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THE INTERACTIONS BETWEEN THE SPOTTED LANTERNFLY, GRAPEVINES, AND GRAPEVINE PATHOGENS

A Thesis in

Plant Pathology

by

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ABSTRACT

Lycorma delicatula (White), or the spotted lanternfly (SLF), is an insect native to Asia and invasive to the US. The spotted lanternfly was first discovered in 2014 in Pennsylvania, and is now established across several states in the Eastern U.S. The rapid spread of SLF populations across the U.S. is a concern due to the threat the insect poses to agriculture as an aggressive phloem-feeder. Notably, infestations of high numbers of adults in vineyards can cause significant damage to grapevines including decreases in vine growth, decreased fruit yield, and in extreme cases vine death if insect populations are not promptly and effectively managed. These detrimental effects could be exacerbated by other factors such as pathogen infection of grapevines. There are many bacterial and viral diseases that commonly affect grapevines across the U.S., and it is unknown how the SLF will interact with the pathogens that cause these diseases and what impact their combined stress will have on grapevines and vineyards as a whole. The goal of this project was to examine the interactions between SLF, grapevines, and grapevine pathogens. First, the ability of SLF to serve as a vector of important grapevine pathogens was examined. Across three annual experiments in 2020, 2021, and 2022, SLF 4th instar nymphs and adults were found to transmit a phytoplasma associated with North American grapevine yellows at rates of 7% and 5%, respectively. SLF were not found to transmit grapevine red blotch virus as 4th instar nymphs or adults in one year (2021) of experiments. *Xylella fastidiosa* subsp. *fastidiosa*, the causal agent of Pierce's disease (PD), was not transmitted by 4th instar nymph or adult SLF in one year of experiments (2022). Second, the effect of combined biotic stressors (SLF infestation and PD infection) on primary metabolites important for grapevine growth and productivity was investigated. Overall, SLF had a negative impact on end-of-the-season carbohydrate storage, in which vines exposed to SLF for seven weeks had lower

starch concentrations in root and cane tissues regardless of PD infection. Notably, PD-infected vines had higher end-of-the-season nitrogen concentration in canes and roots, independent of SLF presence. In addition, the impact of PD-infected vines on SLF development, mortality, and feeding preference was assessed. SLF showed a decreased rate of development from nymph to adult stage and higher overall mortality on PD-infected vines. Lastly, choice tests revealed that female adult SLF preferred to feed on uninfected over PD-infected vines. Taken together, these experiments provide insight into how SLF may influence existing pathosystems as they continue to invade and colonize vineyards across the U.S. This information could be used in future studies to better predict SLF invasion and risk factors associated with grapevines and their pathogens, ultimately leading to improved response preparedness to invasive species like SLF.

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Chapter 1

Introduction

Plant pathogens have unique relationships with their host plants and insect vectors that have been developed through a history of coevolution and which culminate in effective transmission between hosts. Several plant pathogens such as viruses and bacteria, including phytoplasmas, largely rely on insect vector-mediated transmission and have been shown to influence vector behavior to enhance successful cycles of infection. Effects of pathogens on vectors may be direct or indirect, acting upon the insect itself or through infected plant hosts (Eigenbrode et al., 2018). Host plant defense responses to pathogen infection can alter vector performance, and these plant responses have the potential to affect other non-vector insects as well (Shikano et al., 2017). Vector manipulation by phytopathogens has been documented in multiple systems including those of viruses, bacteria, and fungi (Eigenbrode, et al., 2018).

Most insect vectors of plant pathogens are members of the order Hemiptera, which includes aphids, psyllids, leafhoppers, planthoppers, thrips and whiteflies (Heck, 2018). In order for successful acquisition and transmission to occur, pathogens must undergo specific interactions with their insect hosts. Transmission strategies include combinations of the following characteristics: circulative or non-circulative, persistent or non-persistent, and propagative or non-propagative (Hogenhout, 2008; Dietzgen, 2016). Circulative transmission refers to pathogens that travel through insect tissues such as the insect gut, hemolymph and salivary tissue membranes while non-circulative pathogens are not internalized by the insect gut. Persistency describes pathogens, in which insects

are able to transmit pathogens for long periods of time and often throughout their lifespan and life stages. Non-persistent pathogens can be inoculated by the insect over a much shorter period, typically within minutes of acquisition. Non-persistent pathogens are retained in the stylets of insects, while persistent invade deeper insect tissues. Lastly, propagative pathogens replicate within the insect vector while non-propagative do not (Hogenhout, 2008; Dietzgen, 2016). Pathogens may be described using a combination of these characteristics. For example, circulative non-propagative pathogens travel through the insect gut, hemolymph, and salivary glands but do not replicate within the vector.

The mode of transmission plays an important role in vector-pathogen-host interaction, as factors such as acquisition and retention can influence vector behavior to enhance transmission. For example, Carmo-Sousa et al. (2014) demonstrated that aphids did not prefer to migrate from cucumber mosaic virus (CMV)-infected plants to healthy plants during a short time (up to 30 minutes), but after a longer time (60 minutes) a preference to migrate to healthy plants was observed. In addition, choice assays from the same study showed that aphids preferred to settle and feed on CMV-infected versus healthy plants within 30 minutes of release, then changing to prefer healthy plants at later time points. CMV is transmitted by aphids in a non-persistent manner. These changes in vector behavior enhance virus transmission, in which aphids are influenced to acquire the virus through feeding on infected plants for a sufficient time before migrating to healthy plants for transmission.

Plant pathogens have the potential to influence non-vector insects as well. Plant responses to pathogen infection vary widely from changes in metabolites, volatile release, and external symptomology (Shikano et al., 2017). However, it is difficult to predict how

insects will interact with plant pathogens and their host plants when introduced to a pathosystem. Invasive phytophagous insects have the potential to encounter new pathogens as they invade and colonize new areas, and it is unknown how these insects will influence established systems, such as if the insects will be capable of transmitting certain pathogens or how plants will respond to combined pathogen and insect stress.

The spotted lanternfly (SLF), *Lycorma delicatula* (White), is an invasive insect to the U.S. and represents a current example of the concerns associated with the novel interaction between an invasive insect and plant pathogens. SLF is native to Asia and is now established in the Eastern U.S. (Nixon et al., 2021). The first report of the SLF appearing in the U.S. was in 2014 in Berks County, PA (Barringer, 2015). Infestations have since been established across multiple states in the Northeast, including the states directly surrounding PA, and sightings of SLF reach as far west as Iowa and south to Tennessee and North Carolina. (N.Y. State Integr. Pest Manag., 2025). The rapid spread of SLF populations across the U.S. is a concern due to the threat the insect poses to agriculture. A voracious phloem-feeding insect, the SLF is known to feed on over 70 species of plants, including economically important crops, ornamentals, and hardwoods (Urban et al., 2019). Notably, when the SLF is not effectively managed it has been reported to cause significant damage to grapevines, including a decline in vine growth, yield, and in extreme cases complete vine death (Leach & Leach, 2020; Urban, 2019). In addition to these losses, growers are concerned about the increased insecticide applications needed to manage the SLF. Currently, insecticides serve as the only method available for effective control of SLF, and the increased usage poses an economic drain to growers as well as increased environmental risks (Urban & Leach, 2023). These

detrimental effects are associated with SLF feeding alone; however, they could be exacerbated by other factors such as pathogen infection of grapevines or ability of the SLF to spread disease.

Overall Research Goal

There are many diseases that commonly affect grapevines across the U.S., and it is unknown how the SLF will interact with the pathogens that cause these diseases and what impact their combined stress will have on grapevines and vineyards. The overarching goal of this project is to examine the interaction of the SLF with a subset of pathogens of grapevines. This will lead to a better understanding of SLF behavior and impact on grapevines and ultimately aid in the development of management strategies in vineyards. To address this goal, my project had three main objectives: (1) determine if the SLF is capable of transmitting pathogens of grapevine diseases, (2) to examine the response of grapevines to combined pathogen and SLF stress, and (3) to examine the effect of infected grapevines on SLF behavior.

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Chapter 2

Determining the ability of the spotted lanternfly to transmit grapevine pathogens

Abstract

It is currently unknown if the spotted lanternfly (SLF) (*Lycorma delicatula*) is capable of serving as a vector of plant pathogens. This is especially a concern for grape growers, as SLF are known to invade and to heavily infest vineyards where grapevines may also be impacted by multiple diseases. The objective of this chapter was to determine if SLF are capable of transmitting the pathogens of three diseases of grapevines: North American grapevine yellows (NAGY), caused by phytoplasmas; grapevine red blotch disease, caused by grapevine red blotch virus (GRBV); and Pierce's disease, caused by the bacterium *Xylella fastidiosa* subsp. *fastidiosa.* Classic plant-to-plant transmission assays were conducted on grapevines for each pathogen using 4th instar nymph and adult SLF. These experiments showed that SLF nymphs and adults transmit NAGY at a rate of 7% and 5%, respectively. SLF nymphs and adults were not shown to transmit GRBV. Transmission of *X. fastidiosa* subsp. *fastidiosa* was not confirmed via molecular testing, however symptoms of Pierce's disease were observed on plants subjected to transmission by both 4th instars and adults, and thus further testing is warranted.

Introduction

It is currently unknown if the spotted lanternfly (SLF) (*Lycorma delicatula*) (White), is capable of transmitting plant pathogens. The potential for SLF to transmit pathogens of grapevines is of concern due to the invasion and heavy infestation of SLF in vineyards, where the insects may encounter infected vines. Grapevines are susceptible to a wide variety of insect-transmitted diseases, and the introduction of a new vector could be devastating to growers. It is important to identify vectors and understand their role in pathogen spread in order to effectively manage and mitigate the impact of disease. However, it is difficult to predict if an insect will be able to transmit certain pathogens, due to the wide diversity of insect vectors and their unique interactions with the associated pathogen and plant host. While there are no known vector traits that can be used as standards amongst all insects, insect biology and relationships between known vectors and the introduced insect can be used to examine potential transmission capabilities.

Insect biology can indicate if and how pathogens are transmitted. The insect vector must be capable of taking up the pathogen from the host plant and retaining it in some way before transmitting it to another host plant. Pathogens are transmitted in three modes: persistent, in which the pathogen is taken up and enters the insect host tissues and salivary glands; semi-persistent, in which the pathogen enters the gut lining but is not internalized in tissues of the insect; and non-persistent, in which the pathogen is only retained in the stylet (Dietzgen et al., 2016). Vector-borne pathogens can also be

propagative, in which the pathogen replicates in the insect tissues before reaching the salivary glands for transmission, or non-propagative in which the pathogens do not reproduce in the insect host (Dietzgen et al., 2016). The method of transmission by insect vectors may have been influenced by coevolution of the pathogen, plant, and insect. For example, pathogens may have originated as plant-infecting pathogens and have become adapted to insects as an alternative host, or insect pathogens may have adapted to plants as hosts (Nadarasah & Stavrinides, 2011).

Examining the phylogeny of an insect may suggest vector capabilities. The SLF is a member of the order Hemiptera, which contains most of the major known vectors of plant pathogens (Figure 2.1: Phylogeny of Spotted Lanternfly). This includes common pests such as aphids and psyllids. Insects more closely related to the SLF in the Fulgoroidea superfamily have been subjected to limited research regarding pest or vector status in their native habitat. However, some Auchenorrhyncha members such as delphacids and cixiids contain multiple species have been noted as prominent pests and vectors of pathogens that infect crops (Rodríguez-Juárez et al., 2020; Rodrigues et al., 2023). In fact, at least two species of the Cicadomorpha infraorder have been shown to transmit pathogens in grapevines (Rodrigues et al., 2023). The phylogenetic relationships between SLF and known plant pathogen vectors suggest the possibility of SLF to also act as a vector.

The pathogens chosen for this study are the phytoplasmas that cause the disease North American grapevine yellows (NAGY); grapevine red blotch virus (GRBV); and *Xylella fastidiosa* subsp. *fastidiosa*, the causal bacterial agent of Pierce's disease (PD).

These pathogens are insect-transmitted and are economically important in the U.S., causing reduced fruit production or quality and vine death (Beanland et al., 2006; Yepes et al., 2018; Rapicavoli et al., 2017).

NAGY is caused by phloem-residing, unculturable, cell wall-less bacterial pathogens known as phytoplasmas. Phytoplasmas are classified into groups based on the 16S rRNA gene, with the phytoplasmas associated with NAGY belonging to group 16SrI (‘*Candidatus.* Phytoplasma asteris’-related strains) and 16SrIII (‘*Candidatus.* Phytoplasma pruni’-related strains) (Davis et al., 2018). NAGY is present in vineyards across the Northeastern US, creating concern for growers in the area (Davis et al., 2015). Symptoms of NAGY include yellowing and curling of leaves, shoot die-back, fruit abortion, and eventual vine death (Beanland et al., 2006). Phytoplasmas are known for being transmitted by a wide variety of insects, with some vectors being known to transmit multiple phytoplasmas at once (Weintraub & Beanland, 2006). Phytoplasmas are transmitted by insects in a circulative propagative manner, meaning they must be able to colonize throughout and replicate within the insect during a latent period. The range of the latent period for phytoplasmas can vary greatly depending on the insect host and environmental factors such as temperature (Weintraub & Beanland, 2006).

Phylogeny of Spotted Lanternfly

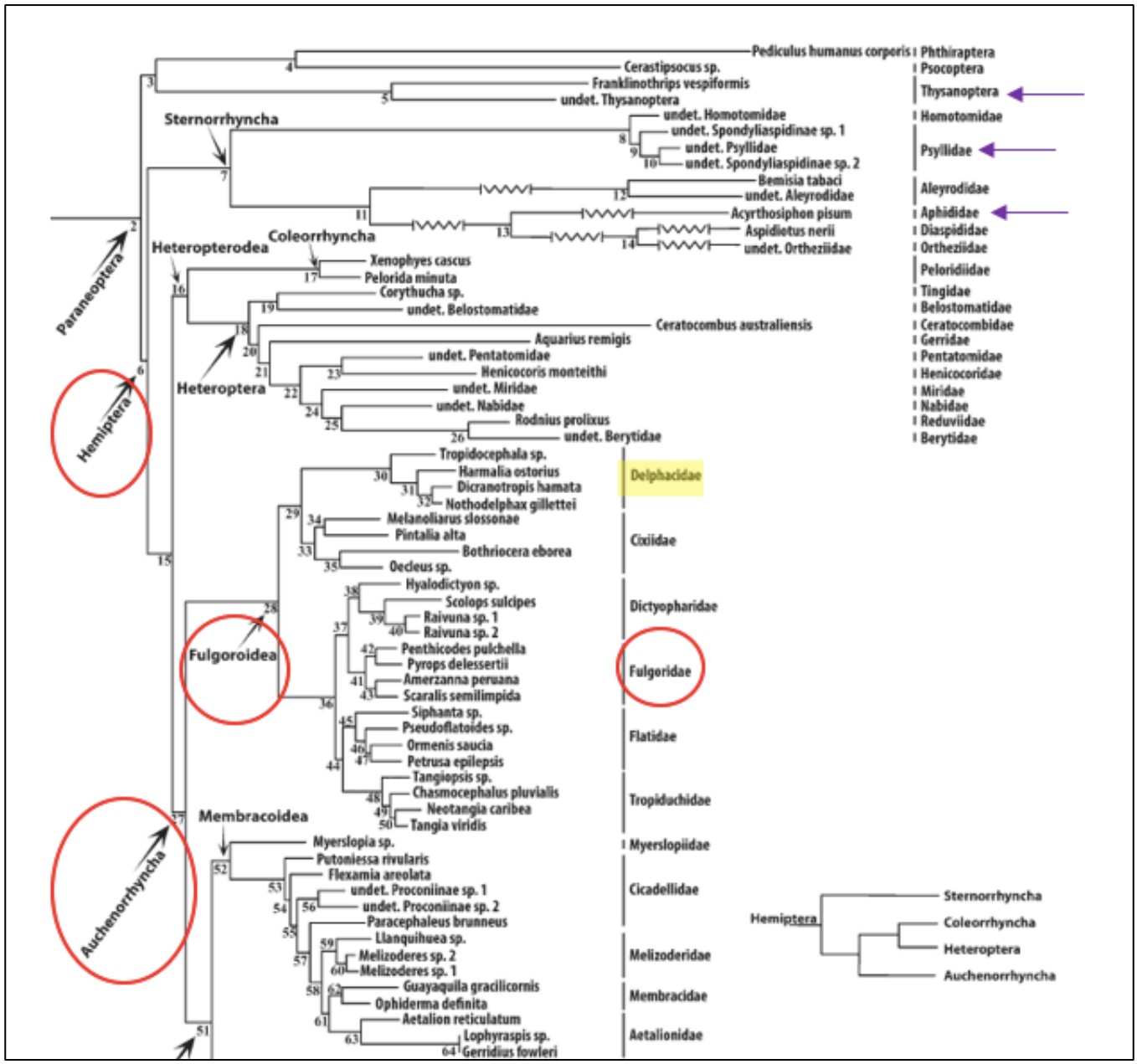

Figure 2.1: (Cryan & Urban, 2012). Phylogeny of spotted lanternfly. Red circles indicate the order (Hemiptera), suborder (Auchenorryhncha), superfamily (Fulgoroidea), and family (Fulgoridae). Delphacidae family is highlighted and purple arrows indicate families of known insect vectors.

There have been multiple potential vectors of NAGY identified, with research mainly focusing on leafhoppers, a group of insects closely related to SLF, and planthoppers, a group of which SLF are members (Lenzi et al. 2019). In fact, planthoppers represent the second-largest group of known phytoplasma vectors, with four families of planthoppers containing known vector species (Weintraub & Beanland, 2006). In addition to those planthoppers that are known vectors, multiple insects from these groups have been found to be dead-end hosts, meaning they are able to take up but not transmit the phytoplasmas from plant to plant (Trivellone & Dietrich, 2021). Thus, it is essential to conduct transmission experiments using the SLF in which both the insects and the recipient plants are tested to determine whether the SLF are capable of not only ingesting the phytoplasmas but completing the disease cycle through transmission.

