PHYTOPLASMA-MEDIATED MANIPULATION OF PLANT-HERBIVORE INTERACTIONS IN THE AMERICAN CRANBERRY

By

NAKORN PRADIT

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And approved by

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ABSTRACT OF THE DISSERTATION

Phytoplasma-mediated manipulation of plant-herbivore interactions

in the American cranberry

by Nakorn Pradit

Dissertation Director:

Dr. Cesar Rodriguez-Saona

The transmission of insect-borne pathogens mainly relies on the foraging and feeding behavior of their insect vectors. Changes in phytochemicals, especially volatiles, nutrients and chemical defenses, of host plants from pathogen infection can facilitate the performance and preference of insect vectors. Moreover, in the ecosystem, these changes in infected host plants can also affect non-vector herbivorous insects. In the American cranberry, *Vaccinium macrocarpon* Ait., false blossom disease is caused by a phytoplasma that requires the blunt-nosed leafhopper, *Limotettix vaccinii* Van Duzee, as a vector. In this study, we hypothesized that (1) phytoplasma infection enhances *L. vaccinii* performance and preference on infected plants, (2) phytoplasma infection enhances the performance of three non-vector leaf feeders, and (3) phytoplasma infection alters levels of phytochemicals and expression of gene related to primary (i.e., nutrients) and secondary (i.e., plant defenses) metabolism in cranberries. Phytoplasma infection had conflicting effects on its vector. *L. vaccinii* had similar survival rates on infected and uninfected cranberries; however, nymphs developed more slowly and adults had higher mass on infected plants than on uninfected plants, indicative of a short-term positive effect. In contrast, female *L. vaccinii* laid less eggs on infected plants than uninfected plants, indicative of a potential negative long-term

effect on population size. In no-choice tests, *L. vaccinii* preferred volatiles from uninfected plants; uninfected plants emit higher volatile emissions than infected plants. Besides these effects on the vector, larvae of three common non-vector herbivores: spotted fireworm (*Choristoneura parallela* Robinson), Sparganothis fruitworm (*Sparganothis sulfureana* Clemens), and gypsy moth (*Lymantria dispar* L.) had 2–3 times higher mass, and damaged 1.5–3.5 times more leaves, when feeding on infected vs. uninfected plants. Larval survival of *S. sulfurena* and *L. dispar* also improved on infected plants. Nutrient levels were higher in infected plants, while defensive proanthocyanidins were lower, which may explain the short-term benefits in performance by vector and non-vector herbivores. Phytoplasma infection induced expression of 132 genes and suppressed expression of 225 genes in cranberries. Expression of genes associated with nutrient metabolism (i.e., carbohydrate) were up-regulated, while those associated with defensive pathways were down-regulated, in the phytoplasma-infected plants. Our study suggests that phytoplasma-infected plants may rely on visual cues and volatiles from neighboring uninfected plants to attract its vector *L. vaccinii*. After attraction, *L. vaccinii* feeding might be facilitated through elevated nutrient and reduced defensive metabolite levels in infected plants. These findings support the "vector manipulation hypothesis." This vector facilitation also benefits other, non-vector, herbivores in the cranberry community. A better understanding of the mechanisms underlying tri-partite interactions among plants, pathogens, and herbivores could help in the development of ways to reduce disease transmission by improving host-plant resistance against diseases and pests of crops.

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CHAPTER ONE

INTRODUCTION

PHYTOPLASMA-VECTOR INTERACTIONS

The transmission of many diseases by arthropod vectors is still considered a mysterious ecological process. Three contributing components, pathogen, host, and vector, add more dimension and make these interactions complex. The transmission of diseases relies on the vector's behavior. Food choice (either infected or uninfected hosts) can influence the spread of vector-borne diseases (Gandon, 2018). Host-plant characteristics and quality are the main factors for food selection by vectors (Brodbeck, et al., 1990; Claridge & Wilson, 1978; Petelle et al., 1979). Many plant diseases are transmitted by arthropods, especially insects. Insects with piercing-sucking mouthparts such as aphids, planthoppers, and whiteflies are common vectors of plant diseases. Many studies have demonstrated the ability of pathogens to manipulate their vector's preference towards infected (Eigenbrode et al., 2002; MacLean et al., 2014) and uninfected plants (Altizer et al., 1998; Daugherty et al., 2011). Changes in some traits of the infected host may result in more attraction towards them by the vector. However, extreme preference for the infected host can also limit or even stop pathogen transmission (Gandon, 2018). Recent studies show that host choice behaviors may rely on the infection status of the vector. Uninfected vectors have been found to be attracted to infected plants, but they prefer uninfected plants after they acquire the pathogen in their body (Blanc & Michalakis, 2016; Fang et al., 2013; Ingwell et al.,2012; Mauck et al., 2016). Although, studies on manipulation of the hosts and vectors by pathogens are of increasing interest, these studies still show inconsistent results on the preference and

performance of insect vectors. The use of different pathogen-host-vector models may provide a better understanding of these interactions.

Phytoplasmas rely on sap-feeding hemipteran insects as the vector of the disease (Weintraub & Beanland, 2005). These bacteria enter the insect vector via the mouthparts and pass into the intestinal lumen of the insect vector. The bacteria replicates in intestinal epithelial cells and adjacent muscle cells (Hogenhout et al., 2008). The bacteria then moves into the hemolymph and infects various tissues of the insect, including the salivary glands. The bacteria in the salivary glands can then access directly the plant cells when the insect feeds on a plant (Nault, 1997; Webb et al., 1999). The time lapse between acquisition and inoculation is called the latency period. The latency period varies by the species of the vector, the age of the vector, and environmental conditions (Carraro et al., 2001; Murral et al., 1996). Interestingly, insect vectors of phytoplasma are specific to groups of phloem feeders such as leafhoppers, planthoppers, and psyllids, but other phloem-feeding hemipterans, such as aphids, do not transmit phytoplasma (Weintraub & Beanland, 2005). This indicates that there is specificity in the acquisition and transmission of these bacteria (Sugio et al., 2011). Many studies show that phytoplasmas can increase the longevity and fecundity of insect vectors (Beanland et al., 2000; Hogenhout et al., 2008).

The food choices of insect vectors can influence the spreading of vector-borne diseases (Gandon, 2018). The foraging behavior of insects relies on various plant traits. The foraging process starts with randomly searching for signal cues from the food source. Volatile and visual cues, such as color, attract insects to host plants (Mauck et al., 2015). For example, some leafhoppers prefer the color red (Lessio & Alma, 2004). Most aphids, some leafhoppers, and some herbivorous beetles prefer the color of leaves (Holopainen et al., 2009; Rodriguez-Saona et al., 2012; Stam et al., 2014). The quantity

and/or quality of plant volatile emissions are also important cues for insects during host location (Eigenbrode et al., 2002; Jiménez-Martínez et al., 2004; Mauck et al., 2010; McMenemy et al., 2012; Shapiro et al., 2012; Srinivasan et al., 2006). After insects locate a plant, they test the plant to check the quality and quantity of nutrition (sugar and amino acid) in plants. The sugar-amino acid ratios and the quantity and quality of secondary metabolites can lead to feeding on plants (Mauck et al., 2014). Plants have evolved strategies to defend themselves from herbivores and pathogens. Plants can create physical barriers, such as trichomes, hairs (Tooker et al., 2010), cuticular waxes and cell walls (Kosma et al., 2010; Pérez-Donoso et al., 2010). Chemical defenses are another strategy that plants use to protect themselves. Constitutive defenses, such as tannin, phenolic compounds (Kaplan et al., 2008) and inducible defenses mediated by the jasmonic acid (JA) and salicylic acid (SA) pathways (Wu & Baldwin, 2010) are produced against herbivores and pathogens. These resistance mechanisms, physical and/or chemical, obstruct the feeding behavior of herbivorous insects on plants (Hougen-Eitzman & Rausher, 1994). Feeding decisions by herbivores depend on plant morphology and phytochemistry (volatile compound, nutritional contents, and chemical defenses) and these traits can be affected by pathogen infection, such as phytoplasma.

PHYTOPLASMA-PLANT-VECTOR INTERACTIONS

Phytoplasmas are bacterial pathogens that belong to the class Mollicutes, Phylum Tenericutes (Hogenhout et al., 2008). These bacteria do not have the cell wall and the majority content of cell membrane is protein. Phytoplasmas contain a minimal genome which lacks coding genes for ATP synthases and sugar uptake and use, making them dependent on their host (Christensen et al., 2005; Kube et al., 2012). In plants,

phytoplasmas mainly remain in the cytoplasm of the phloem sieve cells of vascular systems (Chen, 1971), and have been detected in most organs of host plants (Sugio et al., 2011). Phytoplasmas can induce various morphological and physical changes in the host during infection. There are significant changes in the development of the host plants, including the proliferation of stems (witch's brooms), changes of the flowers into leaf-like structures (phyllody), yellowing (chlorosis), and stunting. These physiological changes result from an effector protein produced by the bacteria. The effector protein has the potential to modulate cellular processes in plant development (MacLean et al., 2014), chlorophyll degradation (Mittelberger et al., 2017), carbohydrate and amino acid translocation (Lepka et al., 1999; Tan & Whitlow, 2001), and plant defense production (Sugio et al., 2011). Phytoplasma infection can lead to the production of defense proteins, increase phenolic compounds, and overproduction of hydrogen peroxide in the host plants (Junqueira et al., 2004; Musetti et al., 2004). For example, the effector protein of Aster Yellows phytoplasma strain witches' broom can suppress SA-mediated defense responses (Lu et al., 2014), but the phytoplasma in apple shows strong induction of SA-mediated defense responses and suppression of the jasmonate biosynthetic pathway (Musetti et al., 2013).

