, Revynthi et al. 2019) have shown promise for controlling other *Brevipalpus* mites. Moreover, it is often possible to integrate different control techniques for improved management, such as combining predatory mites with compatible acaricides and entomopathogenic fungi (Reddy 2001, Midthassel et al. 2016, Andrade et al. 2019). In conclusion, detecting OFV in Florida represents a concern for horticulturists who grow orchids, *Liriope*, *Ophiopogon*, or other susceptible Asparagaceae species which are commonly used in landscaping. Florida is

also home to a plethora of native and naturalized orchid species, many of which are threatened, including cultivated *Vanilla* in southern Florida (Chambers et al. 2019) and the famous Ghost Orchid, [*Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe]. Citrus leprosis was present in Florida during the 1860's and almost eradicated by the mid-1960s (Knorr 1968, Knorr et al. 1968, Childers et al. 2003). An examination of herbarium specimens of Florida citrus found that this historical virus, Citrus leprosis dichorhavirus-N0, is distantly related to the modern isolates of OFV (Kitajima et al. 2011, Hartung et al. 2015, Roy et al. 2020). The recent detection of OFV-Orc1 in South Africa (Cook et al. 2019) in *C. sinensis* (Navel and Valencia orange) and OFV-Orc2 in Hawaii (Olmedo-Velarde et al. 2021) in *C. reticulata* (mandarin) and *C. jambhiri* (rough lemon) associated with leprosis-like symptoms highlights the potential threat of different isolates of OFV on citrus, which will be a definite concern to the US multi-billion-dollar citrus industry already impacted by the Huanglongbing disease. *B. californicus*, *B. yothersi*, and *B. obovatus* are all present in Florida (Childers et al. 2003, Akyazi et al. 2017), and are difficult to identify by non-experts, or without advanced methodologies. DNA barcoding (Armstrong and Ball 2005) or a similarly simple and accurate method for identification of these mite complexes is vital to identify mite populations which need to be monitored or controlled. By doing so, we can determine the risk OFV-Orc represents for the native plants, agriculture and the ornamental/landscaping industries of Florida and the surrounding regions.

5.2.5 Acknowledgements

We would like to give special thanks for the Tallahassee Museum and their patience, cooperation, and support with collecting plant samples. We are grateful for the USDA-APHIS PPQ Beltsville laboratory for their support in the identification and confirmation OFV isolates, as well as *Brevipalpus* mite identification at the USDA-ARS. We also want to thank Drs. Sam Bolton, FDACS and Aline Tassi, Univ. of Sao Paulo, Brazil for checking the mites we have sent for species validation. Furthermore, we are grateful for Dr. Marc S. Frank's identification of the Liriopogons collected. We are especially

indebted to the late Dr. Gary Bauchan for his contributions to this study and the field of acarology, he will be greatly missed. This research was partly funded by the USDA National Institute of Food and Agriculture, Hatch project FLA-NFC-005607. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA; USDA is an equal opportunity provider and employer.

5.2.6 Table 1: List of Asparagaceae (Nolinoidae) species infected with orchid fleck virus, collected from the landscape of northern Florida

Scientific Name	Common Names	County	Strains Detected
<i>Liriope muscari</i> cv. ‘Gigantea’* (Decaisne)	Lilyturf, Orchardgrass, Monkeygrass	Alachua & Leon	OFV-Orc1 & OFV-Orc2
<i>Ophiopogon intermedius</i> ** Don	Aztec Grass, ‘Argenteomarginatus’	Leon	OFV-Orc1 & OFV-Orc2
<i>Aspidistra elatior</i> Blume	Cast Iron Plant, Bar-room Plant	Leon	OFV-Orc1 & OFV-Orc2

Table 1: * *Liriope muscari* cv. ‘Gigantea’ has been traditionally classified as *L. gigantea* Hume by Broussard (2007) and Fantz et al. (2015), although this distinction has been challenged by Wang et al. (2014) and Masiero et al. (2020). ** *O. intermedius* is sometimes misclassified as *Liriope muscari* ‘Variegated Evergreen Giant’ Fantz (2009) or ‘Grandiflora White’ (Fantz 2009). Both OFV-Orc1 and OFV-Orc2 were detected in each species tested, many plants were coinfecte with both strains, see ‘[Virus Detection](#)’.