Grapevine red blotch disease (GRBD) is caused by the phloem-infecting grapevine red blotch virus (GRBV). GRBD is a relatively new disease of grapevines, first reported in California in 2008 (Wilson et al., 2022). Following this report, GRBV was rapidly found and described across both cultivated and wild *Vitis* spp. throughout North America, South America, and parts of Asia, and testing of herbarium samples showed GRBV-infected tissues from as far back as 1940 (Rwahnih et al., 2015). Since this discovery, GRBV has been detected across the U.S. causing concerns for wine grape producers nationwide (Krenz et al., 2014). Symptoms known to be caused by this emerging disease range from red discoloration of leaves, curling and cupping of leaves, chlorosis and necrotic lesions, delayed fruit ripening, and decreased yield (Yepes et al., 2018). GRBV is found across the U.S. due to infected propagative material being

distributed to vineyards (Cieniewicz et al., 2017). In addition, a recent study by Flasco et al. (2023) showed an insect vector to play a role in field transmission. *Spissistilus festinus*, the three-cornered alfalfa hopper, is a member of the order Hemiptera and is a known vector of GRBV, transmitting the virus in a circulative, non-propagative method and transstadially (Flasco et al., 2021). *S. festinus* feeds on the phloem where the virus is colonizing the plants, similar to the SLF. The three-cornered alfalfa hopper also shares the same suborder as SLF, Auchenorrhyncha, which contains treehoppers, leafhoppers, spittlebugs, cicadas, and planthoppers. This relationship and feeding behavior suggest there is a possibility of SLF transmitting GRBV.

Finally, *Xylella fastidiosa* is a xylem-residing bacterium containing multiple economically damaging subspecies. Pierce's disease (PD) of grapevines is one of the most devastating diseases of grapevines, caused by *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*) (Rapicavoli et al., 2017). Symptoms of PD include marginal leaf necrosis and leaf scorching (Rapicavoli et al., 2017). Specific to PD, abscission of leaves from their petioles and development of "green islands" occur, in which the periderm develops irregularly (Rapicavoli et al., 2017). PD is found in the western and southern U.S. As SLF spread, the insects may soon encounter *X. fastidiosa* subsp. *fastidiosa* infected vines in southern areas, and a predictive process-based modeling shows that SLF has a high probability of reaching California by 2033 if no treatments to control populations are applied (Jones et al., 2022). *X. fastidiosa* subsp. *fastidiosa* is transmitted by the insect vector *Homalodisca vitripennis,* the glassy-winged sharpshooter (GWSS), among other vectors (Rapicavoli et al., 2018). The GWSS is a large xylem-feeding leafhopper in the

same suborder, Auchenorrhyncha, as the SLF. *Xff* is transmitted in a propagative persistent manner, allowing the vector to transmit the pathogen over multiple months (Rapicavoli et al., 2017). The bacteria are spread unevenly throughout the infected plant and tend to gather in small colonizing groups or biofilms within the xylem (Almeida, 2016). The limited spread within the plant is compensated for by the long feeding time of the GWSS, which involves probing multiple xylem vessels in one feeding session (Almeida, 2016).

Despite *X. fastidiosa* being a xylem-limited pathogen, SLF has potential to serve as a vector. Many predominately phloem-feeding insects have been shown to feed and probe partially on xylem elements (Chuche, 2017). SLF are phloem feeders but have been shown to make contact with the xylem through electropenetrography (EPG) studies (Lee et al., 2009). Thus, it is a possibility that SLF can act as a vector of PD if the insect continues to spread and reach grape growing regions where *X. fastidiosa* subsp. *fastidiosa* is present.

As stated above, there are known vectors of these important grapevine pathogens that can be used as points of comparison to invasive insects to aid in identifying potential vectors. However, the most definitive method to determine vector status is to conduct transmission experiments. Therefore, the goal of this study is to determine if the SLF is capable of transmitting the pathogens associated with NAGY, GRBV, and PD through controlled transmission experiments. The experiments were conducted using 4th instar nymphs and adult SLF, two life stages found in vineyards (Leach & Leach, 2020). Due to the wide range of insects capable of transmitting phytoplasmas and the phylogenetic

relationships between known vectors of phytoplasmas and SLF, it was hypothesized that SLF was capable of transmitting NAGY at both insect stages.

It was also hypothesized that SLF is capable of transmitting GRBV. The relationship of SLF to the known vector of GRBV suggests the possibility of SLF to transmit the pathogen. As SLF continue to invade vineyards across the northeast U.S. where red blotch disease is prominent, it is important to determine if the SLF can transmit GRBV. Although, it must be considered that SLF feeding behavior may not be conducive to GRBV transmission. Transmission studies using a known vector of GRBV, *S. festinus*, showed that two insects confined to single leaves of grapevines led to higher rates of transmission when compared to either 12 or 20 insects confined to larger areas of shoots (Flasco et al., 2023). This difference in transmission rates is thought to be attributed to the higher feeding damage observed with higher amounts of insects, leading to damaged phloem tissue that could limit GRBV replication and movement within the plant (Flasco et al., 2023). Both fourth instar and adult SLF are aggressive feeders, known to leave wounds on host plants (Urban, 2019). Therefore, it is possible that the aggressive feeding exhibited by SLF could cause phloem damage that is not conducive to GRBV transmission.

Finally, it was hypothesized that SLF is capable of transmitting *X. fastidiosa* subsp. *fastidiosa*, the causal agent of PD. *X. fastidiosa* is a xylem-limited pathogen, however SLF may serve as a vector because the insect could contact the xylem during aggressive probing and feeding (Lee et al., 2009). In addition, the phylogenetic

relationships between the known vectors of PD and SLF indicate the possibility for SLF to serve as a vector.

Materials & Methods

Insect collection and pathogen sources

Fourth instar and adult SLF were manually collected from wild hosts across eastern Pennsylvania as needed between July and October in 2020, 2021, and 2022. Insects were collected, transported, and maintained until use in mesh insect cages containing a potted grapevine or tree of heaven (*Ailanthus altissima*) to serve as a food source. SLF were maintained and all experiments involving SLF were conducted in a greenhouse at the Pennsylvania State University, University Park Campus.

NAGY pathogen-acquisitions were performed using infected vines at the Alson H. Smith, Jr. Agricultural Research and Extensions Center (AREC) at Virginia Tech, located in Winchester, VA. Field-grown mature vines (*Vitis vinifera* cv. Chardonnay) symptomatic for NAGY were selected. Leaves with intact petioles and shoot samples were taken and tested to confirm NAGY.

Infected vines for the GRBV acquisition were field-grown mature vines (*Vitis vinifera* cv. Chardonnay) from a commercial vineyard in Montgomery County, PA. Red blotch symptoms were previously reported by the grower, and vines used in the experiment were sampled and tested to confirm GRBV.

The infected source plants for PD transmission experiments were inoculated grapevines. The infected vines were kept in an APHIS-approved greenhouse facility at

University Park Campus. Three-year old grapevines (*Vitis vinifera* cv. Cabernet franc grafted on 101-14) grown in 19 L pots with custom substrate (1:1:1 field topsoil, perlite, and peat moss) were cultivated in a greenhouse at the Pennsylvania State University, University Park Campus. Vines were trained to one shoot and maintained at approximately five feet. Forty vines were inoculated in April 2022. *Xylella fastidiosa* subs. *fastidiosa* strain Temecula 1 was provided by Dr. C. Roper, UCR, and was grown at 28°C on solid PD3 medium until date of inoculation (Davis et al. 1981). Vines were mechanically inoculated as described by Purcell and Saunders (1999). A 20µl drop of turbid cell suspension in pH 7 phosphate-buffered saline (PBS) was pipetted onto the stem just above the third petiole from the base of the vine. The stem was pierced through the inoculum drop using a 20-gauge needle, so that the droplet was taken up into the xylem. The process was repeated on the opposite side of the stem to the initial droplet placement, for a total of 40 µl inoculum per vine. Control plants were subjected to the same procedure using water instead of bacteria. Vines were monitored for the appearance of symptoms to confirm infection.

Transmission assay experimental layout

For each of the pathogens, SLF were caged on the infected vines for a 72-hour acquisition access period (AAP). SLF were then transferred to uninfected, potted recipient vines for an inoculation access period (IAP) of two-three weeks. The number of recipient vines (replicates), fourth instar SLF per vine, adult SLF per vine, dates of experiments, and timing of IAPs is found in Table 2.1, 2.2, and 2.3.

The NAGY recipient vines were kept at either Peiffer Turf Farm at Penn State Berks (2020 fourth instars and adults) or an APHIS-approved quarantine greenhouse facility at Penn State, University Park Campus (2021 adults; 2022 fourth instars and adults). *Vitis vinifera* cv. Riesling (2020) and Cabernet franc (2021, 2022) vines were used as recipient vines. Recipient vines were tested for NAGY prior to adding the SLF in 2021 and 2022. Riesling recipient vines were not tested prior to adding SLF in 2020; however, all recipient vines were purchased from a nursery with certified phytoplasma-free stock.

The GRBV recipient vines (*Vitis vinifera* cv. Cabernet franc) were kept at Peiffer Turf Farm at Penn State Berks (fourth instar) or an APHIS approved quarantine greenhouse facility at Penn State, University Park Campus (adult). The recipient vines were tested for GRBV prior to adding SLF.

The PD recipient vines (*Vitis vinifera* cv. Cabernet franc) were kept at the APHIS approved quarantine greenhouse facility at Penn State, University Park Campus.

Table 2.1: Experimental Design for NAGY Transmission								
Year	**Number of Recipient Vines**		**Number of SLF per Vine**		**Date of AAP Start**		**Duration of IAP**	
	Nymph	Adult	Nymph	Adult	Nymph	Adult	Nymph	Adult
2020	8	8	50	100	8/07/20	8/22/20	2 weeks	2 weeks
2021	2	8	60	100	8/16/21	9/21/21	2 weeks	2 weeks
2022	4	4	60	100	7/19/22	8/22/22	8 days	2 weeks

Table 2.2: Experimental Design for GRBV Transmission (2021)							
Number of Recipient Vines		**Number of SLF per Vine**		**Date of AAP Start**		**Duration of IAP**	
Nymph	Adult	Nymph	Adult	Nymph	Adult	Nymph	Adult
3	8	50	60	8/18/21	9/7/21	2 weeks	3 weeks

Table 2.3: Experimental Design for Pierce's Disease Transmission (2022)							
Number of Recipient Vines		**Number of SLF per Vine**		**Date of AAP Start**		**Duration of IAP**	
Nymph	Adult	Nymph	Adult	Nymph	Adult	Nymph	Adult
4	4	60	25	7/19/22 7/28/22	8/25/22 8/29/22	2 weeks	3 weeks

Tissue Collection

The plant tissues collected to test for NAGY included leaf petioles, midribs, and phloem tissue scraped from the shoots. Tissues were collected, diced into small pieces, and combined into aliquots of 200 mg mixed tissues. The aliquots were stored at -80°C. Woody roots were also collected from 2020 and 2021 vines, by trimming older, lignified roots from the potted vines during dormancy. Roots were washed and any lignified layers were scraped off. The roots were diced into small pieces and stored in aliquots of 200mg at -80°C. Insects were also collected and stored at -80°C. Table 2.4 includes tissue collection dates. Tissue types were combined petiole, midrib, and phloem unless denoted as SLF (insect tissue only) or root (root tissue only).

Tissues collected for GRBV were petiole and midribs. Leaves and petioles were collected, diced into small pieces, and combined into aliquots of 200 mg which were stored at -80°C. Table 2.5 includes plant tissue collection dates. Nymph and adult SLF were also collected at the end of the AAP and stored at -80°C.

To test for PD, whole symptomatic leaves were collected and separated into petioles, midribs, and leaf margins. Each tissue type was diced into small pieces and kept separated in tubes with approximately 500 mg of tissue per tube. Tissues were stored at -80°C. Nymph and adult SLF were also collected at the end of the AAP and stored at -80°C. Table 2.6 includes plant tissue collection dates.

Table 2.4: Tissue Collection Dates for NAGY		
Year	**Collection Dates** (month/year)	
	Nymphs	Adult
2020	10/2020 9/2021 12/2021 03/2022 (root) 05/2022 12/2022 08/2022 12/2022	10/2020 9/2021 12/2021 03/2022 (root) 08/2022 12/2022
2021	9/2021 10/2021 12/2021 03/2022 (root) 12/2022	10/2021 (SLF) 12/2021 03/2022 (root) 05/2022 11/2022 12/2022
2022	08/2022 (SLF) 01/2023	09/2022 (SLF) 01/2023

Table 2.5: Tissue Collection Dates for GRBV (month/year)	
Nymph	Adult
11/2021 11/2022	12/2021 11/2022

Table 2.6: Tissue Collection Dates for Pierce's Disease (month/year)	
Nymph	Adult
10/2022 11/2022 12/2022 1/2023	11/2022 11/2022 12/2022 1/2023

DNA extraction

Total DNA was extracted from all stored plant tissues using a modified CTAB extraction followed by a Plant Tissue (Mini) protocol from the Qiagen DNeasy Plant Mini Kit. To begin, CTAB extraction buffer (44 mM Tris, 56 mM Tris-HCL, 1.4 M NaCl, 50 mM Na_2EDTA (ethylenediamine tetraacetic acid, disodium dihydrate), 2.5% (w/v) CTAB (hexadecyltrimethylammonium bromide), 1% (w/v) PVP-40 (polyvinylpyrrolidone, MW = 40,000, pH 8) was mixed with 0.2% (v/v) 2-β-mercaptoethanol, at a ratio of 2 µl mercaptoethanol per 1ml CTAB. This mixture was added (1ml) to each tube containing approximately 200 mg of diced tissue. Tubes were incubated at 60°C for 40 minutes and mixed by inversion throughout incubation. Tissues were homogenized using the Tissuelyser II (Qiagen). Homogenized tissues were then processed as described by the Qiagen DNeasy Plant Mini Kit protocol.

To elute, 40 µl prewarmed (65°C) Buffer AE was added to the DNeasy spin column membrane. The samples were incubated for 10 min at room temperature, then centrifuged at 8,000 rpm for 2 min. This step was repeated using an additional 40 µl of prewarmed Buffer AE, for a total of 80 µl elution buffer. DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and stored at -20°C.

Insect tissues were prepared for PCR and qPCR using the method described by Rowhani et al (2000). Stored insects were removed from -80°C and legs and wings (adults) were removed. Insects were individually ground at a ratio of 1:20 tissue to buffer. Two µl of each sample was used immediately in PCR or qPCR.

NAGY polymerase chain reaction and sequencing

Extracted DNA and prepared insects from NAGY samples were used in direct or semi-nested PCR. Primers that were tested are listed in Table 2.7. Invitrogen's AccuPrime™ *Taq* DNA Polymerase High Fidelity kit (#12346-086), including buffers containing dNTPs, $MgSO_4$, and *Taq* polymerase, was used in all rounds of PCR. The volumes of PCR reagents are listed in Table 2.8. These volumes were used as a starting protocol for all primers and rounds; however, template concentration was adjusted during troubleshooting and optimization of PCRs. Extracted DNAs were used as templates for direct PCR or round one of nested PCRs, and the PCR product from the first round was used as template in the second round of nested PCRs. A positive control consisting of DNA extracted from infected leafhoppers was used at a 10% concentration (DNA provided by the lab of T. Wolf, Virginia Tech).

PCR products were visualized on a 1.0% agarose gel. Amplicons at the correct size for the targeted gene were extracted from the gel using the Zymo Gel Extraction Kit (Zymo Research) and sequenced by Sanger sequencing at the Penn State Genomics Core Facility. Sequences were compared to a publicly available reference database (NCBI) to determine the phytoplasma group and identity of positive samples.

Table 2.7: Primers Used in Detection of NAGY

Gene Target		Primer Name	Primer Sequence (5' – 3')	Primer Source
16S of Phytoplasma	Round 1	P1 16S-SR	AAGAGTTTGATCCTGGCTCAGGATT GGT CTG TCA AAA CTG AAG ATG	Deng & Hiruki 1991 Lee et al. 2004
	Round 2	P1A 16S-SR	AACGCTGGCGGCGCGCCTAATAC GGT CTG TCA AAA CTG AAG ATG	Lee et al. 2004 Lee et al. 2004
16S of Phytoplasma	Round 1	R16F2n P7	GAA ACG ACT GCT AAG ACT GG CGT CCT TCA TCG GCT CTT	Gunderson & Lee 1996 Schneider et al. 1995
	Round 2	P5 P7	CGG CAA TGG AGG AAA CT CGT CCT TCA TCG GCT CTT	Schneider et al. 1995 Schneider et al. 1995
spc operon (R1) SecY (R2)	Round 1	L15F1 MapR1	CCTGGTAGTGGYAMTGGWAAAAC ATTARRAATATARGGYTCTTCRTG	Lee et al. 2010
	Round 2	AYSecYF1 AYSecYR1	CAGCCATTTTAGCAGTTGGTGG CAGAAGCTTGAGTGCCTTTACC	Lee, Zhao, & Bottner 2005
spc operon (R1) SecY (R2)	Round 1	L15F1A(III) MapR1A(III)	CTTCTGGTAAAGGACATAAAGG GGTTCTTCGTGCAATTGCAAACC	Lee et al. 2010
	Round 2	AYSecYF1(III) AYSecYR1(III)	CAGCCATTTTAGCAGTTGGTGG CAGAAGCTTGAGTGCCTTTACC	Lee, Zhao, & Bottner 2005
SSU12p	Direct	ItSSU12pF ItSSU12pR	ATGCCTACTRTTTCWCAATTAATTA ATCTTAAACCTAAAGATTGRCGTC	Cui et al. 2021
Cpn60	Direct	H279p H280p	GATIIIGCAGGIGATGGAACMACIAC TGRTTITCICCAAAACCAGGIGCATT	Green, M. et al. 2014
16S of Phytoplasma	Round 1	P1 P7	AAGAGTTTGATCCTGGCTCAGGATT CGT CCT TCA TCG GCT CTT	Deng & Hiruki 1991 Schneider et al. 1995
	Round 2	R16(III)F2 R16(III)R1	AAG AGT GGA AAA ACT CCC TCC GAA CTG AGA TTGA	Cooper et al. 2021

Table 2.8: PCR Reagents for Detection of NAGY

Reagent	Volume (µl) Per Reaction
Water	11.53
AccuPrime PCR Buffer II (10x)	1.5
$MgSO_4$ (50mM)	0.3
Forward Primer (10µM)	0.3
Reverse Primer (10µM)	0.3
AccuPrime *Taq* DNA Polymerase, High Fidelity (5 U/µl)	0.075
Template	1
Total volume	15

GRBV quantitative polymerase chain reaction

Extracted DNAs and prepared insects from GRBV samples were tested by qPCR using the BIO-RAD CFX96 Real-Time PCR Detection System (Bio-Rad). qPCR was set up as described in Setiono et al. (2018), using PerfeCTa SYBR Green FastMix, ROX (Quantabio, Beverly, MA, USA) with thermocycling conditions of 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds. A dissociation protocol with a gradient from 65°C to 95°C (5 seconds and 0.5°C ramp/cycle) was used to generate melt curves. The primers used were P1183 5'- ACA TCT CTG GGT TTG GTG ATA TT -3' and P1184 5'- CTA CAC GCC TTG CTC ATC TT -3' (Setiono et al., 2018). A positive control consisting of DNA extracted from GRBV-infected vines was used (DNA provided by the lab of M. Fuchs, Cornell University).