Phytoplasma effector proteins can also affect plant phytochemistry. Phytoplasmas can increase plant volatile emissions (e.g. E-β-caryophyllene, a sesquiterpene) to attract insect vectors (Mayer et al., 2008a, 2008b; Orlovskis & Hogenhout, 2016). Phytoplasma effector proteins also alter carbohydrate and amino acid levels (Lepka et al., 1999; Tan & Whitlow, 2001), and levels of plant defenses (Sugio et al., 2011). For example, phytoplasma infection can lead to increases in phenolic compounds and hydrogen peroxide in host plants (Junqueira et al., 2004; Musetti et al., 2004). Many studies show suppression of the SA (Lu et al., 2014; Wang

et al., 2015) and JA (Immink et al., 2012; Musetti et al., 2013; Schommer et al., 2008) pathways in phytoplasma-infected plants. Normally plants induce SA against biotrophic and hemibiotrophic pathogens, which rely on plant tissues (Thomma et al., 1998). SA is also induced in plants for protection against piercing-sucking insects (War et al., 2012), while, JA is primarily induced against leaf-chewing insects and necrotrophic pathogens (Liu et al., 2016). Suppression of the JA pathway by phytoplasma infection could facilitate feeding by herbivorous insects.

PHYTOPLASMA IN AMERICAN CRANBERRIES

In American cranberry (*Vaccinium macrocarpon* Aiton), a phytoplasma causes cranberry false blossom (Lee et al., 2014; Polashock et al., 2017). Cranberry false blossom is an important disease that decreases the productivity of cranberries (Beckwith & Hutton, 1929). False blossom disease shows abnormality in plant morphology. The leaves of diseased plants can become apparent by the formation of a witches' broom where several branches appear at the internode. The red coloration of infested plants is also noticeable as is the malformation of the reproductive structures. Flowers can be replaced by a whorl of leaves (phyllody) in severely infected plants. Recent studies show that the phytoplasma is designated as a new subgroup 16SrIII-Y (Lee et al., 2014). Blunt-nosed leafhoppers are the only known vectors of false blossom disease (Chen, 1971; Dobroscky, 1931; Polashock et al., 2014). Previous studies show that blunt-nosed leafhoppers are attracted to green wavelengths, followed by red and yellow. Attraction to red color by this insect may relate to phytoplasma infection (Rodriguez-Saona et al., 2012). Although blunt-nosed leafhoppers have been found in higher density in bogs with greater incidence of cranberry false blossom disease

(Beckwith & Hutton, 1929), there are no studies on the effects of phytoplasma infection on the preference and performance of blunt-nosed leafhoppers.

THE AMERICAN CRANBERRY AND INSECT PESTS

American cranberry (*Vaccinium macrocarpon* Ait.) is a plant native to North America that has been cultivated for over 150 years (Polashock et al., 2014). Cranberry is rich in plant secondary metabolites, particularly phenolic compounds such as chlorogenic acids, benzoic acid, and flavonoids. The major flavonoids in cranberry are proanthocyanins, anthocyanins, and flavonols. The biosynthesis of flavonoids is tissue specific, developmentally-regulated, and can be induced by many factors, one of them being the interaction of cranberries with microorganisms (Polashock et al., 2014). Phenolic compounds are considered a type of chemical defenses in cranberries. There are many herbivores that feed on cranberries including insect vectors of diseases like blunt-nosed leafhoppers. Lepidoptera larvae such as spotted fireworm (*Choristoneura parallela* Robinson (Lepidoptera: Tortricidae)), Sparganothis fruitworm (*Sparganothis sulfureana* Clemens (Lepidoptera: Tortricidae)) and gypsy moth (*Lymantria dispar* L. (Lepidoptera: Erebidae)) are also common leaf feeders of cranberry plants and important pests of cranberries. These herbivores can be influenced by the levels of phenolics in cranberries.

Spotted fireworm and Sparganothis fruitworm are both native to North America. Spotted fireworm is distributed around the continental United State and southern Canada, while Sparganothis fruitworm is widespread in eastern North America. Both moths are polyphagous, feeding on a wide range of plant families. In cranberry, the larvae feed on leaves and fruit. They complete two generations a year. The overwintered larvae feed on the foliage and the second-generation larvae can cause

major damage to the fruit. The phytochemical composition of cranberry plants, especially nutritional content and chemical defenses, can affect the development of these leaf-feeding insects.

Native to Europe and Asia, gypsy moth is an important invasive pest in the US. It has spread throughout the Northeastern US and Canada. Gypsy moth is considered an important forest pest but can occasionally become a pest of cranberries during outbreak years. Neonates accidentally “fly” using the wind (ballooning) from nearby wooded areas into cranberry bogs or, less frequently, when older larvae migrate to bogs due to lack of food in the forest. The larvae can feed on foliage, buds, and flowers of cranberry plants.

HYPOTHESES

Infection of plant diseases, such as phytoplasmas, may influence the development of leaf-feeding insects by two possible ways. First, false blossom disease may increase the quantity and/or quality of food for leaf-feeders. For instance, infection could increase the number of leaves (physical trait), which may result in increased apparency and thus reduced herbivore foraging times. Second, changes in chemical composition of host plants by phytoplasma infection may facilitate leaf feeders to feed on the host plant by decreasing chemical defenses and increasing food quality (higher nutrient levels). For example, chemical defenses in cranberries such as phenolic compounds are known to affect the preference and performance of gypsy moth (Martemyanov et al., 2015; Neto et al., 2010)

The cranberry genome has been under investigation recently (Georgi et al., 2013; Polashock et al., 2014; Sun et al., 2015). Phytoplasmas can affect the expression of defensive genes. Many phytoplasmas have been studied at the genomic level (Kube et al., 2012); however, changes in gene expression due to phytoplasma infection need investigation, especially those genes associated with defense mechanisms. The effects of a phytoplasma that causes false blossom disease in cranberries on manipulating gene expression by suppressing the production of chemical defenses remains unknown.

In this study, I hypothesize that: 1) phytoplasma infection alters volatile organic compound production to attract insect vectors, the blunt-nosed leafhopper; 2) the performance of the vector blunt-nosed leafhopper and non-vector herbivores is enhanced on infected plants; 3) expression of genes associated with defensive pathways are suppressed by phytoplasma infection in cranberries; 4) phytoplasma infection lowers constitutive chemical defenses (i.e., proanthocyanidins and flavonols); and, 5)

phytoplasma infection increases the nutritional quality of cranberry plants, which facilitates herbivore feeding.

OBJECTIVES

The objectives of this study are to:

1) Examine the preference and performance of insect vectors (blunt-nosed leafhoppers) on phytoplasma-infected plants (Chapter 2).
2) Examine the performance of leaf chewers (spotted fireworm, Sparganothis fruitworm, and gypsy moth) on phytoplasma-infected plants (Chapter 3).
3) Evaluate the molecular and biochemical changes related to plant defensive traits in false blossom infected cranberry plants (Chapter 4).

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CHAPTER TWO

PERFORMANCE, HOST SELECTION, AND OVIPOSITION BY THE INSECT VECTOR *LIMOTETTIX VACCINII* ON PHYTOPLASMA-INFECTED CRANBERRIES

ABSTRACT

Vector-borne diseases, such as phytoplasmas, are known to manipulate host plants and vectors to enhance their transmission. In cranberries, phytoplasma infection causes false blossom disease, which is transmitted by blunt-nosed leafhoppers (*Limotettix vaccinii* Van Duzee). Here, we studied the performance and preference of *L. vaccinii* on phytoplasma-infected and uninfected cranberries, and compared volatile emissions and phytohormone levels from these plants. In no-choice performance assays, *L. vaccinii* survival was similar (57%) on infected and uninfected cranberries. However, nymphs on infected plants developed into adults more slowly than on uninfected plants, indicative of a slower developmental rate; this resulted in adults having higher mass on infected plants. In no-choice oviposition tests, *L. vaccinii* laid more eggs on uninfected than infected plants; however, in the choice tests, there was no difference in oviposition preference between the two plant types. In olfactometer studies, *L. vaccinii* nymphs and adults preferred odors from uninfected plants than the no-odor (air) choice. No differences in odor preference were found between infected and uninfected plants. In general, infected plants emitted lower amounts of volatiles, particularly (*E*)-4,8-dimethyl-1, 3, 7-nonatriene, dodecane, and germacrene-D, than uninfected plants but had no effects on phytohormone levels. This study shows conflicting effects of phytoplasma infection on its insect vector: infection improved certain vector performance traits (i.e., adult mass) on plants but reduced oviposition and

attraction under no-choice scenarios. We argue that the overall effect of phytoplasma infection on its vector *L. vaccinii* is thus likely influenced by the presence of surrounding uninfected cranberry plants.

INTRODUCTION

The dramatic increase of vector-borne plant diseases is one of many pest challenges in modern agricultural systems (Almeida, 2008). Vector-pathogen relationships play an important part in the epidemiology of these diseases. Insect vector-borne diseases, particularly those involving viruses or phloem- and xylem-living bacteria, can negatively impact crop productivity. The effectiveness of the transmission of these vector-borne pathogens depends on the insect vector capacity, which is often related to factors such as vector density, feeding activity on hosts, longevity before and after pathogen ingestion, duration of incubation period, and vector competence (Chuche & Thiéry, 2014; Cook et al., 2008). Moreover, vector behaviors related to feeding such as searching, landing, probing, or leaving rely on stimuli from the host plant (Perring et al., 1999). In fact, vector preference to infected plants may increase due to physical and chemical changes caused by infection. Many studies have shown that pathogen infection can alter the plant's chemical composition, often making plants more attractive to the vector (Eigenbrode & Bosque-Pérez, 2016; Ingwell et al., 2012; Stafford et al., 2011), such as increases in volatile emissions (Eigenbrode et al., 2002; Jiménez-Martínez et al., 2004; Mauck et al., 2010) and increases in amino acid content either in the entire plant (McMenemy et al., 2012) or in the phloem (Blua et al., 1994).