5.2.7 Figure captions

Figure 16: Variety of symptoms seen on *Liriope* spp. infected with orchid fleck virus (OFV): (a) symptoms on *Liriope muscari* cv. ‘Gigantea’ (b-c) Details of symptoms on *L. muscari* cv. ‘Gigantea’ (d) rust colored spots on *Ophiopogon intermedius*

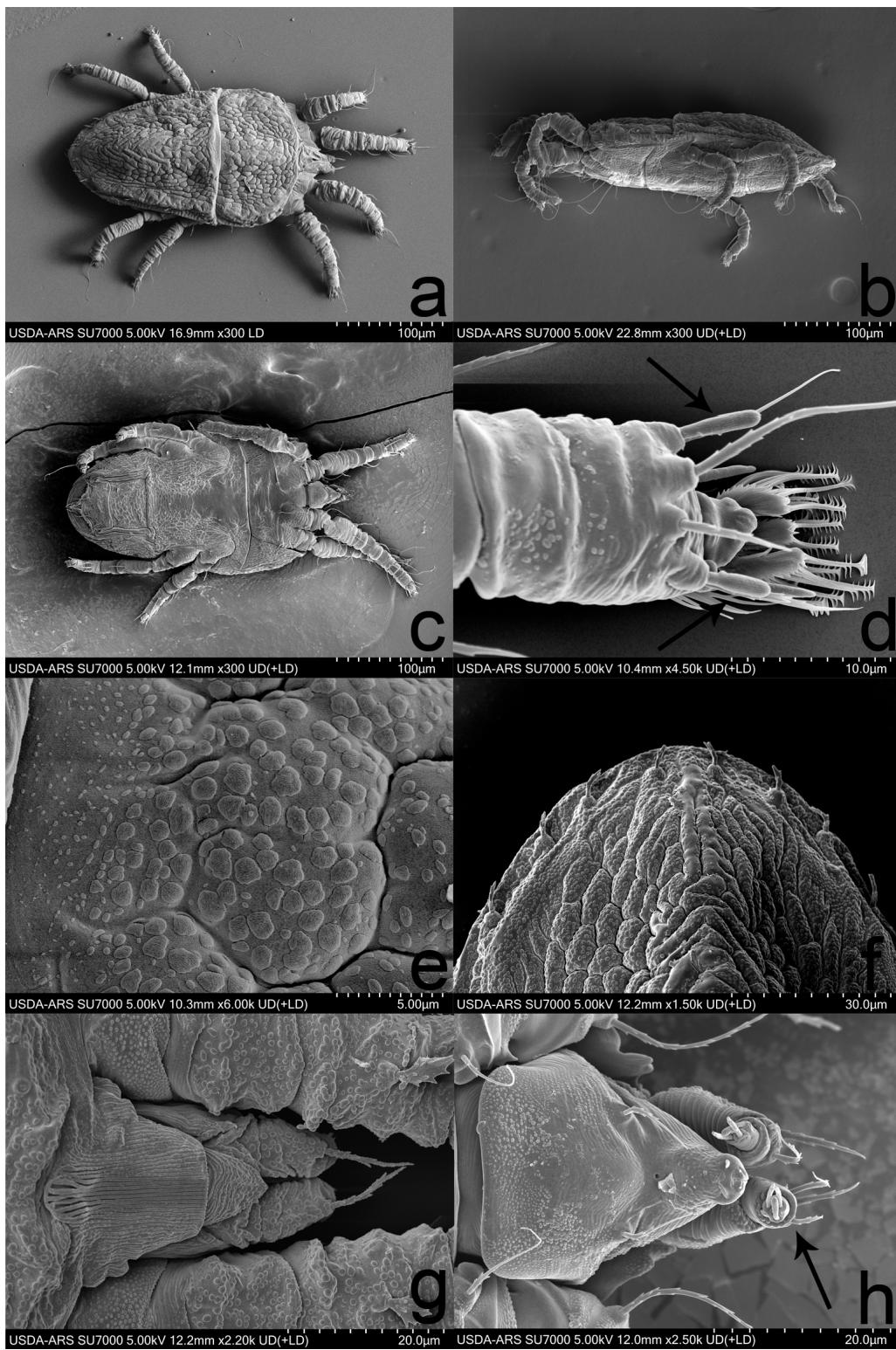
Figure 17: Symptoms seen on *Aspidistra elatior* infected with OFV: (a) Detail of leaf chlorosis (b) Chlorosis appears similar to sun damage (c-d) Chlorotic flecks may indicate early symptoms of OFV

Figure 18: Cryo-SEM images of *Brevipalpus californicus sensu lato* displaying various characters used for identification (Baker and Tuttle 1987, Beard et al. 2015) (a) Dorsum (b) Lateral view (c) Venter (d) Close up of distal end of leg 2, with arrows indicating paired solenidia, characteristic of the genus *Brevipalpus* (e) Enlargement of the microplates of the mite cerotegument (f) Dorsal view of the distal portion of mite abdomen (g) Dorsal view of the mite rostrum (h) Ventral view of mite rostrum, observe 3 distal setae.

5.2.8 Figures







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BIOGRAPHICAL SKETCH

This section is for your biographical sketch. It should be in third person, past tense. Do not put personal details such as your birthday here. Again, to make a full paragraph you must write at least three sentences.