***X. fastidiosa* subs. *fastidiosa* enzyme-linked immunosorbent assay (ELISA)**

The ELISA Reagent Set for *Xylella fastidiosa* subs. *fastidiosa* (*Xff*) and protocol by Agdia was used to initially test for *X. fastidiosa*. In November 2022, petiole samples from symptomatic recipient vines used in the PD transmission experiment as well as nymph and adult SLF were tested by ELISA. Samples from vines that were inoculated with *Xylella fastidiosa* subs. *fastidiosa* were used as a positive control. Optical density was measured at a wavelength of 405 nm (yellow wavelength), with values 3 times the O.D. of the negative control indicating a positive sample.

Xylella fastidiosa subs. *fastidiosa* RPA

Vines were tested for *Xylella fastidiosa* subs. *fastidiosa* using the AmplifyRP XRT+ for Xf kit and protocol by Agdia. Vines used in the 4th instar PD transmission experiment, the 4th instar NAGY 2022 transmission experiment, and vines inoculated with *X. fastidiosa* subsp. *fastidiosa* were each tested separately in October and November of 2022. All tissues used were composite samples of petioles from symptomatic leaves. An additional XRT+ test was run using petioles collected from vines used in the unrelated GRBV transmission experiment, as a negative control.

X. fastidiosa subs. *fastidiosa* quantitative polymerase chain reaction

Extracted DNAs and prepared insects from PD samples were tested by qPCR using the BIO-RAD CFX96 Real-Time PCR Detection System (Bio-Rad). qPCR was set up as described by Deyett et al. (2019), using PerfeCTa SYBR Green FastMix, ROX (Quantabio, Beverly, MA, USA) with thermocycling conditions of 95°C for 15 minutes, followed by 40 cycles of 55°C for 55 seconds, 72°C for 45 seconds, 95°C for 15 seconds. A dissociation protocol with a gradient from 65°C to 95°C (5 seconds and 0.5°C ramp/cycle) was used to generate melt curves. The primers used were XF-ITS-F6 5'-GAG TAT GGT GAA TAT AAT TGT C -3' and XF-ITS-R6 5'- CAA CAT AAA CCC AAA CCT AT -3' (Deyett et al., 2019). Positive controls consisted of DNA extracted from PD-inoculated vines.

In addition, symptomatic tissues and insects were prepared to be used as a template in qPCR using the quick extraction protocol as described by Rowhani et al (2000).

X. fastidiosa subsp. *fastidiosa* isolation from plant tissue

The isolation procedure for *Xylella fastidiosa* subs. *fastidiosa* was provided by the Roper lab (UCR). Leaf petioles were removed from symptomatic PD transmission vines to be tested for *Xylella fastidiosa* subs. *fastidiosa*, and petioles from symptomatic vines inoculated with *Xylella fastidiosa* subs. *fastidiosa* were used as a positive control. The petioles were surface sterilized in 95% ethanol (30s), 20% bleach (30s), sterile water (1min), and again sterile water (1min). The sterilized petioles were then ground in a mesh sample grinding bag with 2ml sterile phosphate-buffered saline (1X PBS). One ml of slurry was used to make serial dilutions to 10^{-8}. To plate, a 20 µl drop was placed onto a PD3 plate (Davis et al. 1981), so that the plate could be tilted to allow the drop to run to the bottom of the plate. The plates were allowed to dry before being wrapped in parafilm and incubated at 28°C for 7-10 days, or until colonies appeared on the positive control plate.

Colonies from the positive control sample were used as morphological comparison to identify colonies from experimental samples. In addition, colonies were analyzed by qPCR. Colonies were collected using a sterile toothpick and mixed into tubes containing 10 µl sterile water. The samples were boiled for 10 min and used directly as template (2 µl) in qPCR.

Results

NAGY was transmitted by nymph and adult spotted lanternflies

In the 4th instar nymph NAGY transmission experiments, testing by PCR revealed one positive recipient vine out of 14 total vines across three years, or a 7% rate of transmission. This vine repeatedly tested positive from plant tissue taken across three sampling dates (October 2020, September 2020, and December 2021) and using different sets of primers. Primer pairs by which one nymph sample tested positive were: R16F2n/P7, P5/P7 (nested PCR) and ItSSU12pF/ItSSU12pR (direct PCR). Sanger sequencing of the PCR products from the nymph sample confirmed the identity as '*Ca.* Phytoplasma pruni', one of the phytoplasmas associated with NAGY (Accession # OQ275133.1, 97.06% Identity).

The adult SLF NAGY transmission experiments revealed one positive recipient vine out of 20 total vines across three years, or a 5% transmission rate, as tested by PCR. Tissue from this vine on one sampling date (December 2021) tested positive by nested PCR using the following primers: P1/P7, R16(III)F2/R16(III)R1. Clear sequencing data was not obtained from the positive adult transmission sample. Table 2.9 summarizes the number of recipient vines that tested positive for NAGY across adult and nymph experiments

Some mild symptoms of NAGY were observed on recipient vines; however, symptoms could not be attributed solely to infection by NAGY. Overall, transmission of NAGY could not be confirmed based on symptomology alone. (Figure 2.2**:** NAGY-infected field vines and experimental recipient vines).

Table 2.9: Number of recipient vines positive for NAGY		
Year	**Number of positive vines out of total vines tested**	
	Nymph	Adult
2020	1/8	0/8
2021	0/2	1/8
2022	0/4	0/4

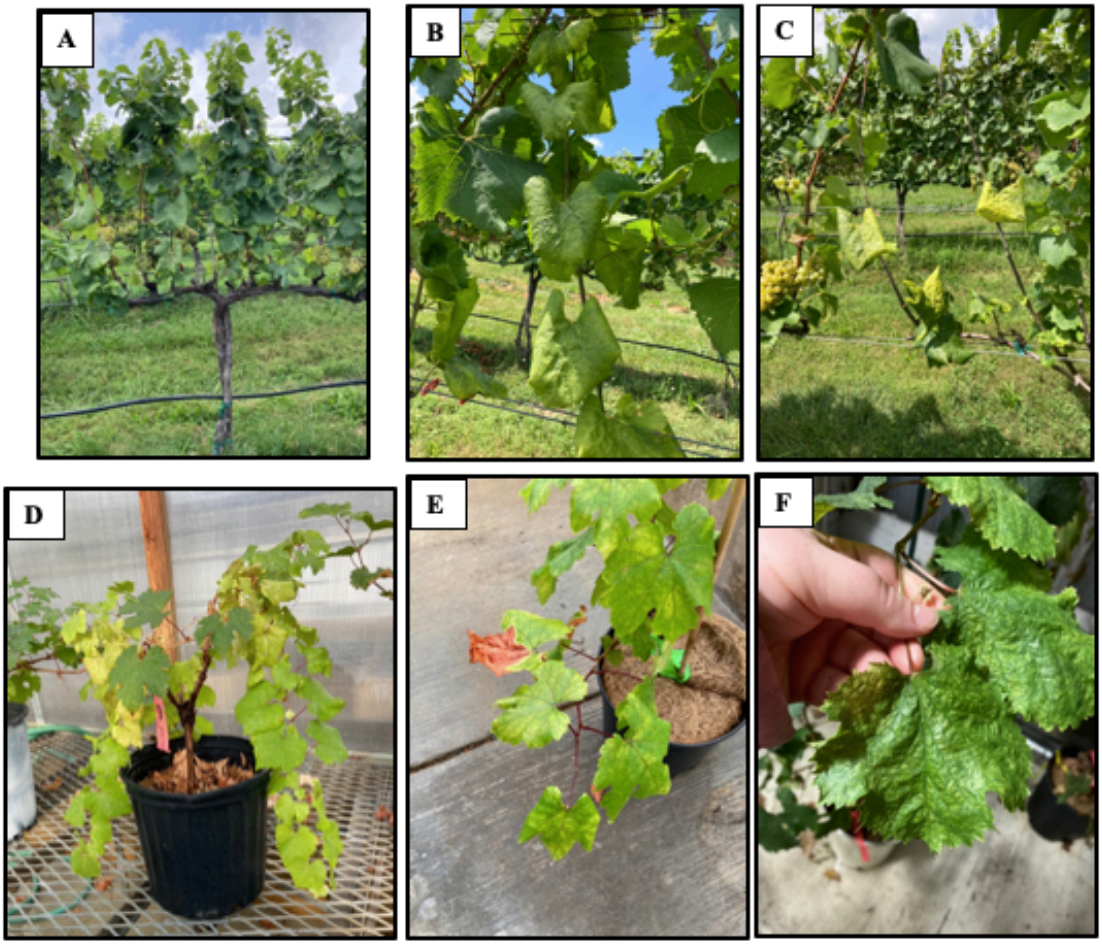

Figure 2.2: Symptoms observed on NAGY-infected field vines (top row) and experimental recipient vines (bottom row). Symptoms on field vines include stunted growth and shortening of internodes (A), downward leaf curling, leaf yellowing, and leaf deformation (B and C). Symptoms observed on NAGY experimental recipient vines include stunted growth, shortening of internodes (D), downward curling of leaves (E), and leaf deformation (F).

GRBV was not transmitted by nymph or adult spotted lanternflies

There was no measured transmission of GRBV by 4th instar nymph or adult SLF. The recipient vines were confirmed to be negative for GRBV when tested by qPCR in 2021 and again in 2022. In addition, the adult insects also tested negative for GRBV (Table 2.10**:** Number of recipient vines positive for GRBV). No symptoms were observed on GRBV recipient vines (Figure 2.3: Symptoms observed on GRBV-infected field vine and experimental recipient vines).

Table 2.10: Number of recipient vines positive for GRBV

Year	**Number of positive vines out of total vines tested**	
	Nymph	Adult
2021	0/3	0/8

Figure 2.3: Symptoms observed on GRBV-infected field vine and experimental recipient vines. Symptoms on field vines include chlorotic lesions and necrotic margins on leaves (A). No symptoms associated with GRBV were observed on recipient vines (B).

Pierce's disease symptoms were observed in nymph and adult transmission experiments

Symptoms of PD were observed in recipient vines of the 4th instar nymph and adult transmission experiment. In both nymph and adult experiments, symptoms on the recipient vines began appearing approximately two months after the date the SLF were transferred to the recipient vines from PD-infected vines. This aligns with the timeline of symptom development observed when inoculating grapevines with *X. fastidiosa* subsp. *fastidiosa*. Symptoms on the recipient vines also aligned with the symptoms observed on the inoculated vines, including scorching of leaf margins, yellowing and reddening along leaf margins, defoliation of dried leaves, and shoot dieback near growing tips (Figure 2.4: PD symptoms in inoculated and experimental recipient vines). In some cases, recipient vines experienced near total defoliation, sometimes leaving petioles attached when the leaf blade fell (matchsticks).

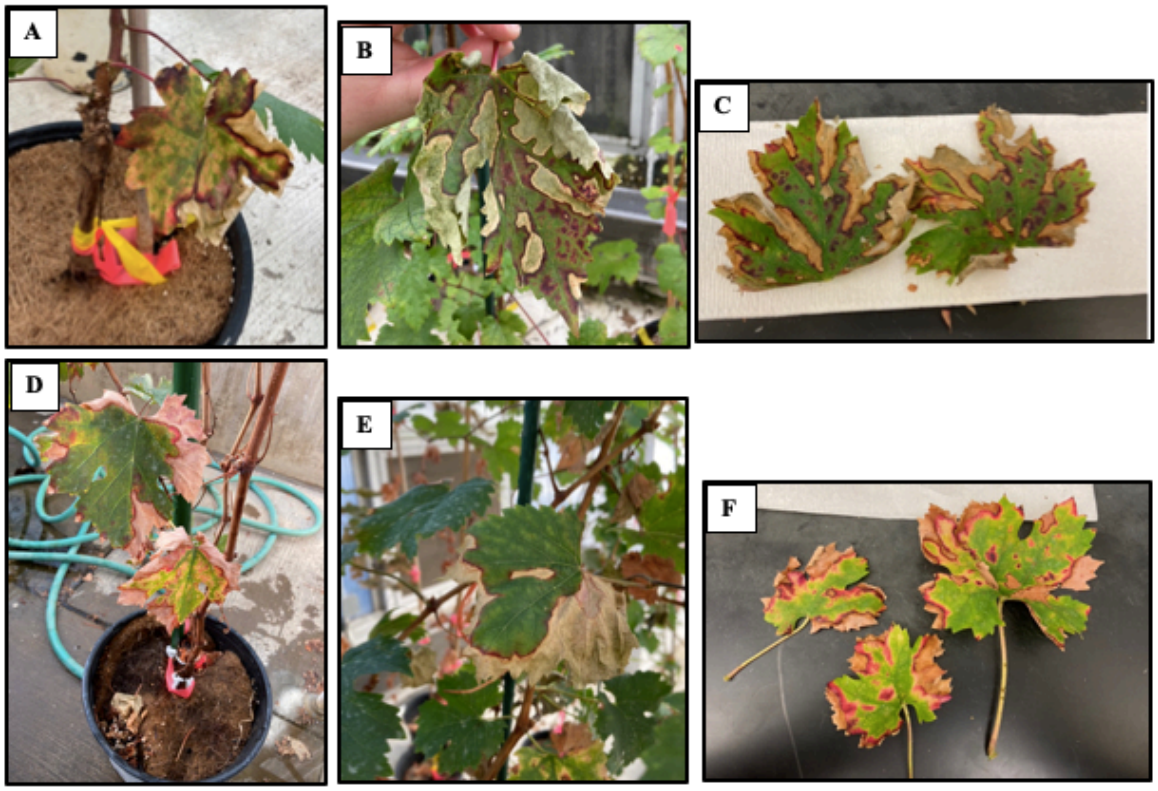

Figure 2.4: Symptoms observed on vines inoculated with *X. fastidiosa* subsp. *fastidiosa* (top row) and experimental recipient vines (bottom row). Symptoms on inoculated vines include scorching of leaf margins and yellowing/reddening of margins (A, B, and C). Similar symptoms were observed on recipient vines (D, E, and F).

Recipient Vines tested negative for Pierce's disease via ELISA

Experimental recipient vines tested by ELISA were considered negative when the O.D. values were recorded after a one-hour incubation, using a threshold of 2x the negative control O.D. value to be considered positive. The positive control samples (grapevines inoculated with *X. fastidiosa* subsp. *fastidiosa*) were positive at 2x the O.D. value of the negative control. While the manufacturer of the ELISA kit recommends reading the plate and interpreting results after one hour of incubation, the plate was also read at two hours of incubation. At two hours of incubation, one experimental sample

tested positive at 3x the O.D. value of the negative control, and the positive control samples tested positive at 3x the O.D. value of the negative control. However, the positive experimental sample was attributed to contamination, as each sample was run in duplicate and only one replicate of this sample appeared positive.

Recipient Vines tested negative for Pierce's disease by RPA

Experimental recipient vines were tested twice by RPA and neither test returned positive. Positive control vines (grapevines inoculated with *X. fastidiosa* subsp. *fastidiosa*) tested positive and negative control vines (grapevines used in GRBV transmission experiment) tested negative by RPA. These results indicate there is no PD infection in the experimental recipient vines. However, further testing is necessary as results may vary based on the timing of tissue collection and the tissue type tested, which could both influence bacterial titer and distribution.

Recipient vines tested negative for Pierce's disease by quantitative PCR

Recipient vines used in the PD 4th instar nymph and adult SLF transmission experiments repeatedly tested negative for *Xylella fastidiosa* subs. *fastidiosa* via qPCR. Vines inoculated with *X. fastidiosa* subsp. *fastidiosa* served as positive controls for each test, and confirmed the qPCR was run successfully. Multiple qPCRs were run, using samples including various symptomatic tissues from recipient vines, insects used in the PD and NAGY transmission experiments, and colonies grown from symptomatic

recipient vines. None of these samples from the experimental recipient vines or insects tested positive by qPCR (Table 2.11).