Phytoplasmas cause over 700 plant diseases worldwide (Maejima et al., 2014); they are obligated phloem-restricted vector-borne bacteria (Chen, 1971). All vectors of phytoplasmas belong to the order Hemiptera and include leafhoppers, planthoppers, and

psyllids (Hogenhout et al., 2008; Weintraub & Beanland, 2005). This bacterial disease relies on nutrient-rich tissues of the host plant (phloem) tissue (Chen, 1971) and of the insect vector (hemolymph) (Hogenhout et al., 2008). Phytoplasma-infected plants develop diverse symptoms such as witches' broom, leaf yellowing or reddening, growth aberrations (proliferations, internode shortening, and stunting), and flower malformations (size reduction, virescence, and phyllody) (Chang, 1998; Namba, 2002). It can also cause phloem tissue aberrations, such as extensive phloem necrosis and excess formation of phloem, resulting in swollen veins (Lee et al., 2000).

In the American cranberry (*Vaccinium macrocarpon* Aiton), a phytoplasma in the subgroup 16SrIII-Y causes cranberry false blossom disease (Lee et al., 2014; Polashock et al., 2017). This disease depends exclusively on the blunt-nosed leafhopper (*Limotettix vaccinii* Van Duzee; Hemiptera: Cicadellidae) as the vector (Beckwith & Hutton, 1929). Cranberry false blossom-infected plants show malformed flowers, enlarged calyx, and shortened, discolored, and streaked petals (Dobroscky, 1931); during bloom, affected flowers fail to set fruit. Other symptoms of cranberry false blossom disease are the appearance of witches' broom and the fact that leaves turn reddish earlier than uninfected plants in the fall (De Lange & Rodriguez-Saona, 2015). In New Jersey (USA), this disease was a major threat to cranberry production and almost wiped out the industry during 1920–1940. However, the use of organic insecticides and the release of improved cultivars that were more resistant to the insect vector subsequently controlled this disease (Chandler et al., 1947). After several years, the disease incidence declined, but it has recently reappeared in many cranberry farms (Lee et al., 2014). This reapperance coincided with an increase in *L. vaccinii* populations because of changes in pest management practices due to stricter regulations

of broad-spectrum insecticide use and increased adoption of new reduced-risk alternatives.

Limotettix vaccinii distribution expands from Nova Scotia and Quebec in Canada to New Jersey and Maryland, USA in the south and to Minnesota and Iowa, USA in the west (Beckwith & Hutton, 1929). This leafhopper has one generation a year; eggs overwinter and hatch in early May, nymphs go through five instars, the adults appear in early July, and populations decline by August (Averill & Sylvia, 1998; De Lange & Rodriguez-Saona, 2015). Previous studies showed that *L. vaccinii* is attracted to green, followed by red and yellow colors, which likely associated with healthy and phytoplasma-infected foliage (Rodriguez-Saona et al., 2012). Additionally, field observations indicate a high density of *L. vaccinii* in false blossom-infected cranberry bogs (Beckwith & Hutton, 1929; Dobroscky, 1929). Although these studies suggest that *L. vaccinii* may be attracted to infected plants, so far none have investigated the effects of false blossom infection on the performance and preference of its vector.

This study tested the hypotheses that phytoplasma infection affects *L. vaccinii* growth, oviposition, and foraging behavior and that these effects on its vector are mediated by changes in volatile emissions and phytohormone levels. Specifically, we conducted a series of no-choice and choice experiments to ask the following questions: (1) Do *L. vaccinii* growth and oviposition differ between phytoplasma-infected and uninfected plants? (2) Is *L. vaccinii* more attracted to phytoplasma-infected plants? and (3) Does phytoplasma infection alter volatile emissions and phytohormone levels in cranberry plants?

MATERIALS AND METHODS

Plants

Phytoplasma-infected and uninfected cranberries (cv. Crimson Queen) were propagated in February 2017 and 2018. Uninfected plants were taken from stolons provided by Integrity Propagation (Chatsworth, New Jersey, USA), whereas phytoplasma-infected plants were taken from a commercial cranberry farm that originated from Integrity Propagation in Chatsworth, New Jersey, USA. Stem cuttings (~7 cm) from uninfected and infected plants were transferred to individual 4 × 4-cm cells and placed in a greenhouse (20 ± 2°C, 70 ± 10% relative humidity [RH], and 15:9 light:dark [L:D]) for rooting. Plants were grown in a 50:50 v/v peat:sand mix, were fertilized once a month from March until May with PRO-SOL 20-20-20 of nitrogen (N)-phosphorus (P)-potassium (K) All Purpose Plant Food (Pro Sol, Inc., Ozark, Alabama, USA) at a rate of 165 ppm N, and were watered daily.

After the cuttings developed a rooting system, five cuttings were transplanted into single pots (7 × 7 cm^2). Plants were grown in the greenhouse until they were used in experiments. Prior to conducting the experiments, 10 plants (five from infected and five from uninfected plants) were randomly tested by DNA fingerprinting using sequence-characterized amplified-region markers (Polashock & Vorsa, 2002) to verify that all plants were genetically the same. Another 10 plants (five plants from infected and uninfected plants) were randomly selected to test for phytoplasma infection by using a nested PCR assay (Lee et al., 2014). These tests showed that all plants belonged to the same genotype (Crimson Queen) and that only infected plants were positive for the presence of phytoplasma (data not shown). Therefore, our methods ensured that the plants were genetically similar, that uninfected plants were free from phytoplasma, and

that growing conditions, propagation methods, and handling were uniform for uninfected and infected plants.

Insects

From the end of May until the end of July of 2017 and 2018, *L. vaccinii* nymphs and adults were collected by sweep net sampling from experimental cranberry beds at the Rutgers P. E. Marucci Center and commercial cranberry beds (Chatsworth, New Jersey). *Limotettix vaccinii* nymphs were collected in May through the end of June, whereas adults were collected in July. Field-collected individuals were used in experiments because all attempts to rear *L. vaccinii* under laboratory conditions failed. To ensure that these field-collected *L. vaccinii* were not infected with the disease, all cranberry beds used for sweeping and their nearby beds were checked for signs of false blossom disease; no evidence of the disease was observed in any of these beds. In addition, *L. vaccinii* individuals were randomly selected to test for phytoplasma infection by using a nested PCR assay (Lee et al., 2014); these tests showed that none were infected with the phytoplasma (data not shown). Therefore, our methods indicate that field-collected *L. vaccinii* were disease (phytoplasma) free. In the laboratory, *L. vaccinii* were maintained in cages on healthy cranberry plants (24 ± 1°C, 65% RH, and 14:10 L:D) for no more than 2 days prior to experiments.

Performance Assays

This study investigated the survival and growth of *L. vaccinii* on phytoplasma-infected and uninfected cranberry plants. The terminal portions of three plants (12 cm length) were placed inside a plastic dialysis tube cage (2.5 cm diameter × 18 cm length), with the ends of each cage closed by cylindrical sponges (2.5 cm diameter × 2.5 cm

length) to prevent the leafhoppers from escaping. Each cage was considered a replicate, and a total of 35 cages with infected and 35 cages with uninfected plants were set up. Five *L. vaccinii* nymphs of similar size were placed in each tube cage at the end of May. The experiment was conducted in a greenhouse under 22 ± 2°C, 70 ± 10% RH, and 15:9 L:D for 20 days. After 20 days, *L. vaccinii* mortality was recorded, and the number of live nymphs and adults was counted and their body mass (to the nearest 0.001 g) measured.

Oviposition Preference

We conducted no-choice and choice experiments to investigate female *L. vaccinii* oviposition preference for phytoplasma-infected and uninfected cranberries. In July 2017, a choice oviposition test between uninfected and infected plants was performed using plastic dialysis tube cages (2.5 cm diameter × 30 cm length). Three terminals of uninfected and three terminals of infected plants were placed at each end of the tube cage and closed at each end with cylindrical sponges (2.5 cm diameter × 2.5 cm length) to prevent the leafhopper from escaping. Each cage was considered a replicate, and a total of 30 cages were set up. Three females and one male *L. vaccinii* were placed through an opening (~1 cm in diameter) in the middle of the tube cage and then closed with clear plastic tape. *L. vaccinii* were allowed to lay eggs on plants for 4 weeks. After 4 weeks, all plant terminals were cut, the leaves were removed, and the number of eggs inserted under the bark counted with the aid of a stereomicroscope.

In 2018, no-choice and choice oviposition tests between uninfected and phytoplasma-infected plants were performed using rearing insect cages. For the no-choice tests, one infected or uninfected plant was placed inside a rearing cage (30 × 30 × 30 cm) (BioQuip Product, Rancho Dominguez, California, USA); a cage was

considered a replicate, and each plant type (infected or uninfected) was replicated five times. For the choice tests, one uninfected plant and one infected plant were placed at each corner (~70 cm in distance) of a BugDorm-2120F insect rearing tent (60 × 60 × 60 cm) (BioQuip Product, Rancho Dominguez, California, USA); each tent was considered a replicate for a total of 10 independent replicates for each plant type. Five female and five male *L. vaccinii* were released into each cage/tent in mid-July and allowed to lay eggs for 2 weeks. After 2 weeks, all plant terminals were cut above the soil level, the leaves were removed, and the number eggs inserted under the bark counted with the aid of a stereomicroscope.

Olfactometer Assays

The behavioral response of *L. vaccinii* nymphs and adults to odors from phytoplasma-infected and uninfected cranberry plants was investigated under a four-arm olfactometer (Volatile Assay System; Rensselaer, New York, USA). The olfactometer consisted of a four-arm star-shape arena within a 10 × 10-cm acrylic chamber. The top of the arena was covered with a glass plate to prevent the insects from escaping. Each arm of olfactometer was connected to a 12 × 16 × 5-cm^3 acrylic chamber via a clear plastic air tube (5 mm diameter × 30 cm length); the acrylic chambers contained the treatment choices. A constant airflow (0.5 l/min/arm) was pushed through charcoal to create odorless air moving into each chamber. Air flow then moved into the arena and exited through a hole in the center of the arena. The entrance and exit holes were covered with mesh Teflon fabric to prevent leafhoppers from exiting the arena. The experiment was conducted in a dark room (22 ± 2°C). A light source illuminated the top and bottom of the arena to allow consistent lighting (~550 lux) in the arena.