Table 2.11: Number of recipient vines positive for Pierce's disease by qPCR		
Year	**Number of positive vines out of total vines tested**	
	Nymph	Adult
2022	0/4	0/4

***X. fastidiosa* subsp. *fastidiosa* was not isolated from recipient vines**

Samples taken from symptomatic tissues of recipient vines used in the PD transmission experiment were plated and yielded no colonies that were morphologically similar to colonies of known *X. fastidiosa* subsp. *fastidiosa*. *X. fastidiosa* subsp. *fastidiosa* was successfully isolated from grapevines inoculated with the bacteria, serving as a positive control and point of morphological comparison (Figure 2.5: Colonies isolated from PD transmission experiment).

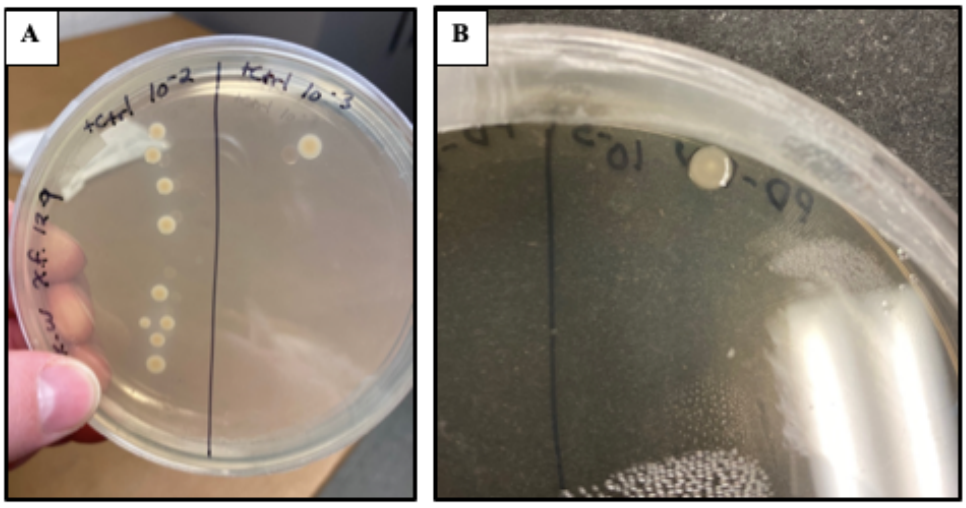

Figure 2.5: Colonies isolated from Pierce's disease-infected vines (A) and experimental recipient vines (B).

Discussion and Conclusion

Results from this study showed that fourth instar nymph and adult SLF are capable of transmitting NAGY but at a low rate, 7% and 5%, respectively, based on testing by PCR. Symptomology was not conclusive in determining infection in vines. Symptoms were observed in the experimental recipient vines. However, the symptoms were much milder compared to infected field vines. The recipient vines also displayed symptoms of nutrient deficiencies, oedema due to temperature fluctuations in the greenhouse, and stress from being kept in pots for multiple growing seasons.

The factors involved in describing a capable insect vector of phytoplasmas are multiple and complex. Insect vectors of phytoplasmas are found within the order Hemiptera, yet it is estimated that only half of all known phytoplasmas have identified vectors (Trivellone, 2019). Weintraub and Beanland (2006) posit that Hemipterans are successful phytoplasma vectors based on certain characteristics, including being hemimetabolous, in which nymphs and adults often feed on similar tissues and locations; and selective and nondestructive feeding on specific plant tissues (i.e. phloem). SLF nymphs have indeed been reported to feed on similar plants and tissues as adults, notably 4th instar nymphs can invade and feed in vineyards where adults later infest (Leach and Leach, 2020). This increases the chances for the phloem-feeding insects to feed on and acquire phytoplasmas from infected plants, and to undergo long latent periods that are associated with phytoplasmas transmission (Weintraub and Beanland, 2006; Wientraub et al., 2019). Another factor that may influence the exposure of vineyards to SLF is climate change. Warmer climates could lead to SLF having more time to encounter infected

plants through earlier hatching, multiple generations per year, and more geographical ranges where SLF and pathogens overlap.

It is important to note that the ability of an insect to uptake phytoplasmas while feeding does not indicate vector capability. Phytoplasmas that are successfully transmitted by an insect will need to pass through the midgut membrane and hemolymph, replicate within various internal organs, then travel across the salivary gland membrane and into the saliva to be deposited when the insect feeds on another plant (Trivellone and Dietrich, 2021; Weintraub et al., 2019).

Around 200 species of hemipteran insects are associated with phytoplasmas, but less than half have been identified as competent vectors (Trivellone and Dietrich, 2021). Lenzi et al. found the leafhopper *Jikradia olitoria* to be capable of transmitting a phytoplasma strain associated with NAGY in artificial feeding assays, yet insect-to-plant transmission has not been shown. Artificial feeding assays are useful to identify putative vectors of pathogens but cannot be employed with insects such as SLF which have not been shown to successfully feed on artificial diets and phytoplasmas cannot be cultured. Due to the potential for unknown NAGY vectors to be present in the research vineyard, there is a possibility that an insect other than SLF could have played a role in the transmission of NAGY found during the present study. An unknown and undetected vector could have been caged with the SLF on the infected vines and transferred to the healthy vines thus allowing them to transmit NAGY. However, this is an unlikely scenario, as plants were closely inspected daily and the leaves were entirely contained by mesh bags preventing outside insects from feeding on the vines. NAGY was detected by

molecular techniques in only one SLF following transmission experiments, thus other methods for detection such as transmission electron microscopy (TEM) could be used. TEM would allow verification of the phytoplasma in the insect body, and show which tissues the pathogens are present in.

Nymph and adult SLF were not shown to transmit GRBV in the one-year trial. Viruses are known to have unique relationships with their vectors and hosts, having developed through coevolution, that may limit a new vector being introduced into an established system (Lefeuvre et al., 2019). *Spissistilus festinus*, the three-cornered alfalfa hopper, was identified by Bahder et al. (2016) to transmit GRBV under greenhouse conditions and later by Flasco et al. (2023) as an epidemiologically relevant vector in vineyards across New York and California. In Missouri, LaFond et al. (2022) found the treehopper *Entylia carinata* to be abundant in vineyards and capable of transmitting GRBV under greenhouse conditions. While these vectors of GRBV have been identified, mechanisms of transmission are still being explored.

Within the family *Geminiviridae,* of which GRBV is a member, the viruses are known to be transmitted in a circulative, persistent manner (Kahl et al., 2021). This aligns with the findings of Flasco et al. (2021) who discovered that GRBV is transmitted in a circulative, nonpropagative manner by *S. festinus.* The virus must travel through the guts, hemolymph, and heads (salivary glands) to be transmitted successfully, and a longer acquisition access period than other *Geminiviridae* members was demonstrated. For this process to occur, geminiviruses must exhibit compatibility between their coat proteins and receptors of insect tissues (Kahl et al., 2021). This specificity is suspected to limit the

range of vectors capable of transmitting a virus such as GRBV and is likely a factor inhibiting SLF in transmitting the pathogen, despite the similarity in anatomy and feeding behavior of SLF and known geminivirus vectors.

While many factors may be involved in determining virus-vector compatibility, it is important to note that the experimental conditions of this GRBV transmission study may influence results as well. There was a high level of 4th instar nymph deaths while caged on the recipient vines. These deaths were likely due to high temperatures inside the mesh bags, caused by sun exposure, high ambient temperature, and limited ventilation of the tightly woven mesh bags. Despite these obstacles, the majority of nymphs were observed to be alive for at least one week of feeding on the recipient vines. After two weeks, some nymphs remained alive, but the majority had died. The optimal acquisition access period, latent period, and inoculation access period is unknown in *S. festinus* and may vary between vectors of GRBV (Flasco et al., 2021). Thus, more experimentation is needed to ensure the SLF have sufficient time to complete the transmission cycle if they are capable.

Finally, PD was not shown to be transmitted by 4th instar nymph or adult SLF through molecular testing, however positive symptomology indicates these results require further testing. *X. fastidiosa* subsp. *fastidiosa* was not detected in recipient transmission grapevines via ELISA, RPA or qPCR, but factors such as low bacterial titers, age of the vine, tissue types sampled, seasonal timing and overall sensitivity of the tests could influence these results.

X. fastidiosa has a wide host range and variety of vectors and does not exhibit high vector specificity within the sharpshooter leafhopper subfamily (Cicadellinae) and spittlebug superfamily (Cercopoidea) (Almeida et al., 2005). As suggested by Almeida and Nunney (2015), this lack of specificity allows *X. fastidiosa* subs*. fastidiosa* isolates to be transmitted by endemic vector species when introduced to a new environment. The mechanisms of transmission of *X. fastidiosa* subsp. *fastidiosa* are well-studied in a vector species of economic importance, *Homalodisca vitripennis,* in which bacteria are transmitted in a non-circulative, persistent, propagative manner in adults (Almeida and Nunney, 2015; Rapicavoli et al., 2017). The bacteria must be taken up from the xylem by the feeding or probing vector, where the cells may form a biofilm and multiply in the foregut (Almeida, 2016). *X. fastidiosa* subsp. *fastidiosa* may also forgo this biofilm formation and be transmitted without an apparent latent period (Almeida et al., 2005). The cells are inoculated into a plant when the vector feeds or probes via an egestion/salivation mechanism (Backus et al., 2015). SLF feed on the phloem of plants but can contact the xylem during aggressive probing and feeding, and other phloem-feeding members of the Fulgoroidea family feed partially on xylem elements (Lee et al., 2009; Chuche et al., 2017). Mixed feeding on phloem and xylem sap has been observed in leafhoppers of the Deltocephalinae subfamily, and the leafhopper *Cicadulina mbila* has been recorded probing the xylem as well as the phloem (Chuche et al., 2017). This interaction with the xylem and lack of specificity within related vectors supports the possibility of SLF transmitting *Xylella fastidiosa* and particularly subsp. *fastidiosa,* which colonizes grapevines that SLF feed heavily and frequently on.

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Chapter 3

Response of grapevines to combined pathogen infection and spotted lanternfly stress, and effect of infected grapevines on spotted lanternfly behavior

Abstract

The spotted lanternfly (SLF) (*Lycorma delicatula)* is known to invade vineyards and feed on grapevines in large numbers. Grapevines are susceptible to multiple diseases and could undergo combined stress from pathogen infection and SLF infestation in a vineyard setting. Similarly, SLF may behave differently towards infected grapevines compared to uninfected grapevines. The goal of this chapter was to examine the interactions between SLF, and grapevines infected with *Xylella fastidiosa* subsp. *fastidiosa,* the bacterium that causes the devasting Pierce's disease (PD). Specifically, the objectives were three-fold: (1) Determine how combined biotic stressors (SLF and PD) affect plant primary metabolites that are important for grapevine longevity and productivity, such as carbohydrate (starch and soluble sugars) and total nitrogen, compared to a single biotic stressor or no stressor; (2) Determine the effect on SLF survival and development from 4th instar nymph to adult stage when feeding on PD-infected vines; and (3) Determine if SLF exhibit a feeding preference between PD-

infected and uninfected (healthy) grapevines. The greatest impact on primary metabolites accumulation was on late season carbon, in which SLF infestation affected most vines regardless of PD infection. Notably, starch was significantly reduced by SLF infestation in both cane and root tissues, regardless of PD infection. On the insect side, there was an increased mortality of SLF when feeding on PD-infected vs non-infected vines. Feeding preference experiments showed that SLF preferred not to feed on PD-infected vines when given a choice between uninfected and infected vines. These results offer some initial insight into the complex interactions between the biotic stressors of PD infection and SLF infestation.

Introduction

Pathogens manipulate their plant hosts by inducing chemical and physical responses that can influence the behavior of their insect vectors, and potentially non-vector insects. Regardless of the ability of the spotted lanternfly (SLF) to transmit grapevine pathogens, it is important to understand how SLF will interact with infected vines and what the combination of SLF infestation and pathogen infection will have on grapevines.

Pierce's disease (PD) is one of the most devastating diseases of grapevines, caused by *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*), and it is found throughout the Southern U.S. PD is prevalent in regions with milder winters, known to cause significant losses in vineyards in Virginia, Florida, Alabama, Texas, and California (Lowder et al., 2019). SLF may soon encounter PD-infected vineyards as the insects spread to regions where PD is prominent. In addition, there is potential for higher PD prevalence in SLF-infested northern states, as these regions experience increasingly milder winters due to climate change. Insight into how the introduction of an invasive insect will interact with a pathogen system such as PD can lead to better planning and preparation for invasive species management. Vineyards that are infected with PD may require different management strategies when they are also infested with SLF. For example, the timing and location of pesticide applications to control SLF may vary depending on how SLF invades and is distributed within a vineyard with PD compared to a non-infected vineyard. Also, since it is unknown if the combination of PD and SLF feeding will have a greater negative impact on the vines compared to PD alone, the level of management of SLF could vary from a vineyard with or without PD.

To examine the interactions between SLF and vines with PD, two main questions were asked in this chapter: (1) How do grapevines respond to combined SLF infestation and infection by *Xylella fastidiosa* subsp. *fastidiosa*? and (2) How do vines infected with *X. fastidiosa* subsp. *fastidiosa* affect SLF development, mortality, and feeding preference?

Plant responses to biotic stressors

It is difficult to predict how combined biotic stressors of SLF infestation and pathogen infection will impact grapevines. The stresses may have an antagonistic or synergistic effect, in which one may interfere (antagonism) or enhance (synergism) the effects of the other. While studies on combined abiotic and biotic factors exist, research on the response of perennial crops to any combination of biotic factors is less common. Thus, the individual effects of pathogens and phloem-feeding insects such as SLF can serve as a basis to move forward with more complex interactions and effects.

Plants are able to respond to attack by biotic stressors, such as pathogens and insects, through induced defenses. Pathogen-associated molecular patterns (PAMPs) are conserved microbial molecules that are recognized by the plant host upon invasion, resulting in PAMP-triggered immunity (PTI) (Miller et al., 2017). PTI can also be induced by host recognition of injury (damage-associated molecular patterns, DAMPs) (Miller et al., 2017). PTI is a form of non-host-specific resistance, acting as a broad-spectrum defense against both adapted and non-adapted attacking organisms (Miller et al., 2017; Walling, 2009). A pathogen or pest is considered to be non-adapted when the plant is able to prevent or overcome colonization and tissue damage due to the invading

organism. Adapted pests or pathogens can have specific alterations that allow them to suppress PTI responses and cause disease (Walling, 2009; Miller et al., 2017). Plants have evolved specific defenses to combat adapted organisms. Following activation of PTI, adapted organisms release effectors that disrupt PTI and cause disease. While PTI is associated with general plant defense pathways, plants have coevolved resistance genes to activate effector-triggered immunity (ETI) in response to specific effector proteins as well (Miller et al., 2017). Defense responses may be shared during simultaneous attack by pathogens and insects, or differ depending on the type of stressor, timing of attack, and intensities of the stress (Walling, 2009). Hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) mediate long-distance responses, with SA usually being triggered by phloem feeders and biotrophic pathogens, and JA by chewing insects and necrotic pathogens. Combinations of these attacking organisms can affect hormonal production, and can result in a benefit to the insect, the pathogen or both. In fact, JA and SA are antagonistic in many plant species.

In the case of *Xff* infection in grapevine, initial detection of bacterial lipopolysaccharides (LPS), a known PAMP in phytopathogenic bacteria, is delayed (Rapicavoli et al., 2018). This delay in detection is partially due to shielding of the bacterial cells by an O-antigen polysaccharide chain (a component of the LPS) and allows the pathogen to avoid initial PTI responses and colonize the host (Rapicavoli et al., 2018). The ability of *Xff* to avoid initial detection and early elicitation of PTI greatly changes the plant's response to infection. Rapicavoli et al. (2018) found that grapevines (cv. 'Cabernet Sauvignon') inoculated with a mutant *Xff* strain that lacked the O-antigen shield had systemic and long-lasting SA-mediated defense pathways activated, plus other

PTI-related responses. Vines inoculated with the wild type *Xff* had no SA-mediated defense responses at any time point in the 18-week period of infection but instead had systemic JA-mediated responses that lasted for the duration of the infection. Supporting these results, Zaini et al. (2018) found that grapevines (cv. 'Thompson Seedless') at 12 weeks post-inoculation with *Xff* activated defense responses including PTI but had limited SA-mediated responses. These studies show that the defense responses of grapevines to *Xff*, including JA-mediated responses, persist throughout the duration of the infection. In addition, *Xff* is able to cause PD in plants by delaying the elicitation of PTI and the related activation of SA-mediated defense pathways.

Another component of plant defense response to *Xff* is the production of secondary metabolites, notably phenolic compounds. A number of phenolic compounds have been identified to play a part in host response to pathogen infection. Wallis and Chen (2012) found heightened levels of phenolic compounds in 'Thompson Seedless' xylem sap and xylem tissue at two months post-inoculation with *Xff*. However, production decreased over time, with an ultimate reduction in phenolic compounds compared to uninfected control plants six months post inoculation. This study shows that while *Xff* may initially cause an increase in phenolic compound production, it ultimately causes a reduction due to the lack of resources available for secondary metabolite production. As the disease progresses, plant resources are reallocated, with a shift to production of primary metabolites to support necessary metabolic processes as photosynthetic rates are reduced (Wallis & Chen, 2012).

In susceptible grape varieties, there is a decline in photosynthesis, starch storage, and stem hydraulic conductivity (Fanton et al., 2022). The study by Fanton et al. (2022)

attributed the loss of hydraulic conductivity to the formation of tyloses. Tyloses are vascular occlusions produced by the plant that limit the movement of pathogens throughout the xylem, but also inhibit water transport, which can induce water stress responses (Sun et al., 2013). Stomatal closure occurs as a response to water stress as a way to reduce transpiration and water loss, but prolonged closure leads to significant reductions in photosynthesis (Ingel et al., 2020). Infected plants must then rely on starch reserves to sustain essential processes and defense responses, resulting in depletion of stored starch and further reductions in photosynthesis (Fanton et al., 2022; Ingel et al., 2020). In PD-susceptible vines, starch depletion was specifically noted in stems (Fanton et al., 2022). Transcriptional analysis by Ingel et at al. (2020) supported these findings of starch-depletion and reduced photosynthesis, in which an upregulation of genes associated with starch, carbohydrate, and sucrose metabolism was reported while a suppression of genes related to photosynthesis and carbon fixation was found.

On the outside of the vines, symptoms characteristic of *Xff* appear, including marginal leaf necrosis and leaf scorching (Rapicavoli et al., 2017) (Figure 3.1: Symptoms of Pierce's Disease). Specific to PD, abscission of leaves from their petioles and development of "green islands" occur, in which the periderm develops irregularly (Rapicavoli et al., 2017). Below ground, root mass and root length can be negatively affected by high titers of *Xff*, and decreasing root mass and length are associated with increasing above ground symptoms (Wallis et al., 2013).

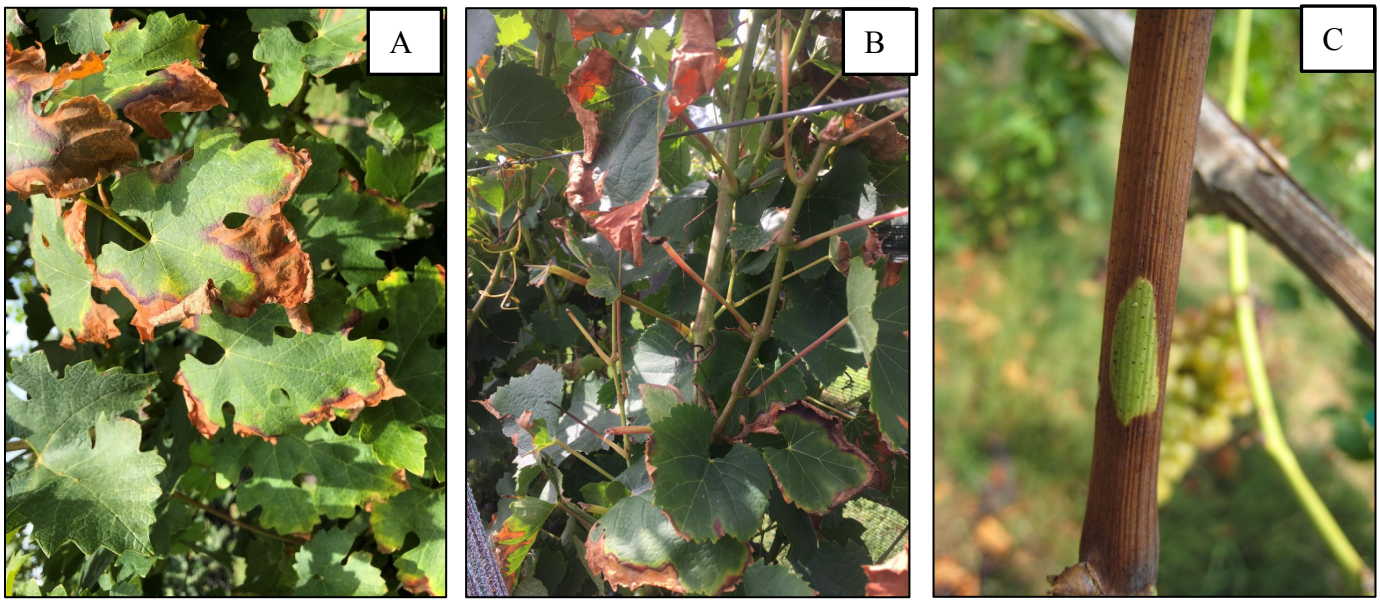

Figure 3.1: (Lowder et al., 2019). Symptoms of Pierce's Disease. Marginal necrosis and leaf scorching (A), abscission of leaves with "matchstick" petioles (B), and a "green island" formed by irregular development of the shoot tissue (C).

Phloem-feeding insects are known to elicit specific plant responses as well. Hormonal responses associated with chewing versus piercing and sucking insects can vary, particularly in the antagonism between JA and SA pathways (War et al., 2012; Broekgaarden et al., 2011). Chewing insects are known to suppress SA production and in turn upregulate JA as a defense. JA regulates many genes responsible for plant defenses against herbivores, and jasmonates induce the formation of antioxidative enzymes, alkaloids, and even production of trichomes that act as a physical protection against chewing insects (War et al., 2012). Conversely, phloem-feeding results in the opposite- SA is upregulated while JA is suppressed (Sing et al., 2021; Broekgaarden et al., 2011). The differences in response may be partially attributed to the stealth of phloem-feeding insects, in which damage is often barely perceptible by the plant (Walling, 2008). In

contrast, chewing herbivores cause greater physical damage to the plant (Walling, 2008; Broekgaarden et al., 2011). Additionally, phloem-feeding insect attacks may cause a restructuring of plant cells. Changes such as cell wall thickening, lignification, stomatal closure, and formation of a waxy cuticle have been documented (War et al., 2012).

Plant responses to insect feeding is highly specific to the attacking insect and host plant (Broekgaarden et al.2011; Timm & Reineke 2014). In grapevines (cv "Riesling"), mealybug (*Planococcus ficus)* feeding elicited a relatively low defense response compared to uninfested control vines, quantified by the limited number of differentially expressed transcripts. (Timm & Reineke 2014). In the case of the SLF, a wide variety of biological pathways were activated in a *Vitis* hybrid cultivar (Marquette) in response to phloem feeding, including an increase in cell-wall remodeling and detoxification (Islam et al., 2022). Pathways induced by SLF feeding may be unique from other phloem-feeders due to their aggressive feeding. SLF can cause wounding to stems and trunks of vines, potentially leading to more destruction in plants and greater host perception, unlike smaller phloem-feeding insects such as aphids and whiteflies (Islam et al, 2022).

Parameters important to grapevine growth and fruit production are also impacted by SLF attacks. SLF accumulate and feed in large numbers on single vines, leading to reductions in carbon assimilation and resource storage in roots at the end of the season (Harner et al., 2022, 2025) and reduction in water use that persists after SLF removal from the plants (Harner et al., 2025). The reduction in carbon assimilation can be linked to stomatal closure, induced by the molecular signaling pathways triggered in response to insect herbivores (Lin et al., 2021). However, the specific mechanisms leading to

stomatal closure in response to SLF feeding are unknown but may be associated with phytohormonal changes (Harner et al., 2022).

Overall resource allocation in vines is affected by SLF, with a greater negative impact on storage of starch and nitrogen in roots than in above-ground tissues (Harner et al., 2022). These findings are supported by transcriptional analysis, in which SLF feeding has been noted to alter the gene expression for the processing of nitrogen assimilation and translocation in *Vitis* hybrid cultivar (Marquette) grapevines (Islam et al., 2022). Indeed, long exposure to SLF led to depletion of carbohydrate (starch) reserves, and in extreme cases this may lead to insufficient carbon reserves for supporting dormant vines and seasonal growth, potentially causing vine collapse (Harner et al., 2022).

Effects of SLF have also been examined on other woody perennials. Long-term negative effects of intense SLF attack on young silver maples (*Acer saccharinum*), weeping willows (*Salix babylonica)*, river birches (*Betula nigra*), and trees of heaven (*Ailanthus altissima*) were observed by Hoover et al. (2023). They found that heavy feeding by SLF over four consecutive seasons significantly reduced diameter growth and below-ground starch storage in roots in these species. Additionally, nutrients essential to photosynthesis and overall growth, including iron, sulfur, and phosphorus, were reduced in the leaves of all species compared to control plants within the second year of attack. In a shorter time frame, Lavely et al. (2022) investigated the impacts of fourth instar and adult SLF feeding when confined to either a single branch of or a whole young host tree sapling. They found that high numbers of adults (40 SLF) confined to a single branch of

red or silver maple suppressed gas exchange and reduced nitrogen concentration in leaves and branch wood.

Overall, SLF have been shown to have negative effects on perennial plant health when feeding in large numbers. It is unknown if these impacts will be exacerbated with the addition of infected host plants, or what complex interactions may come into play.

Insect responses to pathogen-infected plants

Insect vectors have specific relationships with associated pathogens and host plants. Unique responses in vector behavior can have significant effects on the insects, including effects on insect survival, development, or fecundity. Host manipulation by pathogens favoring multiplication and dispersal has been reported in a number of systems. Vectors may be manipulated directly or indirectly by plant pathogens, and interactions are specific and complex (Eigenbrode et al., 2018). For example, larval thrips (*Frankliniella occidentalis)* exposed to tomato spotted wilt virus (TSWV) had shorter development times, and reproductive rates were higher in thrips exposed to TSWV (Shalileh et al., 2016). In the same study, adult thrips that were exposed to TSWV as larvae preferred to feed on non-infected leaf disks. TSWV is only acquired in the larval stage and then transmitted throughout adulthood. A shorter development time and increased reproductive rates could be conducive to virus transmission. Similarly, the preference of viruliferous thrips to feed on non-infected plants as adults increases chances of transmission. Further, adult thrips may then lay eggs on the plants they have infected, allowing the next generation of larva to acquire the virus and thus continue the disease cycle.

Insects more closely related to SLF, such as leafhoppers, have also been shown to be manipulated by plant pathogens. Known vectors of phytoplasmas, including leafhoppers and planthoppers, preferentially feed on young green leaves and shoot tissues. The accumulation of phytoplasmas in these younger tissues may increase the likelihood of vectors acquiring the pathogens due to preferential feeding (Marcone, 2014). In addition, characteristic phytoplasma symptoms include proliferated growth of young green and yellow tissues in plants, resulting in phyllody, witches' broom, virescence, and overall yellowing. These changes may also increase attractiveness to vectors, leading to higher chances of insects feeding and acquiring phytoplasmas from plants (Marcone, 2014). Phytoplasmas have the potential to directly influence their insect vectors as well. Beanland et al. (2000) described the influence of the aster yellows phytoplasma on leafhopper fitness. Aster leafhoppers (*Macrosteles quadrilineatus*) exposed to the aster yellows phytoplasma lived longer and laid more eggs than those insects not exposed, increasing the time for inoculation and potentially increasing insect vector populations on or near infected host plants (Beanland et al., 2000).

Another hemipteran, the Asian citrus psyllid (*Diaphorina citri*), is affected by the bacterium '*Candidatus* Liberibacter solanacearum' (CLso), the causal agent of economically important diseases in solanaceous crops. CLso alters the volatile organic compounds of the host plant, causing non-infected psyllid vectors to preferentially settle on infected plants (Mas et al., 2014). Following this initial settling behavior, psyllids that become infected carriers of the bacteria then change preference towards non-infected plants, increasing the chance of pathogen transmission (Mas et al., 2014). A closely related pathogen, '*Candidatus* Liberibacter asiaticus' (CLas), the causal agent of citrus

greening disease, has been shown to directly impact *D. citri* (Pelz-Stelinski & Killiny, 2016). The fecundity of *D. citri* was found to have increased in infected insects, although nymphal development rates and adult survival were reduced in infected insects (Pelz-Stelinski & Killiny, 2016).

Much is still unknown about the requirements for SLF growth and development; however, studies have been conducted on SLF feeding behavior. Mason et al. (2020) investigated the feeding behaviors of SLF on ornamental trees. They found that SLF consistently attacked specific trees within a landscape, choosing specific red and silver maple trees over other trees of the same species. The reasons for this preference may be associated with nutritional variations in the trees. The study utilized single-timepoint foliar elemental composition measurements to evaluate the components that could be playing a part in SLF attraction. Foliar nitrogen, calcium, and sulfur concentrations had a positive relationship with SLF attack, while phosphorus, potassium, iron, and sodium had a negative relationship. Of these elements, nitrogen has been identified as a common rate-limiting nutrient for insect growth and development (Mattson, 1980). Indeed, plants with higher nitrogen content are often more attractive and supportive to phloem-feeding insects, as is the case with the brown planthopper (*Nilaparvata lugens*), an insect related to the SLF through membership to the Fulgoroidea superfamily (Rashid et al., 2017).

Further insight into the factors influencing host plant selection can be found in studies regarding plant volatile production. Plant volatiles can play a role in the attraction of phytophagous insects to host plants, including the SLF. Research by Derstine et al. (2020) found isolated host plant volatiles that served as attractants to SLF and discovered certain blends of volatiles increased attraction beyond individual effects. Similarly,

Cooperband et al. (2019) showed that methyl salicylate collected from volatiles of grapevines and tree of heaven was the strongest attractant to all life stages of SLF in the lab, out of the kairomones tested. Methyl salicylate is a defense-associated hormone found to be increased in the xylem sap of grapevines infected by *Xff*, thus it may play a part in how SLF will respond to PD-infected vines (Wallis et al., 2013).

The documented responses of insects to PD-infected grapevines specifically is limited to vector studies, thus it is unknown how insects of different trophic levels may respond to PD-infected vines. As a pathogen, *Xff* has established an evolutionary relationship with its insect vectors, and elements of this relationship may play a role in the interaction of SLF with PD regardless of the ability of SLF to act as vectors. A study by Perring et al. (2009) found a seasonal effect of *Xff* on vectors, in which the glassy-winged sharpshooter (*Homalodisca vitripennis)* (GWSS) preferred to feed on PD-infected grapevines in winter, when they are dormant, speculatively due to the biochemical composition of the plant during that phenological stage. Changes in xylem sap, such as induction of phenolic compounds, have been described to fluctuate throughout the course of infection. Wallis & Chen (2012) examined the changes in phenolics in potted *V. vinifera* 'Thompson seedless' vines inoculated with *Xff*. They found that the concentration of a variety of phenolic compounds was higher during early *Xff* infection (within two months of inoculation), while four months post infection there were no differences compared to healthy control plants, and at six months post infection phenolic levels ultimately had a significant reduction. These phenolic responses in xylem sap and tissues of *Xff* -infected vines are one potential factor contributing to vector feeding preference.

Further support for PD infection altering vector feeding preferences was found by Daugherty et al. (2011), who reported that GWSS were more likely to feed on PD-infected vines that were painted to look healthy (non-symptomatic) versus symptomatic PD-infected vines. Similarly, the insects preferred healthy grapevines compared to infected, symptomatic vines and compared to healthy vines artificially colored to look symptomatic. These findings indicate that visual cues may play a role in feeding selection of vectors, in which insects discriminate against symptomatic grapevines, likely as infected vines are visually perceived to be of poorer quality.

Taken together, the studies by Perring et al. (2009) and Daugherty et al. (2011) suggest that both biochemical and visual cues play a part in insect feeding preference. Interestingly, during the winter season when no visual symptoms of infection are present (no foliage), GWSS chose to feed on infected vines over healthy. When symptoms are present on foliage later in the season, sharpshooters preferred to feed on healthy (asymptomatic) vines. These findings could have great implications for bacterial acquisition and transmission throughout the year. Since *Xff* is transmitted persistently by GWSS (adults retain *Xff* for life), the likelihood of pathogen transmission is enhanced by vectors feeding more heavily on infected vines early in the season, and then switching to preferentially feed on healthy vines later in the season. While this relationship has been studied specifically with vectors, the changes in host plant chemistry and visual appearance in response to pathogen infection can trickle down to alter the relationships between the infected plant and all other microbes and insects that feed on it.

Objectives and hypotheses

The goal of this work was to examine the interactions between SLF and grapevines infected with PD. Specifically, objectives were three-fold: (1) Determine how combined biotic stressors (SLF and PD) affect plant primary metabolites that are important for grapevine longevity and productivity, such as carbohydrate (starch and soluble sugars) and total nitrogen, compared to a single biotic stressor or no stressor; (2) Determine the effect on SLF survival and development from 4th instar nymph to adult stage when feeding on PD-infected vines; and (3) Determine if SLF exhibit a feeding preference between PD-infected and uninfected (healthy) grapevines.

As noted above, there is limited information on grapevine responses to combined stress by pathogens and insects. This research aimed to elucidate the combined effects of SLF and PD on grapevines, in hopes of gaining an understanding of how SLF may influence pathogens as insect populations spread towards areas with vineyards affected by PD. Knowing the progression of vine responses to both PD and SLF can help develop a more targeted management approach. It was hypothesized that *Xff* infection and SLF infestation would have a synergistic effect. SLF have been shown to reduce carbohydrate (starch) reserves in roots of perennial plants and cause a reduction in photosynthesis and growth (Harner et al, 2022; Hoover, et al, 2023; Lavely et al., 2022). Similarly, *Xff* infection can negatively impact photosynthetic rates, leading to a reduction in stored starch as resources are reallocated to support essential metabolic processes (Fanton et al., 2022; Ingel et al., 2020). It was expected that reductions in carbohydrate reserves and inhibited photosynthesis would be exacerbated due to *Xff* infection and SLF infestation, resulting in a synergistic effect from the combined biotic stressors. Synergism may also

occur in part due to the persistent suppression of SA-mediated pathways by *Xff* (Rapicavoli et al., 2018). SA plays a part in the defense response to certain phloem feeding insects; thus, the suppression of SA-mediated defense pathways may lead to SLF imparting greater feeding damage on infected vines than non-infected vines (Sing et al., 2021; Broekgaarden et al., 2011).

While most information involving pathogen-insect interaction is regarding vectors, pathogens are able to indirectly influence non-vector insects as well. SLF may respond differently to infected plants compared to healthy, regardless of whether SLF are capable vectors or not. SLF survival and development on PD-infected grapevines was tested, to provide insight into how SLF may respond as they are introduced into diseased vineyards. It was hypothesized that SLF would have a higher rate of mortality on *Xff* -infected vines compared to uninfected (healthy), and that SLF would exhibit a decrease in successful development from nymph to adult when feeding on infected vines. The reduction of plant resources such as starch, secondary metabolites, and potentially nitrogen during late-season *Xff* infection was expected to negatively impact SLF as the insects rely on plant nutrients for survival.