A single nymph or adult *L. vaccinii* was released in the center of the olfactometer arena and allowed to move for 20 mins. The time *L. vaccinii* spent at the end of each arm of the olfactometer (i.e., near the odor source) was recorded and analyzed using the Ethovision XT software (Noldus Information Technology, Wageningen, Netherlands). Treatments in the olfactometer arms were tested as a choice between: (1) uninfected plants vs air (blank), (2) phytoplasma-infected plants vs air (blank), and (3) uninfected plants vs infected plants. Two opposite arms of the olfactometer were randomly selected for one of the treatments, whereas the other two arms contained the second treatment. Treatments were replaced after each replication. A total of 20, 18, and 48 nymphs were tested between uninfected plants vs air (blank), infected plants vs air (blank), and uninfected vs infected plants, respectively. A total of 20, 16, and 48 adults (mixed sexes) were tested between uninfected plants vs air, infected plants vs air, and uninfected vs infected plants, respectively. Because we were particularly interested in whether *L. vaccinii* discriminates between infected and uninfected plants, the number of individuals tested was almost double that of those in other choice tests.

Volatile Collection and Analysis

Volatiles from uninfected and phytoplasma-infected plants were collected in the greenhouse (22 ± 2°C, 70 ± 10% RH, and 15:9 L:D) by using a push-pull system (Heath & Manukian, 1994; Rodriguez-Saona et al., 2001). The charcoal-purified air entered the top of glass chamber over the plant at a rate of 2 l/min. Headspace volatiles were collected by pulling air at a rate 1 l/min from the chamber through HayeSep Q (30 mg) volatile traps (Volatile Assay Systems, Rensselaer, NY, USA). Volatile collections started at 7:00 AM and were continuously collected for 12 hr (time when leafhoppers

are mostly active; e.g. Mahmoud, Amr, & Ebadah, 2011). Each treatment was replicated eight times.

Volatile samples were analyzed as described previously (Rodriguez-Saona et al., 2001). The collected volatiles were eluted from adsorbent traps with 150 µl of methylene chloride. An internal standard (2.28 µl of n-octane in 200 µl of methylene chloride) was added to the extract. The volatile extractions were injected into a gas chromatograph (GC) (Hewlett-Packard chromatograph GC 6890) equipped with a capillary injector, a flame ionization detector, an auto injector, and a HP-1 methyl siloxane column (10 m × 0.53 mm internal diameter 2.67-µm film). The GC was programmed in a split mode (25:1), and the carrier gas was helium at the 40 cm/sec flow velocity. After infection, the oven temperature was maintained at 50°C for 3 mins, programmed to rise to 190°C at 5°C/min, and maintain at 190°C for 5 mins. Data were analyzed with Hewlett-Packard ChemStation software. For each sample, the amount of detected volatiles was based on a comparison of their peak areas with that of the internal standard. To confirm the identities of the different peaks, a subset of samples was analyzed using a Hewlett Packard 5890 Series II GC coupled to a mass spectrometer (Hewlett Packard 5972 Mass Selective Detector). An aliquot of 1 µl was manually injected in splitless mode onto the same type of column as described above, and the temperature program was similar as the one described above. The detected volatiles were identified by comparison of their mass spectra with those in the National Institute of Standards and Technology library and by comparison of their spectra and retention times with those of commercially available authentic standards. After volatile collections, plant material was harvested to determine fresh biomass, and data were reported as the amount of emissions per gram per hour.

Phytohormone Analysis

We also quantified the effects of infection on three important phytohormones involved in plant defense responses against insect herbivores (Erb et al., 2012) salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA). Samples were collected from five phytoplasma-infected plants and five uninfected plants and stored at −20°C before extraction (N = 5 replicates per plant type). The leaves of each sample were randomly selected from the whole plant. Phytohormone levels in these samples were analyzed by liquid chromatography–mass spectrometry (LC-MS) at the Department of Environmental Systems Science at ETH Zürich (Zürich, Switzerland). Briefly, dried samples (10–20 mg) were placed in a 2-ml round bottom Eppendorf tube and frozen in liquid nitrogen. Frozen samples were ground to powder by using the Genogrinder, and a 100-µl extraction solution (80:20 isopropanol:methanol) supplemented with isotopically labeled standards of the phytohormones (4 ng/µl) was added to each sample. The samples were mixed by vortexing and then sonicated in a water bath for 30 min. After sonication, samples were centrifuged at 10,000 rpm at 4°C for 15 min, and the supernatant was transferred to a 2-ml Eppendorf tube. The remaining sample was extracted via the same process mentioned above two more times, using 40 µl of the extraction solution each time. The combined supernatant (approximately 180 µl) was centrifuged at 13,000 rpm for 30 min to remove any solids. After centrifugation, the supernatant was transferred to a vial for liquid chromatography–mass spectrometry analysis. The amount of phytohormone was calculated using the Masshunter Quantitative analysis software (Agilent, Santa Clara, CA, USA)

For LC-MS analysis, separation was done with ultra-high-performance liquid chromatographs equipped with a Zorbax SB-C18 column (1.8 µm, 2.1 × 100 mm; Agilent, Santa Clara, CA, USA). The analysis of phytohormones was done with

Quadrupole Time-of-Flight LC-MS (Agilent, Santa Clara, CA, USA) with positive and negative ion modes. In the positive ion mode, water + 0.1% formic acid was used as solvent A and acetonitrile + 0.1% formic acid was used as solvent B. In the negative ion mode, water + 5 mM ammonium formate was used as solvent A and acetonitrile as solvent B. The elution gradient in the positive ion mode was 99.5% A/0.5% B at 1 min, 97.0% A/3.0% B at 5 min, and 1.0% A/99.0% B at 15 min and 17 min, whereas the elution gradient in the negative ion mode was 99.5% A/0.5% B at 1 min, 97.0% A/3.0% B at 5 min, 1.0% A/99.0% B at 15 min, and 0.2% A/99.8% B at 17 min. The flow rate was 0.6 ml/min, and the injection volume was 5 µl. Column temperature was at 50°C. The diode-array detector detected UV wavelengths between 190 and 640 nm.

Statistical Analysis

Differences between phytoplasma-infected and uninfected plants on *L. vaccinii* survival, mass, oviposition, and time spent in olfactometer tests, as well as volatile emissions and phytohormone levels were analyzed either by analysis of variance (ANOVA) or using Mann-Whitney U-tests. The decision for using ANOVA or Mann-Whitney U-tests was based on results from normality and homoscedasticity tests. Square root or $\log_{10}$ transformations were used to make the data meet the assumptions for ANOVA; otherwise, nonparametric tests such as Mann-Whitney U-tests were used. All parametric and nonparametric tests were performed using IBM® SPSS® version 24. Because each cage/tent was considered a replicate, the mass of *L. vaccinii* for each cage/tent was averaged prior to statistical analysis. Additionally, principal component analysis (PCA) was used to visualize differences in volatile emissions and phytohormone levels between the uninfected and phytoplasma-infected plants (Minitab® version 18).

RESULTS

Performance Assays

After 20 days, *L. vaccinii* survival did not differ between phytoplasma-infected and uninfected plants (U = 579, p = 0.684; Fig. 2.1a). There was no significant difference between the number of nymphs and adults on the uninfected plants (mean number of nymphs ± SE = 2.09 ± 0.20; mean number of adults ± SE = 2.21 ± 0.23; U = 267.5, p = 0.849; Fig. 1a). The number of nymphs was, however, 46% higher than the number of adults on phytoplasma-infected plants (mean number of nymphs ± SE = 2.48 ± 0.23; mean number of adults ± SE = 1.70 ± 0.14; U = 182, p = 0.047; Fig. 2.1b), indicating a slower development rate on infected plants.

Limotettix vaccinii nymphal mass did not differ between phytoplasma-infected and uninfected plants ($F_{1,48}$ = 2.858, p = 0.094; Fig. 2.2a); however, adult mass was ~15% higher on phytoplasma-infected than on uninfected plants ($F_{1,42}$ = 4.85, p = 0.03; Fig. 2.2b).