Lastly, to further examine the response of SLF to PD-infected vines, feeding preferences of adult SLF between PD-infected and healthy vines was determined. Regardless of the ability of SLF to act as a vector of PD, the pathogen may indirectly influence the behavior of the insects. SLF response to infected vines could provide insight into SLF distribution and feeding patterns that might occur as SLF enter vineyards infected with PD. It was hypothesized that SLF would prefer to feed on healthy versus PD-infected vines, based on the known visual and chemical changes that occur during

PD-infection that could be perceived as a reduction in quality of feeding material to the SLF.

Materials and Methods

Experiments 1, 2, and 3 were conducted using vines inoculated with *Xylella fastidiosa* and SLF collected as follows:

Mechanical inoculation of grapevines with *Xylella fastidiosa*

Three-year old grapevines (*Vitis vinifera* cv. Cabernet franc) grafted on 101-14 were grown in 19 L pots with custom substrate (1:1:1 field topsoil, perlite, and peat moss) and cultivated in an APHIS approved greenhouse facility at the Pennsylvania State University, University Park Campus. Vines were trained to one shoot and maintained at approximately five feet. Forty Cabernet franc vines were inoculated in April 2022 with *Xff* strain Temecula 1, which was provided by C. Roper, UCR. The strain was grown at 28°C on solid PD3 medium until date of inoculation (Davis et al. 1981). Vines were mechanically inoculated as described by Purcell and Saunders (1999): A 20 µl drop of turbid cell suspension in pH 7 phosphate-buffered saline (PBS) was pipetted onto the stem (shoot) just above the third petiole from the base of the vine. The stem was pierced through the inoculum drop using a 20-gauge needle, so that the droplet was taken up into the xylem. The process was repeated on the opposite side of the stem to the initial droplet placement, for a total of 40 µl inoculum per vine. Twenty control plants were subjected to the same procedure using water instead of bacteria. Vines were monitored for symptom development to confirm infection. Vines were periodically irrigated throughout the experiments and fertilized with 35g urea in July 2022, prior to beginning the experiments.

Spotted lanternfly collection

Fourth instar and adult SLF were manually collected from wild hosts across eastern Pennsylvania as needed between July and October 2022. Insects were collected, transported, and maintained until use in mesh insect cages containing a potted grapevine or tree of heaven (*Ailanthus altissima*) to serve as a food source. SLF were maintained and all experiments involving SLF were conducted in a greenhouse at the Pennsylvania State University, University Park Campus.

Experiment 1: Combined effects of *X. fastidiosa* infection and SLF infestation on grapevines

An experiment to assess the combined effects of PD infection and SLF infestation was conducted from July-September 2022 on potted vines (cv Cabernet franc) maintained under greenhouse conditions. PD-infected vines were inoculated with *Xff* as described above. The experiment was set up using a 2x2 factorial design: two levels of SLF infestation (yes or no), and two levels of pathogen infection (yes or no). Six potted vines were used for each treatment combination. All vines were individually covered with a mesh insect bag, modified to add a zipper (AgFabric, Wellco Industries, Inc., Coronoa, CA, USA). Vines were arranged in a randomized complete block design with six blocks, each one containing one vine from each treatment. Forty fourth instar SLF nymphs were added to each vine on July 28, 2022.

Following initial infestation, adult SLF were added to vines approximately weekly to replace dead SLF and maintain the infestation at 40 SLF per vine for

approximately seven weeks. SLF were removed from all vines on September 16, 2022. A few days later, on September 22, the vines were moved to the Horticulture farm at the Russell E. Larson Agricultural Research Center and left outdoors to acclimate to decreasing ambient temperatures until December 22 and then stored in a walk-in cooler during the winter months.

Quantification of nonstructural carbohydrates and nitrogen in woody tissues

Cane (lignified shoot) and root tissues were sampled at the end of the experiment for quantification of total nonstructural carbohydrates (TNC), the sum of starch and soluble sugars. Cane samples were collected twice, on the day SLF were removed from the vines, September 16, and while the vines were stored outdoors on November 17. On the first date, three ~8cm long sections were taken from the bottom, middle, and top portions of the main cane. All cane sections from each vine were combined for analysis. On the second date, samples were taken from the remaining tissue of the main cane, ~20cm total.

Lignified roots were sampled from each vine on November 14, 2022. Vines were removed from their pots and soil shaken to expose roots. Lignified roots were collected from the outer edges of the root mass. Roots were rinsed with water to remove soil particles. Cane and root samples were frozen with liquid nitrogen and stored at -80°C until processing. Both canes and root samples were lyophilized for one week at -50°C and under 0.100mbar pressure (FreeZone 12-liter Freeze Dryer, Labconco, Kansas City, MO, USA). Cane and root tissues were then milled to a fine powder (<1mm mesh; UDY

Cyclone Mill, UDY Corporation, Fort Collins, CO, USA) and stored at room temperature until TNC and N analysis.

TNC and soluble sugar concentrations were quantified in each tissue using an enzymatic assay according to Comas et al. (2005) with a spectrophotometer at 520 nm (UV1600, VWR International). Starch was estimated by subtracting soluble sugars from TNC. Concentrations were expressed in glucose equivalents. Ground cane and root tissues samples were analyzed for total nitrogen via combustion (Horneck & Miller, 1998) by The Pennsylvania State University Agricultural Analytical Services Laboratory.

Disease ratings of PD-infected vines

To assess the impact of SLF on PD-infected vines, disease ratings were taken on vines infected with PD. Ratings were recorded five times throughout the experiment. Ratings were assigned as follows: 0 = No PD symptoms; 1 = one or two leaves with significant marginal necrosis; 2 = two to three leaves with significant marginal necrosis; 2.5 = more than two to three leaves symptomatic, but majority are healthy; 3 = one half or more leaves showing marginal necrosis and match sticks; 4 = all leaves showing heavy scorching and numerous matchsticks; 5 = a dead vine.

Disease severity was calculated using the following formula from Chiang et al., 2017:

Disease Severity (%)/Treatment (PD or PDSLF)=

$$\frac{\Sigma \text{ (Class frequency X Score of rating class)}}{\text{(Total number of symptomatic plants) X (Maximal disease index)}} \text{ X } 100$$

Class frequency is the number of plants with the same disease rating, and score of rating class is the disease rating score (1, 2, 2.5, 3, or 4).

Experiment 2: Effect of *Xff*-infected vines on SLF mortality and development

SLF mortality was measured by counting the number of dead SLF on each vine used in Experiment 1. SLF were counted and replaced on six dates throughout the experiment (8.10, 8.17, 8.26, 9.5, 9.8, and 9.12, 2022). To assess if the development of SLF varied between infected and not infected vines, the number of 4th instar nymphs that developed into adults was measured. The total number of dead nymphs on each vine was counted and subtracted from 40, the initial number of nymphs added per vine. The resulting number represented the number of nymphs that did not die before molting into adult SLF.

Experiment 3: Spotted lanternfly feeding preference between *Xff*-infected and noninfected grapevines

SLF feeding choice experiments were conducted in September-October 2022. Choice experiments were set up with pairs of vines, using either two uninfected (healthy) vines (*Vitis vinifera* cv. Chardonnay) or one healthy vine (cv. Cabernet franc) and one *Xff* -infected vine. *Xff* -infected vines were grapevines (cv. Cabernet franc) inoculated with *Xylella fastidiosa* subs. *fastidiosa* as described above and denoted as "Pierce's disease (PD)-vines" throughout the experiment. Paired vines were of similar age and growth stage and were kept under the same environmental conditions.

Preliminary choice tests using two healthy vines (healthy vs. healthy) were conducted using a combined five female and five male adult SLF. It was observed that female SLF determined feeding choices, in that male SLF followed female SLF locations

and feeding patterns in cage. Therefore, only female SLF were chosen to conduct the feeding preference experiments. A total of five healthy vs. healthy and six PD vs. healthy replicates were conducted, using 10 SLF per replicate.

Choice tests were performed in a greenhouse on paired vines by inserting one shoot from each vine into opposing ends of a tubular cage. Cages were constructed using two metal tomato cages tied together by zip-ties and covered in a homemade zippered mesh tube (Figure 3.2: Feeding preference cage set-up). The cages were elevated on a rolling cart. The shoots were suspended using wire so that they did not touch the surrounding cage, and both ends of the cage were sealed tightly around the shoots using zip ties. The shoots from each vine were inserted a similar distance into the cage and overlapped slightly in the middle of the cage. Pairs of vines were chosen for each replicate by comparing shoot length, lignification, and number of leaves per shoot, so that the two shoots inserted into the cage were as similar as possible. Shoots from infected vines showed foliar symptoms but still had active growing tips, and infected shoots were selected to have a similar number of leaves as their healthy counterparts. Fresh pairs of vines were used for each replicate and the tomato cages were rinsed in between each replicate. Clean mesh tubes were used each replicate.

For each replicate, 10 adults female SLF were starved for one hour prior to starting the experiment. The SLF were then added together into the middle of a cage, equidistant from either shoot. SLF were placed directly onto the mesh cage. SLF were added in the morning or early afternoon and monitored across two days. Observations were made approximately every hour during daylight, recording how many SLF were on each vine or the cage, and how many SLF were actively feeding on each vine. SLF were

considered to be actively feeding if the stylet was visibly inserted into the plant (Figure 3.3: SLF feeding in choice test). Notes including the number of dead SLF at each observation were recorded as well.

Figure 3.2: Feeding preference cage set-up. Each cage represents one replicate of either the healthy vs healthy (A) or PD vs Healthy (B) trial.

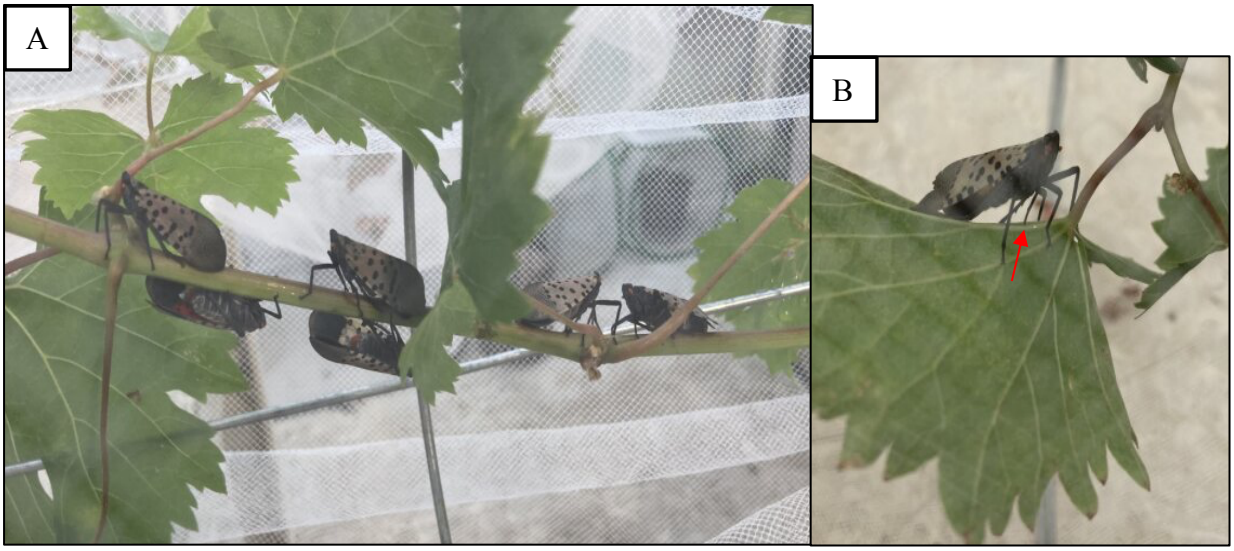

Figure 3.3: SLF feeding in choice test. SLF settled and feeding on shoot of healthy vine (A). SLF feeding on leaf vein of healthy vine (B). Red arrow indicates where stylet is inserted into leaf vein.

Statistical Analyses

Statistical analyses to assess SLF feeding preference, disease ratings, SLF development, and SLF mortality were performed using R v. 4.1.2 (http://www.R-project.org/). For the SLF feeding preference experiment, the proportion of SLF actively feeding on a vine to the total number of SLF actively feeding in the cage was calculated for each time point. This proportion was calculated to account for differences in SLF numbers due to insect deaths. The mean proportions from all time points were then calculated for each vine. The mean proportions for each vine were subjected to a pairwise Wilcoxon test to calculate the pairwise statistical differences between each vine treatment pair, i.e. between two healthy vines or between one PD-infected and one healthy vine.

Data from disease ratings were subjected to a one-way analysis of variance (ANOVA) with a block as a random factor. For date-wise analysis, data were subjected to one-way ANOVA at each time point. All data from SLF mortality and development experiments were subjected to a one-way ANOVA with a random block variable. Statistical analyses to assess the quantification of TNC and nitrogen were performed using SAS v. 9.4 for Windows (SAS Institute, Cary, NC, USA). Data were subjected to 2-way ANOVA to assess the effect of the main factors (PD and SLF) and their interaction via MIXED procedure, with block as a random factor.

Results

PD and SLF effects on starch, soluble sugars, and nitrogen

Overall, there was significant reduction in starch due to SLF feeding in both cane and root tissues after prolonged feeding, regardless of PD presence (Tables 3.1 and 3.2). There was not a significant interaction effect between SLF and PD treatments, meaning that regardless of PD infection SLF caused a reduction in starch concentrations. For example, starch concentration varied from 79.38 mg/g in PD only vines to 25.25 mg/g in PDSLF vines, and from 54.73 mg/g in noPDnoSLF vines to 28.1 mg/g in PDSLF vines.

The concentration of soluble sugars in cane tissues was found to depend on both SLF and PD factors. The interaction effect between SLF and PD stressors was significant, indicating that SLF reduce soluble sugar concentration only when combined with PD infection. Again, this was apparent when comparing the average soluble sugar concentrations across treatments. There was a significant difference in soluble sugars with 61.53 mg/g in PD vines compared to 24.8 mg/g in PDSLF vines. In contrast, the presence of SLF did not affect soluble sugars in canes of vines with noPD (noSLF vines= 57.41 mg/g and SLF = 56.77 mg/g). Conversely, SLF and PD did not have a significant interaction effect on root soluble sugar concentrations. In the roots, PD vines had a lower concentration of soluble sugars than noPD vines, regardless of SLF infestation. Meanwhile, SLF caused an increase in root soluble sugars, independent of PD infection.

Lastly, late-season analysis of cane and root nitrogen showed limited effects due to SLF and PD (Table 3.3). The interaction between SLF and PD stressors was not significant in neither canes nor roots. The effect of SLF on nitrogen concentration was

found to be moderate to nonexistent in cane and root tissues, regardless of PD infection. However, PD vines had higher nitrogen concentration on both canes and roots than noPD vines, regardless of SLF presence.

Table 3.1 Average cane carbohydrate measurements from vines sampled September 16, 2022.

	Starch (mg/g)		Soluble Sugars (mg/g)	
	PD	**NoPD**	**PD**	**NoPD**
noSLF	79.38 ± 10.49	54.73 ± 11.02	61.53 ± 4.97	57.41 ± 5.29
SLF	25.25 ± 11.07	28.1 ± 3.95	24.8 ± 2.72	56.77 ± 2.15
***p*-value PD**	0.2489		0.0035	
***p*-value SLF**	0.0005		0.0003	
***p*-value PD X SLF**	0.1515		0.0004	

Table 3.2 Average root carbohydrate measurements from vines sampled November 11, 2022.

	Starch (mg/g)		Soluble Sugars (mg/g)	
	PD	**NoPD**	**PD**	**NoPD**
noSLF	252.65 ± 11.94	191.45 ± 35.30	51.82 ± 5.13	70.95 ± 5.45
SLF	129.45 ± 29.03	152.367 ± 32.26	75.78 ± 3.66	85.73 ± 5.56
***p*-value PD**	0.3994		0.0061	
***p*-value SLF**	0.0022		0.0007	
***p*-value PD X SLF**	0.0760		0.3294	

Table 3.3 Average percent nitrogen measurements from vines sampled September 16 (cane) and November 11 (root) 2022.

	Canes		Roots	
	PD	**NoPD**	**PD**	**NoPD**
noSLF	0.61 ± 0.02	0.46 ± 0.29	1.37 ± 0.03	0.94 ± 0.15
SLF	0.53 ± 0.04	0.44 ± 0.03	1.30 ± 0.60	1.09 ± 0.07
***p*-value PD**	0.0003		0.0030	
***p*-value SLF**	0.0900		0.6809	
***p*-value PD X SLF**	0.1809		0.2636	

SLF effects on PD disease ratings

Disease ratings were taken on two dates prior to SLF introduction on July 28, 2022. Prior to adding SLF to PDSLF vines, no significant difference in ratings was observed between the vines assigned to PD and PDSLF treatments (p=0.417). When ratings on individual dates were compared, only one date showed a significant difference between treatments (Figure 3.4 Average disease ratings on vines infected with Pierce's disease). On September 5, 2022, the average disease ratings were lower in vines under combined PDSLF treatment (p =0.073). Figure 3.5 shows the area under the disease progress curve, in which the disease severity percentage of PD vines and PDSLF vines are similar prior to adding the SLF to the vines on September 28th. The difference in disease severity percentage increased on the last three dates, in which PD vines had a higher percentage than PDSLF.

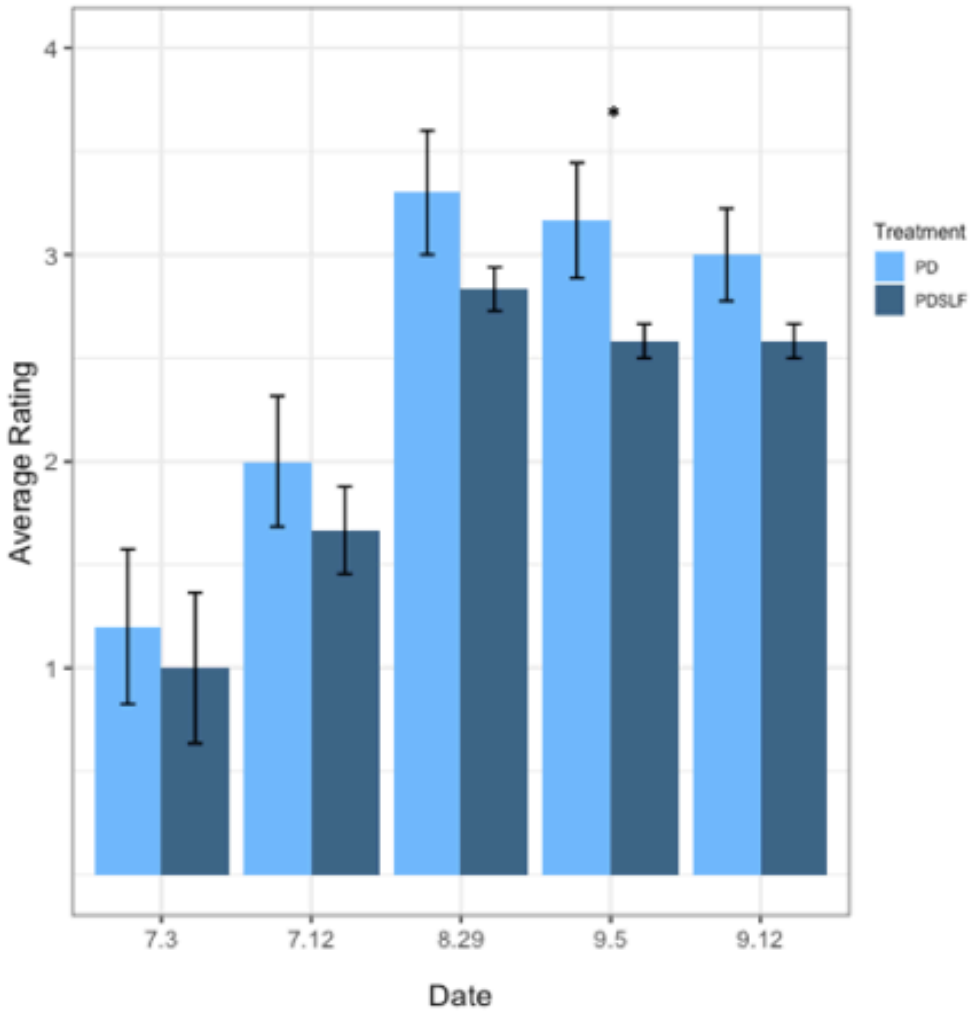

Figure 3.4: Average disease ratings in vines infected with Pierce's disease. PD vines were exposed to PD infection treatment, PDSLF vines were exposed to PD infection and SLF infestation treatment. Ratings of 1, 2, 2.5, 3, and 4 were recorded to assess disease severity on five dates in 2022. SLF were added to SLF infestation treatment on 7.28.22. PD vines had a significantly higher disease rating only on one date, 9.5.22 ($p = 0.073$).

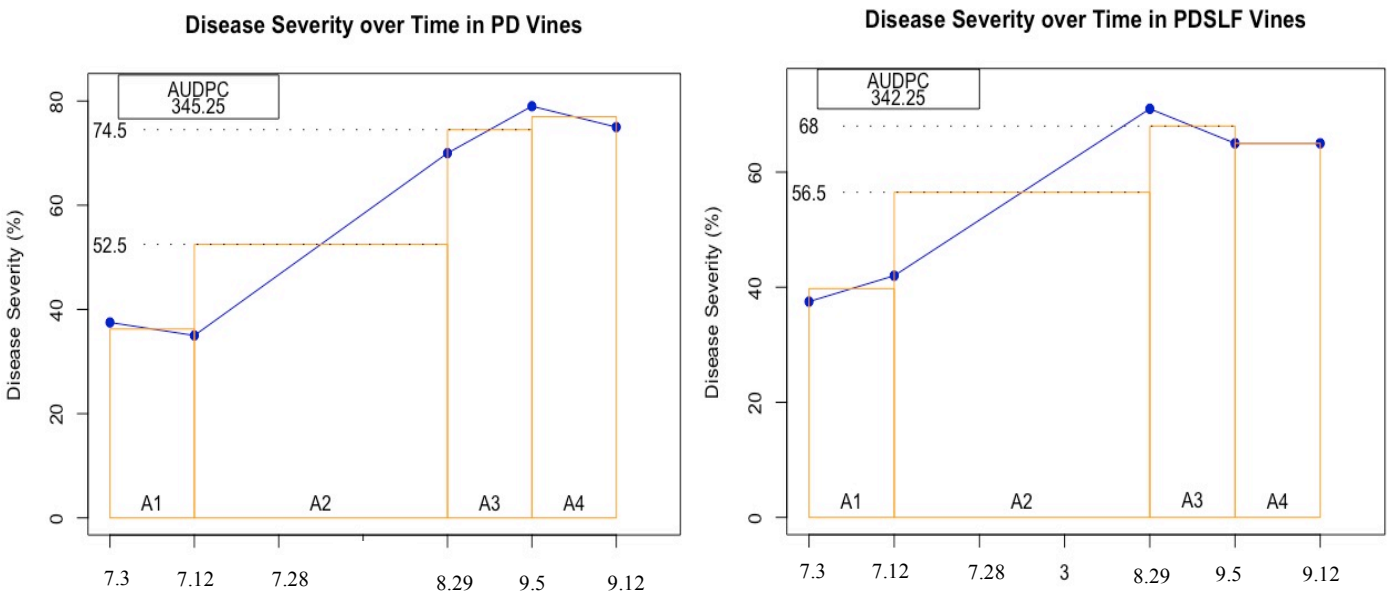

Figure 3.5: Disease progress curves for vines under PD treatment (A) and vines under PDSLF treatment (B). Ratings were taken on five dates in 2022, listed on the y-axis in month.day format. SLF were added to the PDSLF vines on 7.28.22. Area under the disease progress curve (AUDPC) is included. Dotted lines indicate average disease severity (%) between two time points.

PD effects on SLF development and mortality

Weekly SLF mortality was only significantly affected by PD infection on one date at the end of the experiment (Figure 3.6: Mortality of SLF on healthy (uninfected) and PD-infected grapevines). At 46 days after infestation (DAI), SLF on PD-infected vines had a significantly higher number of mortalities than those on healthy (uninfected) vines ($p = 0.072$). When cumulative SLF deaths were analyzed, three dates showed a significant increase of SLF deaths on PD-infected vines compared to healthy. Like weekly mortality, effects on cumulative mortality were observed late in the experiment, at 39, 42, and 46 DAI ($p = 0.097$, 0.041, and 0.278, respectively).

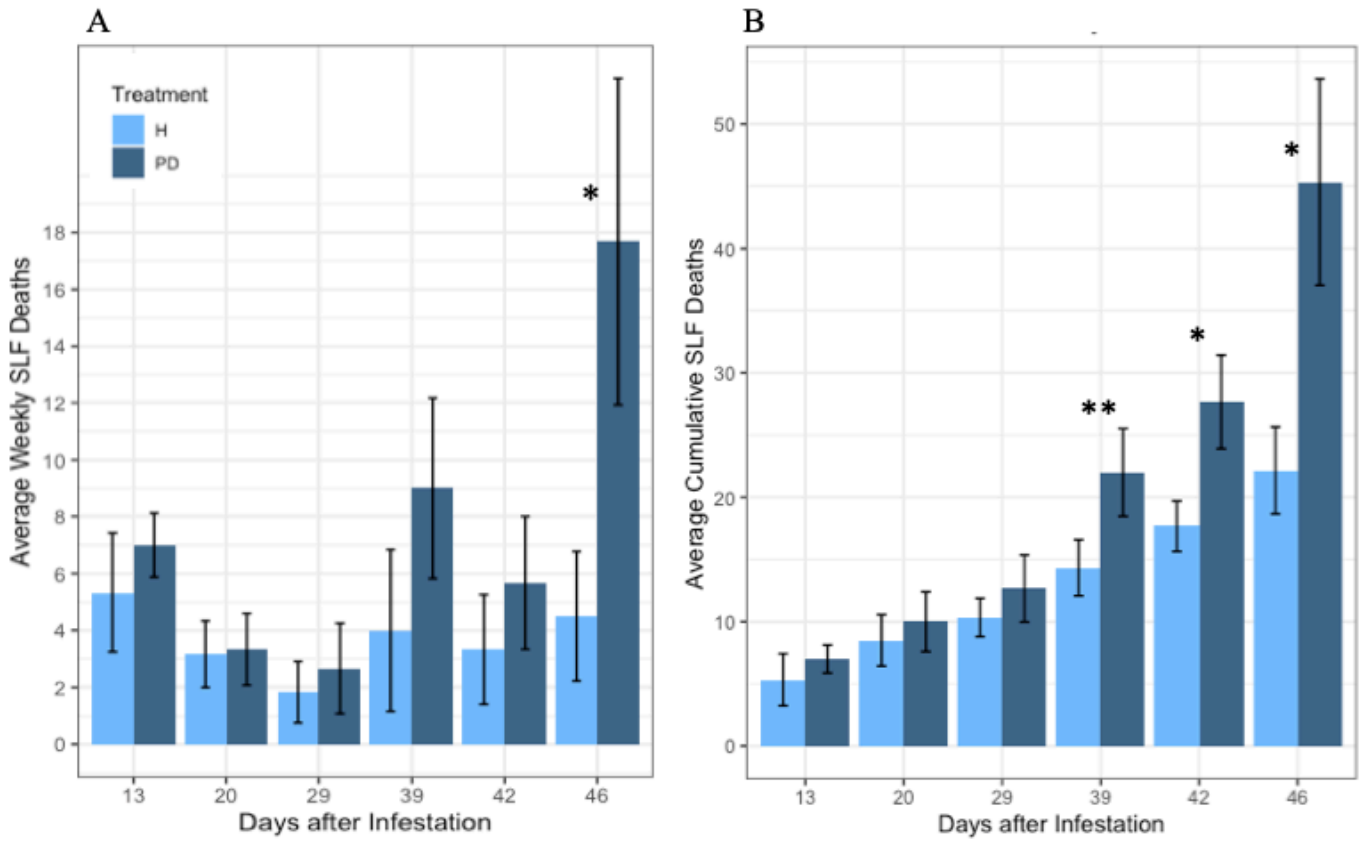

Figure 3.6: Mortality of SLF on healthy (uninfected) and PD-infected grapevines. Weekly mortality (A) of SLF. Bars represent the average (± SE) number of SLF that died each week when caged on healthy or PD-infected vines. When compared at each date, SLF exhibited a higher mortality on PD-infected vines at 46 days after infestation (p=0.0717). Cumulative mortality (B) of SLF. Bars represent the average (± SE) cumulative number of SLF that died at each date when caged on healthy or PD-infected vines. When compared at each date, SLF exhibited a higher cumulative mortality on PD-infected vines at 39 (p=0.097), 42 (p=0.041) and 46 days after infestation (p=0.0278).

The development of 4th instar nymphs to adult SLF was negatively influenced by PD infection (Figure 3.7: Development of 4th instar SLF nymphs to adults on healthy (uninfected) and PD-infected grapevines). A significantly higher number of nymphs developed into adults when exposed to healthy (uninfected) vines compared to PD-infected vines (p =0.055).

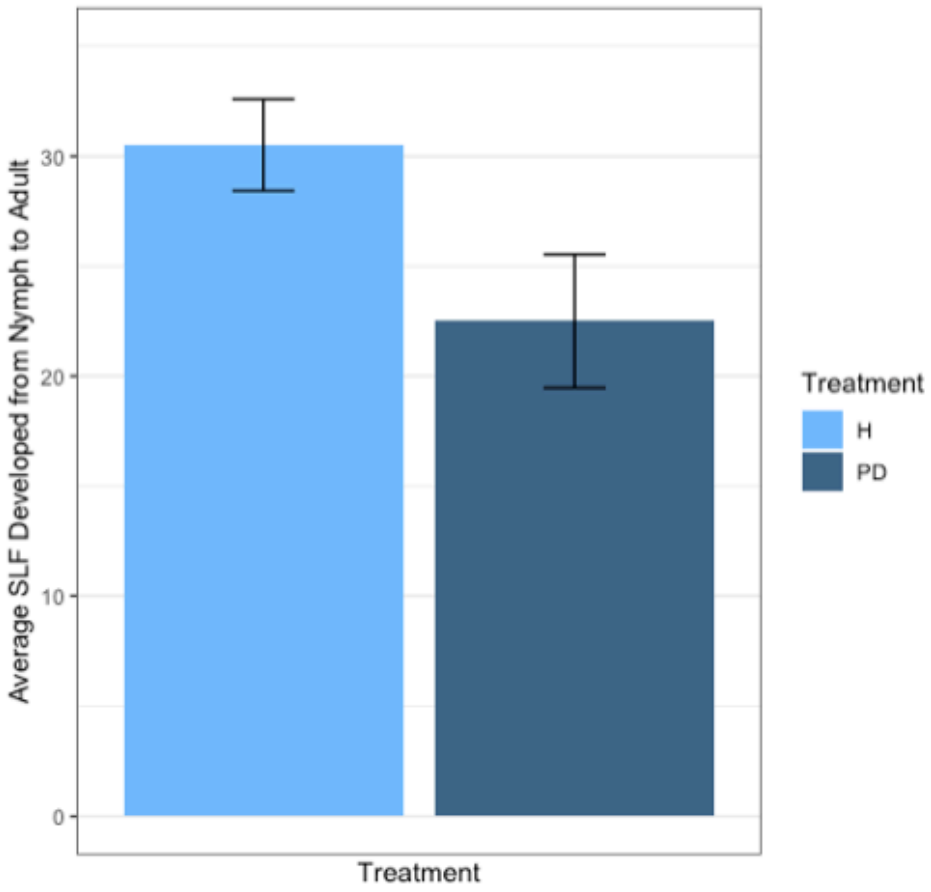

Figure 3.7: Development of 4th instar SLF nymphs to adults on healthy (uninfected) and PD-infected grapevines. Bars represent the average number of SLF nymphs that molted into adults when caged on PD-infected or healthy vines. Healthy vines displayed a higher number of successfully developed adult SLF compared to PD-infected vines (p=0.0545).

SLF prefer to feed on healthy over PD-infected grapevines

When given a choice between a PD-infected and healthy (uninfected) vine, adult female SLF chose to feed on the healthy vine (Figure 3.8: SLF choice experiment on PD-infected and healthy (uninfected) grapevines). The average proportion of SLF actively feeding on the healthy vine out of the total number of SLF in the cage was higher than those feeding on the PD-infected vine ($p = 0.03$). Similarly, the proportion of SLF actively feeding on a healthy vine out of the total number of SLF feeding was higher than those feeding on the PD-infected vine ($p = 0.03$). Thus, SLF overall chose to feed on healthy vines compared to PD-infected vines.

When given a choice between two healthy vines, SLF did not show a preference between either vine in proportion of SLF feeding on a given vine/total SLF in cage nor SLF feeding on a given vine/total SLF actively feeding across the experiment ($p = 0.813$, $p = 1.0$, respectively).

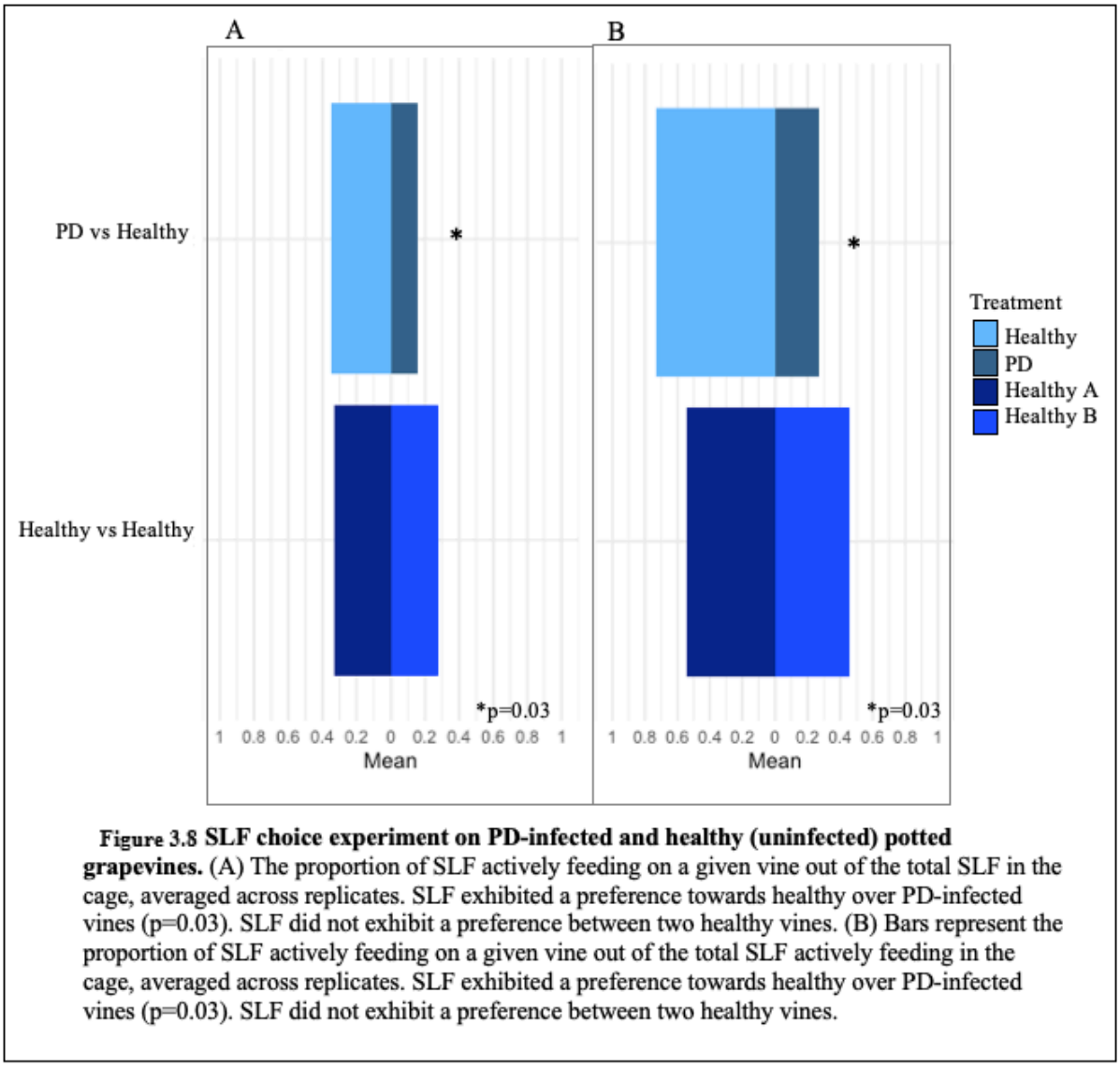

Figure 3.8 SLF choice experiment on PD-infected and healthy (uninfected) potted grapevines. (A) The proportion of SLF actively feeding on a given vine out of the total SLF in the cage, averaged across replicates. SLF exhibited a preference towards healthy over PD-infected vines (p=0.03). SLF did not exhibit a preference between two healthy vines. (B) Bars represent the proportion of SLF actively feeding on a given vine out of the total SLF actively feeding in the cage, averaged across replicates. SLF exhibited a preference towards healthy over PD-infected vines (p=0.03). SLF did not exhibit a preference between two healthy vines.