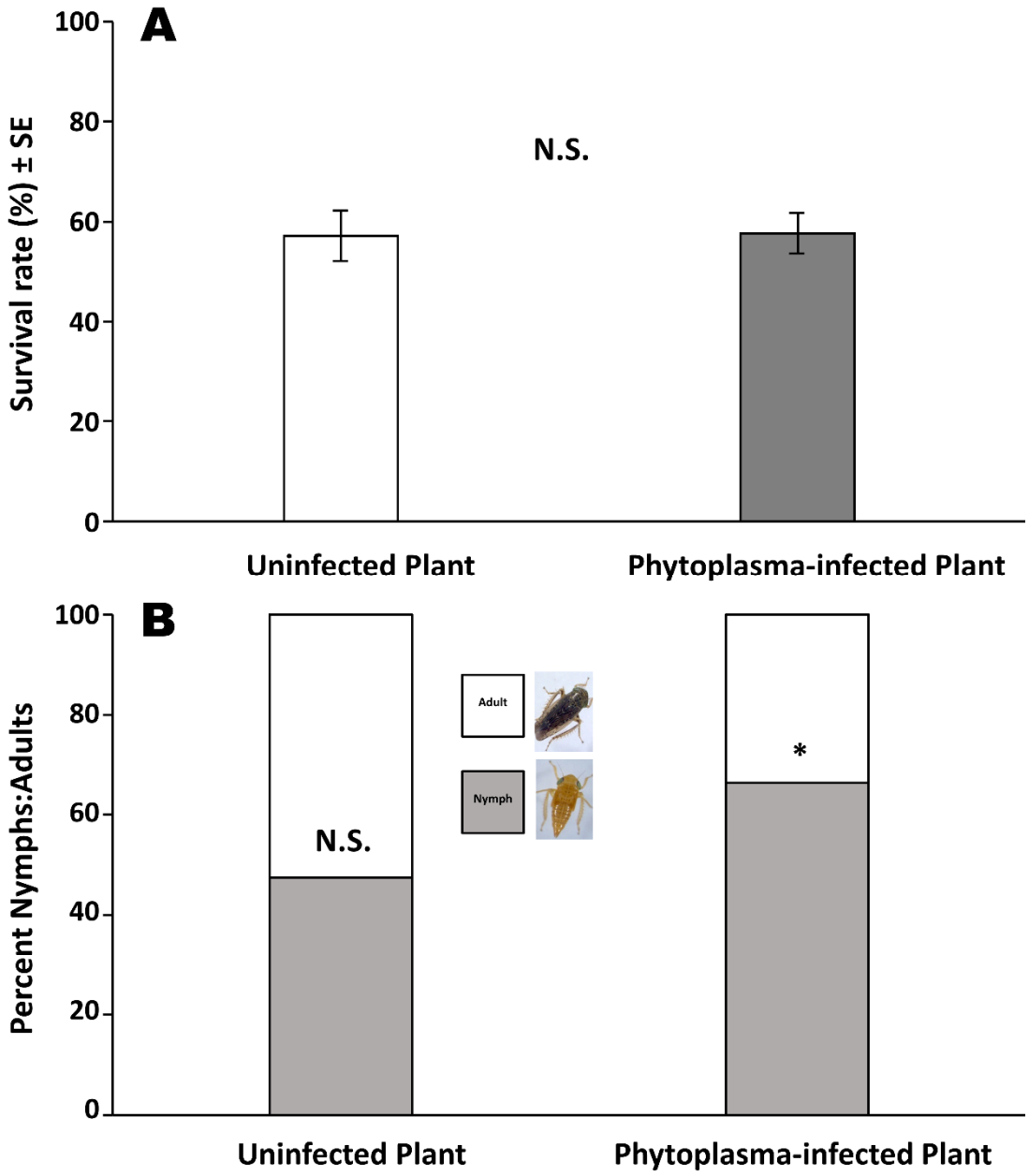

Figure 2.1. **Survival (A) and percent of nymphs:adults (B) of *Limotettix vaccinii* on uninfected and phytoplasma-infected cranberry plants**. An asterisk (*) indicates statistical significance ($p \leq 0.05$); N.S. = no statistical difference ($p > 0.05$). $N = 35$.

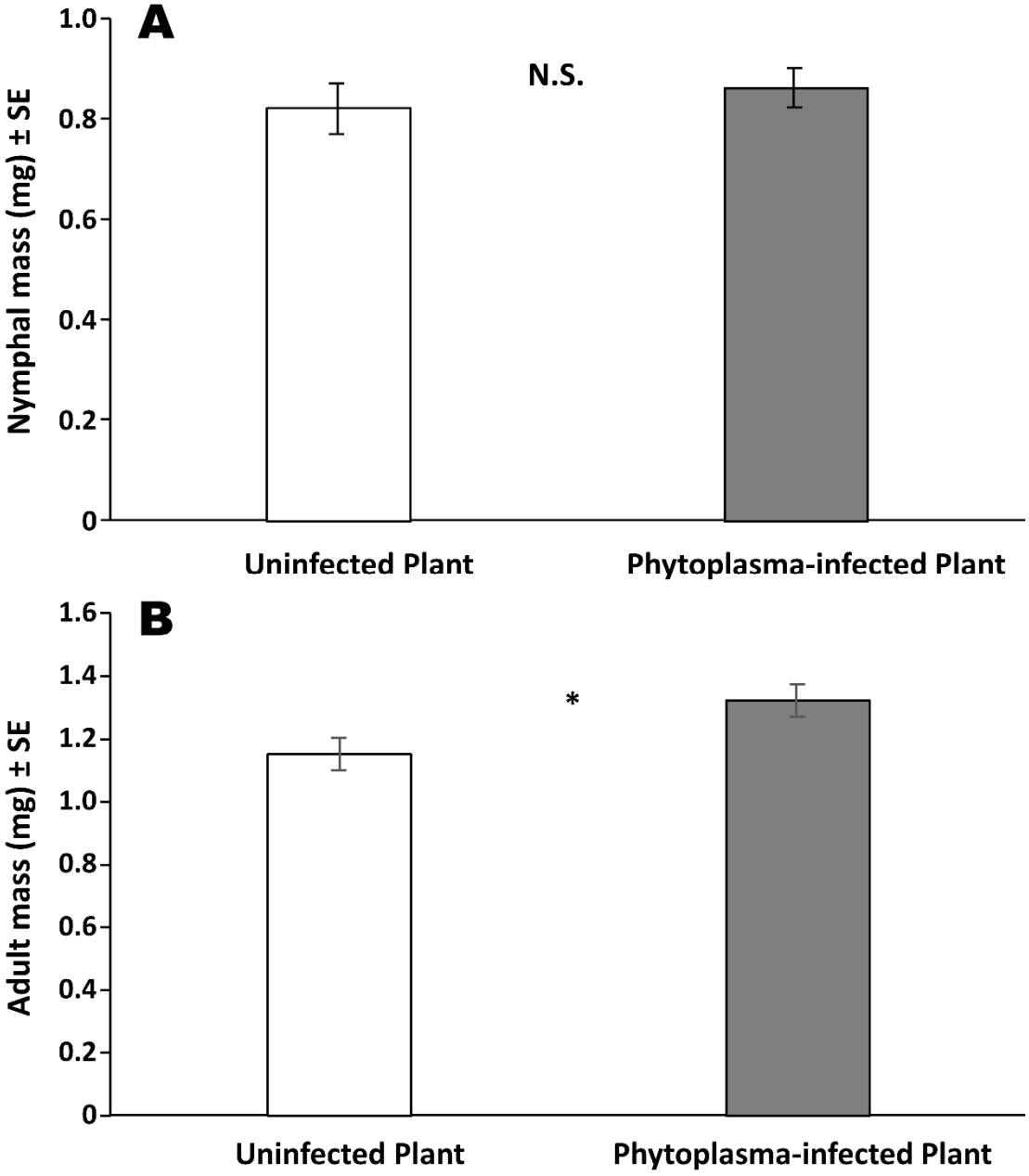

Figure 2.2. Mass (mean ± SE) of *Limotettix vaccinii* nymphs (A) and adults (B) on uninfected and phytoplasma-infected cranberry plants. An asterisk (*) indicates statistical significance ($p \leq 0.05$); N.S. = no statistical difference ($p > 0.05$). $N = 70$ nymphs and 70 adults.

Oviposition Preference

Out of 30 tube cage choice tests, *L. vaccinii* laid no eggs on 46.67% of them. Among those that had eggs, the number of eggs laid on uninfected plants was 1.8 times higher than on phytoplasma-infected plant ($U = 33.5$, $p = 0.044$; Fig. 2.3a). In rearing cage choice tests, *L. vaccinii* laid no eggs on 60% of them and laid only 4 eggs on uninfected plants and 5 eggs on phytoplasma-infected plants. In rearing cage no-choice tests, the number of eggs laid on uninfected plants was 7 times higher than on phytoplasma-infected plants ($U = 0$, $p = 0.014$; Fig. 2.3b).

Olfactometer Assay

Limotettix vaccinii nymphs spent significantly more time on odors from uninfected plants than on odors from the empty (air only) arms (mean time ± SE: uninfected plants = 118 ± 58 sec, air = 39 ± 9 sec; $U = 27$, $p = 0.018$; Fig. 2.4a). However, nymphs spent the same amount of time on odors from phytoplasma-infected plants and on the empty arms (infected plants = 58 ± 12 sec, air = 57 ± 10 sec; $U = 103$, $p = 0.708$; Fig. 2.4a) and when given a choice between odors from infected and uninfected plants (infected plants = 84 ± 17 sec, uninfected plants = 76 ± 15 sec; $U = 476$, $p = 0.955$; Fig. 2.4a).

Similarly, adult *L. vaccinii* spent significantly more time on odors from the uninfected plants than on the empty arms (mean time ± SE: uninfected plants = 274 ± 67 sec, air = 97 ± 29 sec; $F_{1,24} = 5.921$, $p = 0.023$; Fig. 2.4b). However, they spent the same amount of time when given a choice between phytoplasma-infected plants and the empty arm (infected plants = 217 ± 37 sec, air = 153 ± 38 sec; $F_{1,23} = 2.024$, $p = 0.168$; Fig. 2.4b) and between infected and uninfected plants (infected plants = 273 ± 55 sec, uninfected plants = 280 ± 39 sec; $U = 345$, $p = 0.742$; Fig. 2.4b).

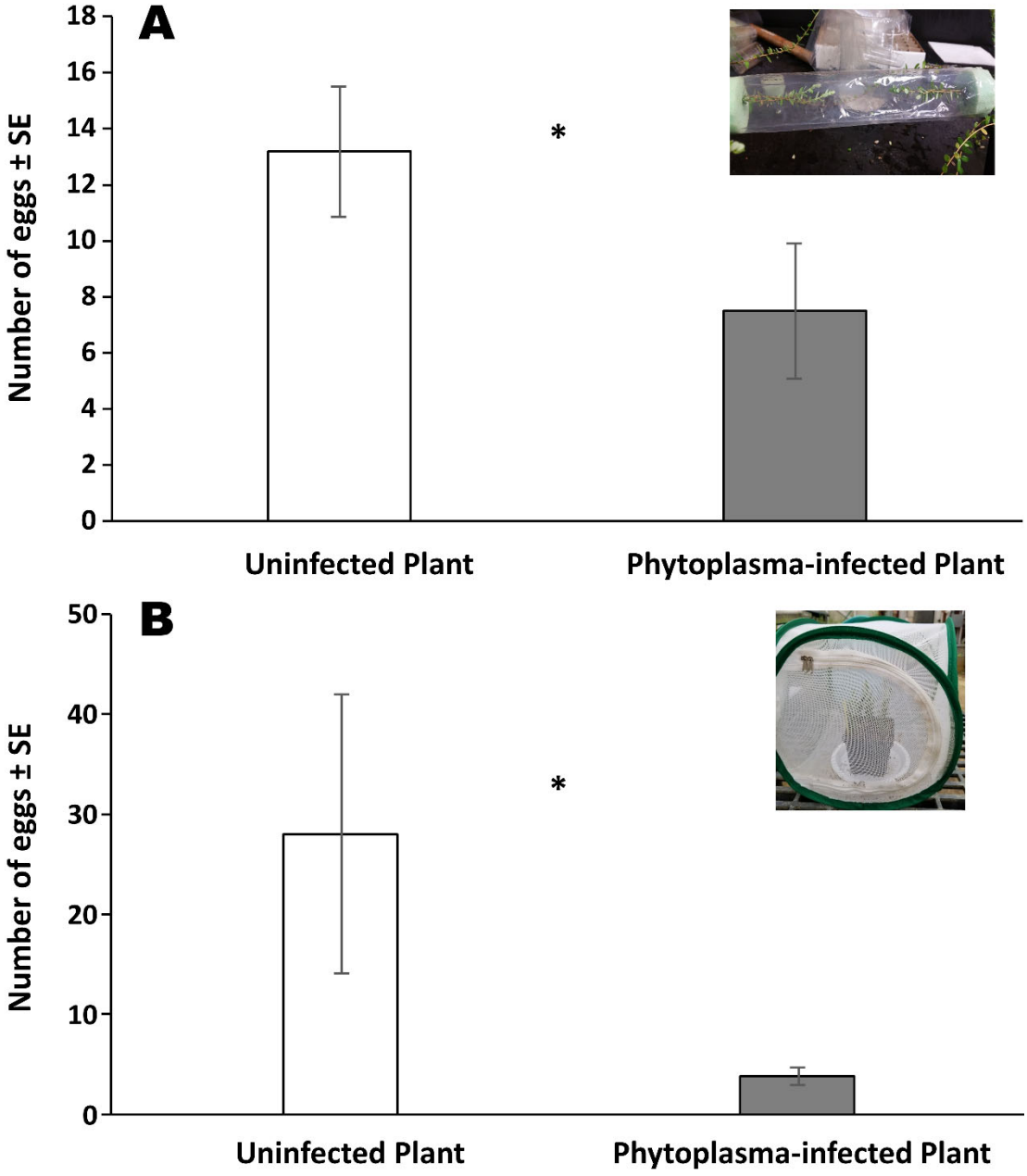

Figure 2.3. Number (mean ± SE) of eggs laid by *Limotettix vaccinii* on uninfected and phytoplasma-infected cranberry plants in choice tube cages (A) and no-choice rearing cages (B). An asterisk (*) indicates statistical significance ($p \leq 0.05$). $N = 30$ for choice tests; $N = 5$ for no-choice tests.

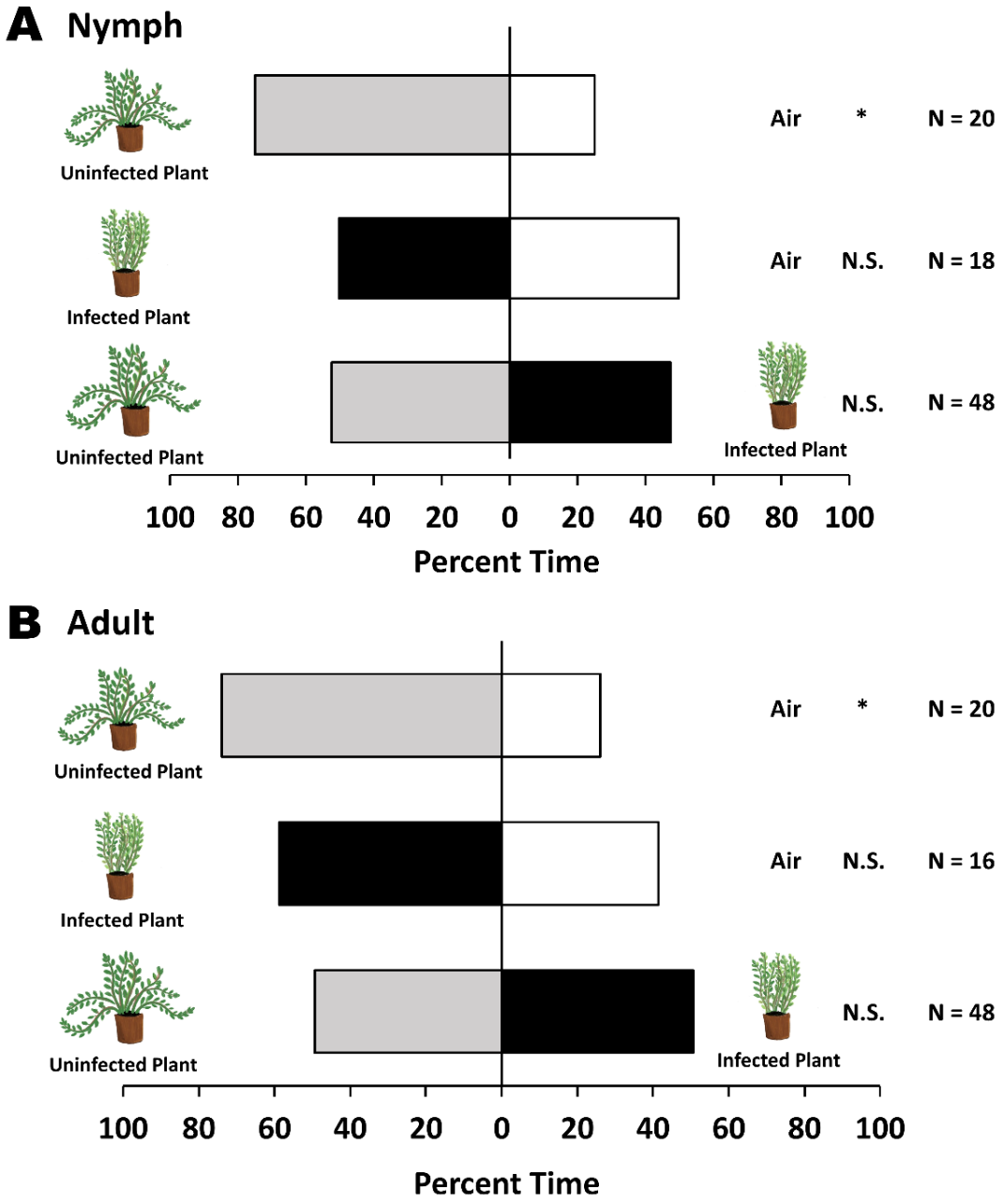

Figure 2.4. Percentage of time (mean ± SE) spent by *Limotettix vaccinii* of leafhopper nymphs (A), adult and females (B), and adult males (C) on each choice in olfactometer assays. Choice consisted of: uninfected cranberry plants vs air, phytoplasma-infected cranberry plants vs air, and uninfected plants vs infected plants. An asterisk (*) indicates statistical significance ($p \leq 0.05$); N.S. = no statistical difference ($p > 0.05$). *N* = number of individuals tested in each choice test.

Volatile Analysis

The Principal component analysis (PCA) shows a clear separation in volatile composition between phytoplasma-infected and uninfected plants (Fig. 2.5a). The first two components explained 49.9% of the total variation, with the first and second components explaining 35.3% and 14.6% of the variation, respectively. Uninfected plants emitted 1.8 times more total amounts of volatiles than phytoplasma-infected plants (Table 2.1). In particular, emissions of three volatiles, namely, (*E*)-4,8-dimethyl-1, 3, 7-nonatriene; dodecane; and germacrene-D, were significantly lower in phytoplasma-infected than uninfected plants (Table 2.1).

Phytohormone Levels

The PCA revealed little to no separation in phytohormone levels between phytoplasma-infected and uninfected cranberry plants, with the first two components explaining 94.3% of the variation (Fig. 2.5b). Consistent with this, amounts of SA ($F_{1, 8} = 0.173$, $p = 0.689$), JA ($F_{1, 28} = 0.553$, $p = 0.479$), and ABA ($F_{1, 8} = 3.029$, $p = 0.120$) did not differ significantly between infected and uninfected plants (Fig. 2.6).

Table 2.1. Volatile Emissions from Uninfected and Phytoplasma-Infected Cranberry Plants.

Volatile Compounds	Mean ± SE (ng/h/g)		Statistical test value[a]	*p* value[b]
	Uninfected plant	Phytoplasma-infected plant		
cis-3-Hexen-1-ol	0.674±0.145	0.468±0.137	$F_{1,14}$=1.056	0.322
Hexanol	0.469±0.126	0.696±0.125	$F_{1,14}$=1.628	0.223
2,5-Hexane-dione	1.287±0.204	1.450±0.248	$F_{1,14}$=0.258	0.619
α-Pinene	0.888±0.196	0.951±0.244	$F_{1,14}$=0.041	0.842
Camphene	1.189±0.198	1.422±0.244	$F_{1,14}$=0.634	0.439
β-Pinene	0.649±0.203	0.322±0.164	*U*=19	0.166
Myrcene	0.188±0.092	0.432±0.181	*U*=22	0.195
cis-3-Hexenyl acetate	1.772±0.235	1.437±0.230	$F_{1,14}$=1.039	0.325
Hexyl acetate	0.436±0.137	0.397±0.124	$F_{1,14}$=0.045	0.836
Decane	1.260±0.294	0.789±0.149	$F_{1,14}$=2.046	0.175
Eucalyptol/limonene	0.960±0.149	0.701±0.102	$F_{1,14}$=2.056	0.174
cis-Linalool oxide	0.477±0.089	0.413±0.122	$F_{1,14}$=0.177	0.681
Nonanal	4.191±0.521	2.905±0.445	$F_{1,14}$=3.526	0.081
Linalool	0.568±0.076	0.353±0.116	$F_{1,14}$=2.399	0.144
(*E*)-4,8-Dimethyl-1, 3, 7-nonatriene	15.056±2.527	4.202±0.960	*U*=3	0.001*
cis-3-Hexenyl butyrate	0.336±0.087	0.519±0.142	*U*=19	0.195
Methyl salicylate	0.742±0.087	0.412±0.113	$F_{1,14}$=2.399	0.144
α-Terpineol	1.464±0.665	1.139±0.231	*U*=24	0.442
Dodecane	1.040±1.487	0.618±0.094	*U*=10	0.021*
Unknown	1.290±0.088	1.453±0.203	$F_{1,14}$=0.545	0.473
Indole	1.338±0.197	0.823±0.214	$F_{1,14}$=3.133	0.098
Phenylethyl ester	0.575±0.097	0.443±0.125	$F_{1,14}$=0.692	0.419
Tridecane	0.342±0.071	0.342±0.077	$F_{1,14}$=0.000	0.998
α-Cubebene	0.213±0.058	0.443±0.125	*U*=17	0.110
Copaene	0.120±0.048	0.101±0.064	*U*=27	0.563
Muurolene	0.463±0.138	0.359±0.096	*U*=21	0.655
Tetradecane	0.393±0.130	0.231±0.074	*U*=24	0.397
Caryophellene	0.906±0.196	0.453±0.107	$F_{1,14}$=4.126	0.062
Humulene	0.613±0.217	0.168±0.070	*U*=18	0.125
Germacrene D	7.927±1.529	2.301±0.991	*U*=31	0.002*
δ-Cardinene	2.445±0.290	1.379±0.502	$F_{1,14}$=3.385	0.087
Total	50.213±5.269	27.714±4.760	$F_{1,14}$=10.041	0.007*

[a]Statistical values based on one-way ANOVA tests or Mann Whitney U tests.
[b]An asterisk (*) indicates significance at α = 0.05.