Discussion and Conclusion

One of the goals of this work was to determine how two biotic stressors (SLF and PD) when combined affect important plant metabolites, such as carbohydrate and nitrogen, in woody tissues compared to a single stressor or no stressor. Vines were exposed to SLF for approximately seven weeks, and SLF were replaced regularly to maintain a heavy feeding pressure. Overall, the biggest effect was on late season TNC by the SLF, as shown by the significant reduction in starch concentrations due to SLF stress in both cane and root tissues. This reduction in starch was independent of PD presence and supports the reduction of carbon resources that has been reported in vines under heavy SLF infestation. Harner et al. (2022) found a reduction in starch accumulation in woody roots of *V. vinifera* cv. Riesling vines, as plants use up carbohydrate reserves in response to the insect attack.

Soluble sugars in canes were lower in SLF vines only in presence of PD. This is consistent to what has been reported in previous work in another vinifera cultivar (Riesling), in which SLF alone did not affect soluble sugar concentrations in cane tissues (Harner et al., 2022). Therefore, SLF alone might not be enough to elicit a response. As noted by Fanton et al. (2022), limited research has been conducted on the role of soluble sugars in *Xff* defense responses. However, they suggested that there is a breakdown of starch reserves into soluble sugars, which are then used for production of secondary metabolites associated with *Xff* defense responses. It is unclear in what tissues this reduction may occur, and at what point during infection. For example, in the present study a reduction in cane soluble sugars during late-season infection was not observed in PD vines; however, further testing at earlier time points and in multiple tissue types could

lead to different results. A reduction in above-ground soluble sugars could have an impact on the following year's vine growth, especially if the reduction extended to the buds.

Non-structural carbohydrate reserves play a key role in facilitating early season vegetative growth, as well as maintaining basal metabolism during winter dormancy (De Rosa et al., 2022). Soluble sugars are related to cold tolerance, and sugar metabolism is important in dormancy transition, acting as an energy reserve and signaling factor (De Rosa et al., 2022). Specifically, higher levels of soluble carbohydrates are associated with cold hardiness in grapevines (Ershadi et al., 2015). Following winter, during bud deacclimation, these soluble carbohydrates decrease as they are reallocated to metabolic processes like cell growth and conversion to starch (Ershadi eta al., 2015). These essential processes involved in overwintering vines depend on the proper storage and utilization of carbohydrates, thus future studies examining the impact of combined *Xff* and SLF stress on bud development and relation to cold hardiness is warranted.

Late-season analysis of cane and root nitrogen showed the effect of SLF infestation to range from limited to nonexistent in cane and root tissues. This is somewhat in contrast to the findings of Harner et al. (2022), who found that high densities of SLF reduced nitrogen concentration in root tissues of Riesling vines grown under field conditions as well as in potted Marquette vines. Their study also measured leaf nitrogen, which was found to be significantly reduced due to high densities of SLF in both cultivars, and cane nitrogen, which was unaffected by SLF infestation in both cultivars.

Conversely, PD presence was associated with an increase in nitrogen concentration in both canes and roots, regardless of SLF stress. These results are

interesting, as the impact of PD on nitrogen concentrations in grapevines has not yet been established. However, significant alterations in the nitrogen metabolism of susceptible sweet orange plants (*Citrus sinensis*) were caused by *Xylella fastidiosa* infection (Purcino et al., 2007). Grapevines can have reduced root biomass due to *Xff* infection (Wallis et al., 2013). It is possible that the PD-infected vines had reduced root biomasses compared to non-infected vines. Thus, the nitrogen concentration of the sampled roots in PD vines may have been higher than in uninfected vines, yet the overall nitrogen content in the PD vine roots could be equal to or less than uninfected vines. Similarly, the PD vines had notably less leaves than the uninfected vines. The increased nitrogen in cane tissues of PD vines could be due to the lack of leaves available for nitrogen accumulation. Repeating the experiment to include measuring the root biomasses and overall root nitrogen concentration, as well as leaf biomass and leaf nitrogen concentration would help decipher how nitrogen storage and accumulation is impacted by *Xff*.

The higher amounts of nitrogen found in PD vines could be a beneficial host response for the bacteria, as nitrogen availability is associated with increased bacterial growth (Purcino et al., 2007). Further examination of how these alterations in nitrogen concentrations affect long-term growth of vines is needed, and to determine what processes are occurring in the nitrogen metabolic pathways that lead to these increases in both above and below-ground tissues of grapevines.

PD-infected vines with and without SLF showed a trend of increasing disease ratings over time, before leveling out at the end of the season. Towards the end of the experiment, all vines were showing age-related symptoms and a reduction in growth that could contribute to increased disease ratings overall. Disease ratings were based on visual

foliar symptoms. External PD symptoms are related to internal plant responses to pathogen infection. While the exact cause for the difference in disease ratings between PD and PDSLF treated vines is uncertain, it is possible that some responses to SLF feeding have an antagonistic effect to *Xff* infection and vice versa. Symptom severity is positively related to bacterial titers (Wallis et al., 2013). If SLF feeding interrupted the ability of the bacteria to colonize the plant, it is possible that a reduction in or slowing of symptom development could occur.

Changes in nutrient availability or induction of plant defenses by SLF feeding have the potential to impact *Xff* colonization. In *Vitis* hybrid 'Marquette' grapevines, transcriptional responses to SLF feeding include the upregulation of genes involved in cell wall strengthening, which could lead to fortification of plant structural barriers such as wax and lignin (Islam et al., 2022). These defenses overlap with responses to *Xff*, in which induction of pathways involved in lignin production and xylem cell wall thickening have been reported (Zaini et al., 2018). An increase in production of physical defenses against pathogen colonization induced by SLF feeding could be one factor involved in the reduced symptom severity observed in PDSLF vines. However, future investigation is needed to establish what is causing this effect.

The negative impact of pathogen stress on grapevines is mirrored in the negative impact of infected vines on SLF. An overall trend of increased SLF mortality on PD-infected vines compared to healthy vines was observed, with significant differences found at dates towards the end of the season when symptoms of PD vines were at a height. In addition, development from nymphs to adult stages was reduced in SLF subjected to PD vines compared to healthy. This confirms the hypothesis that SLF would

have a general decrease in fitness when feeding on PD-infected vines compared to healthy, perhaps due to an overall decline in available nutrient resources in infected vines. Despite the decreased fitness, it is important to note that the SLF were observed to be actively feeding throughout the experiment. SLF must feed constantly to survive and cannot survive much longer than three days without feeding (Personal communication, Julie Urban). Phloem feeders like the SLF consume carbohydrates and nutrients from their host plants (Lavely et al., 2022). PD is known to cause starch depletion as infected vines rely on carbohydrate reserves to support primary metabolic processes (Fanton et al., 2022). This reduction could lead to reduced SLF fitness on PD-infected vines. Although a reduction in starch due to PD was not observed in the present study, it is important to note that whole cane pieces were sampled, including bark, phloem, and xylem. Starch reductions may be tissue specific, and further analysis of tissue-specific starch concentrations could lead to different results (Ingel et al., 2020).

Limited information is available on the specific nutrients required by SLF for growth and development. Nitrogen is commonly identified as a rate-limiting nutrient for insect growth and development (Mattson, 1980), and Mason et al. (2020) reported that foliar nitrogen, calcium and sulfur concentrations in woody ornamentals were positively related to SLF feeding preference. PD-infected vines had higher nitrogen concentrations in both cane and root tissues compared to noPD vines by the end of the experiment, but foliar nitrogen concentrations were not examined in the present study. Despite the increase in nitrogen under PD infection, SLF performed worse overall on PD vines. This indicates that a multitude of factors are playing a part in SLF development and mortality.

PD vines may have overall lowered plant health, which could directly impact SLF. For example, PD vines had less leaves available for SLF to feed on compared to noPD vines. *Xff* -infected vines have been shown to have a loss in hydraulic conductivity, which could lead to lowered sap pressure and inhibit the ability of SLF to feed as the insects rely on turgor pressure to passively feed (Fanton et al., 2022; Uyi et al., 2021). In addition, the induction of secondary metabolite production as a response to PD infection could also impact SLF. As previously mentioned, fortification of plant structural barriers through lignin production and xylem cell wall thickening occurs in response to *Xff* infection. These responses overlap with SLF induced defenses, such as the upregulation of genes involved in cell wall strengthening, which could lead to fortification of plant structural barriers such as wax and lignin (Islam et al., 2022). While SLF feeding may reduce symptom severity in PD-infected vines, the converse may also be true- PD infection may reduce the ability of SLF to feed on PD-infected vines, via reduced SLF development and increased mortality, as a response to lowered plant health and *Xff* -induced plant defenses.

Feeding preference experiments showed female SLF choose to feed on healthy versus PD-infected, symptomatic grapevines. These results overall indicate that SLF do not perform well on PD-infected vines, and that their behavior changes accordingly. PD is known to cause changes in both internal and external symptomology that alter feeding behavior of its vectors. For example, increased phenolic compounds in xylem sap of infected vines and the appearance of visual symptoms have been suggested to alter vector feeding behavior. GWSS have been shown to prefer infected vines over healthy at an early stage of infection when no foliage or visual symptoms are present, but when

secondary metabolites in the xylem are increased (Perring, 2009). Yet, in late-season experiments, vectors preferred healthy vines regardless of visual symptoms (Daugherty et al., 2011). Unlike GWSS which feed on the xylem, SLF are phloem-feeders, thus it is likely that there are changes in vine health and nutrient composition occurring outside of the xylem that impact SLF survival, development, and feeding behavior. Secondary metabolites induced by *Xff* infection seemingly repel GWSS; however, it is unclear what effect these defense compounds will have on SLF. SLF are known to consume host plants that produce metabolites that result in high toxicity in other phytophagous insects, such as tree of heaven and black walnut (Mason et al., 2020). Thus, it is possible that changes in visual, volatile, or other chemical cues may be more involved in SLF avoidance of PD-infected vines than phenolic defense compounds.

In addition, as SLF invade southern regions of the U.S where PD is present, feeding behavior may change. Warmer climates accelerate the appearance and increase the severity of PD symptoms, which could deter SLF from feeding on infected vines (Varela et al., 2001). However, the warmer climate could also impact SLF lifecycles, leading to earlier hatching and potentially multiple generations per year. This would increase the overlap between PD symptom development and SLF presence, possibly increasing the chance of SLF feeding on PD vines during the earlier stages of PD infection when symptoms are milder and increased secondary metabolites are present. Further investigation involving the separation and elucidation of visual and chemical (volatile and internal) cues is needed to understand the factors driving SLF responses to infected vines.

Overall, the interactions between the biotic stressors of PD infection and SLF infestation are complex. The greatest impact on primary metabolites accumulation was on late season carbon. In most cases, SLF affected vines regardless of PD infection. Notably, starch was significantly reduced by SLF infestation in both canes and roots, regardless of PD infection. This is interesting to consider in conjunction with the results of SLF performance on PD-infected vines, in which there was an increased mortality of SLF. SLF caged on PD-infected plants were not often observed to be actively feeding, while those caged on healthy vines were frequently feeding. This observation is supported by the results of the feeding preference experiment, in which SLF preferred not to feed on PD-infected vines when given a choice between uninfected and infected vines. When not given a choice, it seems SLF may have fed less vigorously on infected vines. This indicates that SLF are able to influence carbohydrate production even when feeding less aggressively, and suffering from decreased developmental rates and increased mortality.

Future work investigating changes in primary plant metabolites (i.e., carbohydrates, nitrogen) and secondary metabolites (phenolic compounds) due to SLF and PD stressors at different time points throughout the season could help elucidate the plant responses involved in these changes. In addition, longer-term impact of combined SLF and PD stress on various grapevine measurements of growth could provide insight into how these interactions may affect grape production in a field environment. For example, future research could include evaluation of cold tolerance, early season growth, and fruit production in grapevines exposed to SLF and infection with *Xff*, in comparison to vines exposed to only SLF or PD stress or no stress at all.

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Chapter 4

Conclusion

The goal of this study was to examine the interactions between the SLF and pathogens of grapevines. Being an invasive insect, it is unknown what role the SLF will play in affecting established pathogen systems. As the SLF continues to spread across and feed in vineyards in the U.S., it is likely to encounter common grapevine pathogens.

This study showed that SLF nymphs and adults transmit North American grapevine yellows (NAGY) at a rate of 7% and 5%, respectively. This result shows that SLF are capable of transmitting phytoplasmas, but future testing is needed to examine the impact of SLF transmission of NAGY in a vineyard setting. Further examination of the mechanisms of transmission of phytoplasmas by SLF is needed as well. SLF are currently established in areas with known NAGY infection throughout the Northeastern U.S. (Beanland et al., 2006; N.Y. State Integr. Pest Manag., 2025). As such, it will be important to monitor the spread of NAGY in these areas, as it is unknown how impactful SLF transmission of the phytoplasmas will be in a field environment. Furthermore, it is unknown how SLF will respond to infected grapevines in vineyards. Examination of SLF attraction and feeding preferences towards or against NAGY-infected vines could influence SLF invasion and distribution in the vineyard, leading to changes in management tactics and timing of pesticide applications.

SLF nymphs and adults were not shown to transmit grapevine red blotch virus (GRBV). Transmission of *X. fastidiosa* subsp. *fastidiosa* (*Xff*), the causal agent of

Pierce's disease (PD), was not shown via molecular testing, however symptoms of PD were observed on plants after transmission trials and thus further testing is warranted.

This study has also shown that combined biotic stressors can alter grapevine responses as measured by metabolites and symptom severity. Plant responses to infection and insect stress can vary widely depending on a number of factors and are often specific to the organisms involved (Walling, 2008). The greatest impact on metabolite production in this study was on late season carbon measurements. In most cases, SLF affected vines regardless of PD infection. Notably, starch was significantly reduced by SLF infestation in both canes and roots, regardless of PD infection. Reallocation of carbohydrates in the source-sink system of grapevines could lead to detrimental effects on the next season's growth and fruit production. For example, grapevines rely on soluble sugar reserves for freeze tolerance and survival of winter dormancy (Harner et al., 2022).

Another significant interaction of PD and SLF found in this study was the overall reduction in disease ratings, or visual foliar symptoms, on PD-infected vines treated with heavy SLF infestation compared to PD-only vines. This effect could be due to a number of factors that require further research. One possible contribution is the overlap in plant defense responses to SLF attack and PD infection. SLF may have induced or exacerbated plant defense responses, such as fortification of physical barriers (wax and lignin production) and restructuring of cell walls. These responses to SLF overlap with plant defenses to *Xff* and could lead to a reduction in bacterial titers or a slowing of bacterial growth, resulting in less severe symptoms.

Future experiments to continue examination of PD and SLF interaction effects could involve a wider range of grapevine varieties to provide further insight into how

these biotic stressors interact in a vineyard setting. In addition, further investigations into overwintering and impacts on multiple future seasons caused by combined PD and SLF stresses could be an important part of determining long-term effects in a production environment.

SLF were shown to have a decline in development and survival on PD vines and chose to feed on healthy vines versus PD-infected vines. These results provide insight into the interactions between SLF and PD, in which underlying factors including changes in nutritional and volatile composition may be at play in affecting SLF performance on PD-infected vines. In fact, while SLF feeding may play a part in reducing symptom severity in PD-infected vines, the converse may also be true- PD infection could reduce the ability of SLF to feed on PD-infected vines, via reduced SLF development and increased mortality, as a response to *Xff*-induced plant defenses that overlap with plant defense responses to SLF. These interactions warrant further investigation.

Future studies could involve identifying specific factors involved in SLF avoidance of PD-infected vines, for example through finding what chemical and/or visual cues are involved in feeding preference. In addition, examining the changes in primary and secondary metabolites of vines under combined SLF and PD stress throughout the growing season could provide insight into specific processes leading to poor SLF performance. Overall, understanding how SLF respond to infected vines could lead to better predictions of insect distribution in vineyards with diseased vines and to improving integrated pest management in vineyards.