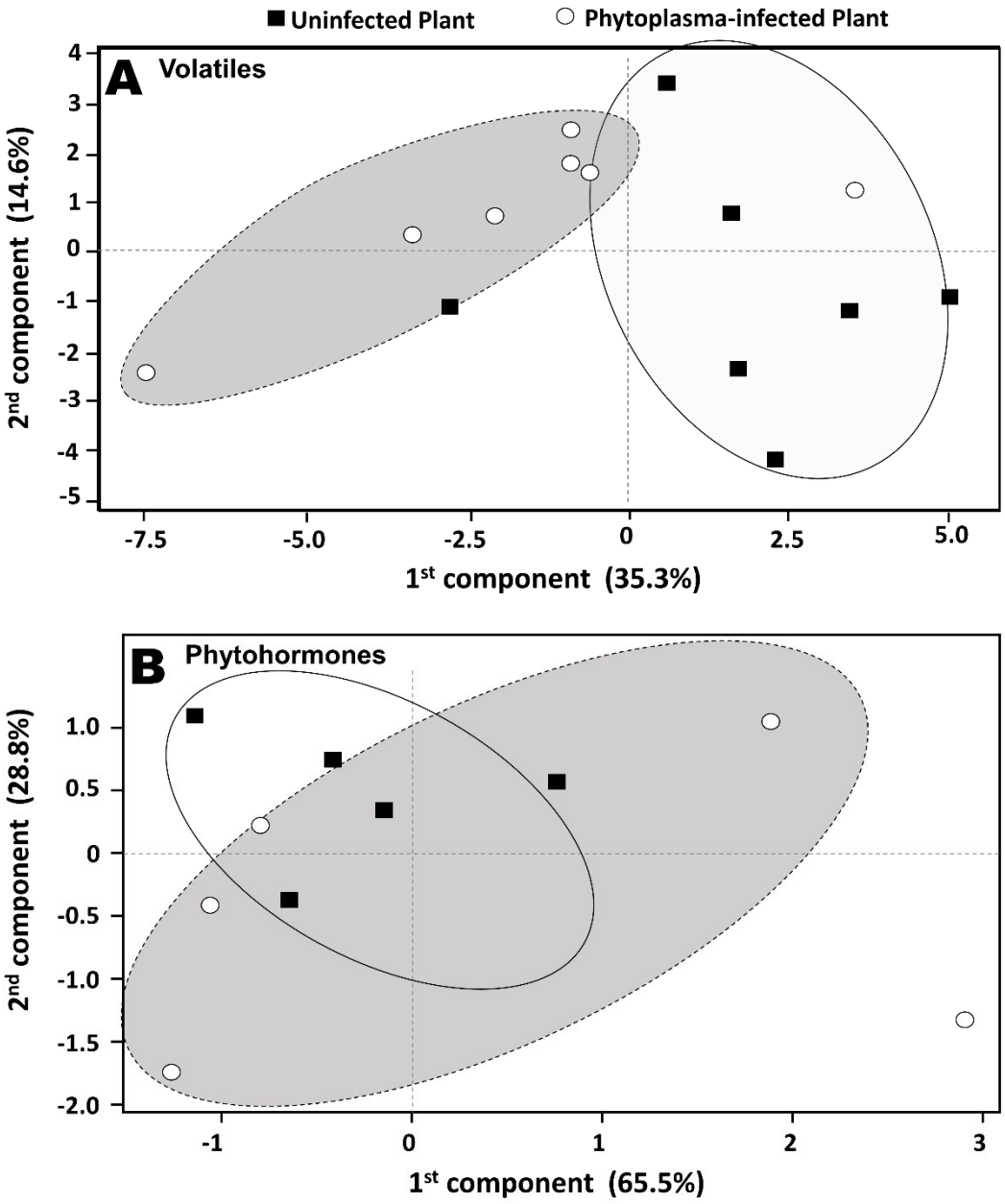

Figure 2.5. Principal component analysis (PCA) for volatile pattern of emissions (*N* = 8) (A) and phytohormone levels (*N* = 5) (B) from uninfected and phytoplasma-infected cranberry plants. First and second component plotted against each other and percentage variation explained between parentheses.

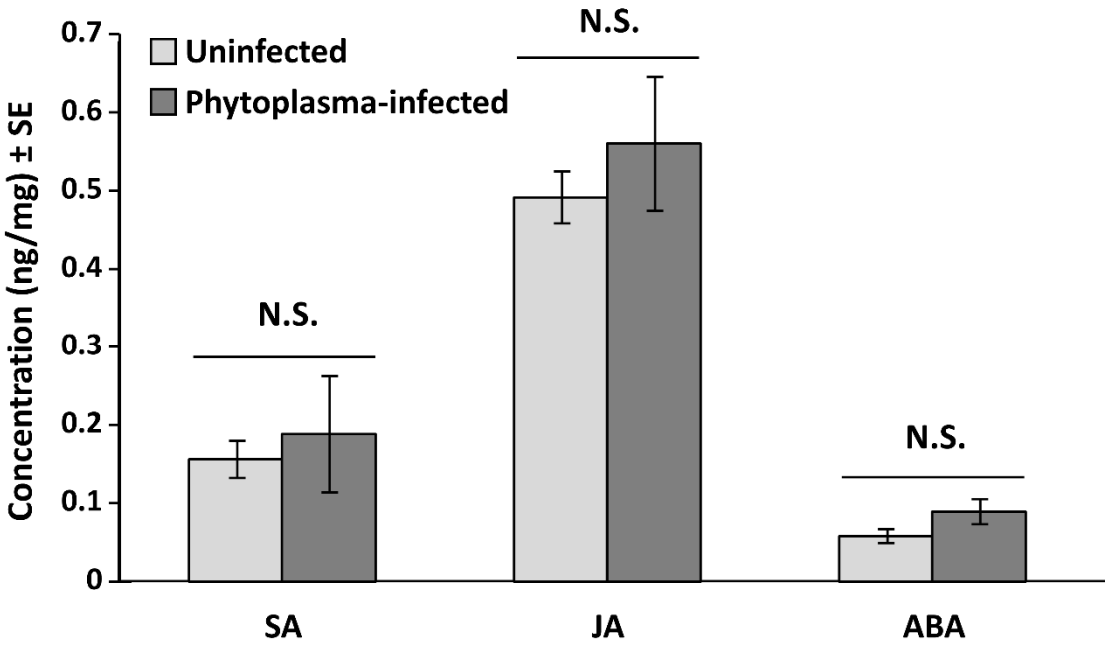

Figure 2.6. Mean (± SE) amounts of phytohormones in uninfected and infected cranberry leaves. SA = salicylic acid, JA = jasmonic acid, ABA = abscisic acid. n.s. = not significant ($p > 0.05$). $N = 5$.

DISCUSSION

This study demonstrated the effects of infection by false blossom disease on the performance and preference of its insect vector *L. vaccinii* in cranberries. We found that (1) infected plants increase *L. vaccinii* development (i.e., prolonged immature stages) and growth (i.e., bigger adult mass), (2) infected plants reduce *L. vaccinii* fecundity, and (3) infection did not affect *L. vaccinii* attraction to cranberries but reduced their volatile emissions.

Infection by phytoplasmas can have beneficial, detrimental, or neutral impacts on the insect vectors (Table 2.2). For example, *Dalbulus maidis* DeLong & Wolcott, a maize specialist, can survive on aster yellows phytoplasma-infected aster (Purcell, 1988) and live longer on bushy stunt p]hytoplasma-infected maize (Ebbert & Nault,

2001). In contrast, the leafhoppers *Scaphoideus titanus* Ball and *Euscelidius variegatus* Kirchbaum have lower lifespans on phytoplasma-infected plants compared to uninfected plants (Bressan et al., 2005a; Bressan et al., 2005b). The effects of phytoplasmas on insect vectors can also vary from positive to negative. For example, the jumping plant louse *Cacopsylla picta* has higher mortality and slightly increased development times but reduced adult size on phytoplasma-infected apples (Mayer, et al., 2011). Moreover, the outcome of these effects can depend on specific plant-phytoplasma-vector interaction (Table 2.2). For example, the aster yellows phytoplasma improves survival and increases longevity of its vector *Macrosteles quadrilineatus* (Forbes) (Beanland et al., 2000; Kingdom & Hogenhout, 2007); however, this same disease had no effects on female adult survival of *M. quadrilineatus* on Arabidopsis (Sugio et al., 2011). In our study, phytoplasma infection improved some aspects of the performance of the vector *L. vaccinii* on cranberries: infected plants increased immature developmental times, which resulted in bigger adult size. Prolonged development times may help increase *L. vaccinii* acquisition of the phytoplasma, thus benefitting transmission. Bigger *L. vaccinii* adult mass may result in greater ability to disperse (Casey, 1976; Ellington, 1991) and higher mating success (Gross et al., 2004). In fact, *M. quadrilineatus* males were more mobile on aster yellows phytoplasma-infected aster and lettuce plants than on uninfected plants under laboratory conditions (Hoy et al., 1999). Although, *S. titanus* infected with "flavescence dorée" phytoplasmas tended to disperse less than uninfected leafhoppers (Papura et al., 2009). Phytoplasma infection increased total nitrogen and overall nutrient content in cranberries (Pradit et al., 2019), which may explain the observed improved *L. vaccinii* performance on infected plants. High nitrogen and amino acid content were found in phytoplasma-infected maize (Junqueira et al., 2004) and periwinkle (*Catharanthus*

roseus) leaves (Lepka et al., 1999), respectively. Higher nutrient content in plants has been shown to extend the duration of the nymphal stage (Prestidge, 1982) and produce larger leafhoppers (Brodbeck et al., 1999).

Even though we expected bigger adults to produce more offspring (Brodbeck et al., 1999), our study shows that phytoplasma infection reduces *L. vaccinii* oviposition on cranberries. Previous studies have shown variable effects of phytoplasma infection on the reproduction of insect vectors (Table 2.2). For example, the bolt strain of aster yellows phytoplasma increases *M. quadrilineatus* fecundity; however, a more severe strain did not affect the leafhopper's fecundity (Beanland et al., 2000). In contrast, the "flavescence dorée" phytoplasma decreases the fecundity of *E. variegatus* (Bressan et al., 2005a) and *S. titanus* (Bressan et al., 2005b). During and after feeding on infected plants, the phytoplasma can move through the vector's hemolymph and infect the reproductive organs (Dickinson et al., 2013). In fact, transovarial transfer of phytoplasmas to offspring has been reported in *S. titanus* (Alma et al., 1997), *Hishimonoides sellatiformis* Ishihara (Kawakita et al., 2000), and *Cacopsylla pruni* Scopoli (Tedeschi et al., 2006), indicating that phytoplasmas can move into the reproductive organs of vectors.

Table 2.2. Studies on the Influence of Phytoplasmas on Insect Vector Performance.[a]

Vector	Host plant	Phytoplasma/disease		Survival	Growth and development	Reproduction	References
Family Cicadellidae *Limotettix vaccinii* (Van Duzee)	Cranberry	False blossom disease		0	+	–	This study
Macrosteles quadrilineatus Forbes	Romaine lettuce, *oat*	Aster yellows phytoplasma		+		+/0	Beanland et al, 2000
Macrosteles quadrilineatus Forbes	*Arabidopsis*	Aster yellows phytoplasma		0		+	Sugio et al, 2011
Macrosteles quadrilineatus Forbes, *Dalbulus maidis* DeLong & Wolcott	*Arabidopsis*, *Nicotiana benthamiana* L., lettuce, China aster, and maize	Aster yellows phytoplasma		+			Kingdom & Hogenhout, 2007
Dalbulus maidis DeLong & Wolcott	Aster	Aster yellows phytoplasma		+			Purcell, 1988
Dalbulus maidis DeLong & Wolcott	Maize	Maize bushy stunt phytoplasma		+			Ebbert & Nault, 2001
Euscelidius variegatus Kirschbaum	Broad bean, and maize	Flavescence dorée phytoplasma		– (♀), 0 (♂)		–	Bressan et al, 2005a
Scaphoideus titanus Ball	Grapevine	Flavescence dorée phytoplasma		–		–	Bressan et al, 2005b
Macrosteles quadripunctulatus (Kirschbaum)	Daisy	Chrysanthemum yellows phytoplasma		–		0	D' Amelio et al, 2008
Euscelidius variegatus (Kirschbaum)	*Oat*	Chrysanthemum yellows phytoplasma		0		+	D' Amelio et al, 2008
Family Psyllidae *Cacopsylla picta* (Förster)	Apple	Apple proliferation phytoplasma		–	–	–	Mayer et al, 2011
Family Cixiidae *Hyalesthes obsoletus* Signoret	*Urtica dioica* L.	Bois Noir stolbur phytoplasmas			0 (adults)		Kaul et al, 2009

[a]Effects were classified as positive (+), negative (−), or neutral (0, i.e., no effects).

It is, thus, possible that the phytoplasma could have a direct effect on egg production of infected *L. vaccinii*, resulting in reduced oviposition. Reduced oviposition may be also affected by oviposition-deterrent compounds in phytoplasma-infected plants. Further studies are needed to elucidate the mechanism for this reduction in *L. vaccinii* oviposition, which may help develop strategies to increase host-plant resistance against this pest.

Surprisingly, *L. vaccinii* was more attracted to uninfected cranberry plants than to phytoplasma-infected cranberries under no-choice scenarios. This is contrary to our expectations, as we expected phytoplasma-infected plants to emit more volatiles to attract its insect vector. For instance, a previous study showed attraction of the vector *C. picta* to odors from phytoplasma-infected apples (Mayer et al., 2008b). However, the attractiveness of the vector to infected host plants might not depend solely on volatiles; visual cues might also be important. For example, *Mgenia fuscovaria* Stål are attracted to aster yellows-infected grapevine (Kruger et al., 2011), but this attraction is not mediated by volatile emissions (la Grange, 2017). Instead, the yellow color of infected leaves appear to play a critical role in *M. fuscovaria* attraction (Krüger et al., 2015). Phytoplasma-infected cranberries have a distinctively red coloration in the Fall and red is attractive to *L. vaccinii* adults (Rodriguez-Saona et al., 2012). Other characteristics in infected cranberries, such as the bushy characters and anomaly of flower (Polashock et al., 2017), could also attract *L. vaccinii*. The role of visual cues in *L. vaccinii* host finding needs investigation.

Our study shows that the total volatile emissions were lower in phytoplasma-infected than in uninfected cranberry plants. Emissions of three volatile compounds were, in particular, reduced: (*E*)-4,8-dimethyl-1, 3, 7-nonatriene, dodecane, and germacrene D. Previous studies have shown the ability of phytoplasmas to change

volatile emissions from infected plants. In apples, phytoplasma infection induced a higher production of β-caryophyllene, ethyl benzoate, and methyl salicylate (Mayer et al., 2008a, 2008b; Rid et al., 2016). Ethyl benzoate was also detected in the apple proliferate disease-infected pears and infected tobacco plants (Rid et al., 2016). Infection of witch's broom disease caused by a phytoplasma in acid lime increases limonene, β-ocimene, and trans-caryophyllene emissions but also reduces emissions of other compounds, such as citral, citronellal, cis-verbenol, neryl acetate, and linalool in leaves (Al-Yahyai et al., 2014). Our results show that *L. vaccinii* is attracted to volatiles from uninfected plants. It is, thus, likely that this insect uses volatiles to locate the habitat where host plants, such as cranberries, are located and then, as indicated above, use visual cues to locate infected plants among uninfected plants.

Previous studies have shown that cranberry volatiles are induced by methyl jasmonate, a volatile derivative of JA (Rodriguez-Saona et al., 2013). Therefore, because of lower emissions of these volatiles in phytoplasma-infected cranberries, we expected infection to cause a reduction of JA levels. Based on the literature, phytoplasma infection often suppresses JA and either induces or suppresses SA; however, our study found no effects of phytoplasma infection on neither of these phytohormones nor ABA, which often modulates JA and SA biosynthetic pathways (Fan et al., 2009). For example, phytoplasma infection causes a strong induction of the SA-mediated defense responses and suppression of the jasmonate biosynthetic pathway in apple (Musetti et al., 2013). Similarly, the phytoplasma effector SAP11 protein leads to the downregulation of lipoxygenase expression and JA synthesis in Arabidopsis (Sugio et al., 2011). However, Lu et al. (2014) found that transgenic *Arabidopsis thaliana* (L.) Heynh. expressing the secreted aster yellows phytoplasma strain witches' broom protein11 (SAP11) effector suppresses SA-mediated defense responses.

In sum, many phytoplasma-infected plants are often found surrounded by uninfected plants in cranberry fields (commonly referred to as bogs or marshes). To locate infected plants in these scenarios, the insect vector *L. vaccinii* may use volatile cues from uninfected plants during long distance host searching and then switch to visual cues. Infected plants, through changes in nutrient content and secondary metabolites (Pradit et al., 2019), are better hosts for the vector. As a result, the vector *L. vaccinii* might spend more time feeding on infected plants, which could explain the observed prolonged times for immature development and increases in adult mass. Bigger adult size may enhance their dispersal capacity. However, these benefits on the vector are only for one generation because infection by the phytoplasma results in lower egg production. Understanding the mechanisms by which pathogens manipulate host plants for their own benefit and the benefit of insect vectors, such as changes in chemical and visual cues, will help in findings ways to protect crops against vector-transmitted plant diseases.

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CHAPTER THREE

PHYTOPLASMA INFECTION OF CRANBERRIES BENEFIT NON-VECTOR PHYTOPHAGOUS INSECTS

ABSTRACT

Despite increasing knowledge about the impacts of pathogens on the interactions between plants and insect vectors, relatively little is known about their effects on other, non-vector, organisms. In cranberries, phytoplasma infection causes false blossom disease, which is transmitted by leafhoppers. We hypothesized that changes in plant chemistry induced by phytoplasma infection might affect the performance and feeding behavior not only of vectors but also of other phytophagous insects. To test this, we measured growth, survival, and the number of leaves damaged by larvae of three common non-vector herbivores: spotted fireworm (*Choristoneura parallela* Robinson), Sparganothis fruitworm (*Sparganothis sulfureana* Clemens), and gypsy moth (*Lymantria dispar* L.) on phytoplasma-infected and uninfected cranberries (*Vaccinium macrocarpon* Ait.). We also assessed the effects of phytoplasma infection on nutrients and phytochemistry related to defenses. In general, larvae of all three herbivore species grew 2–3 times bigger, and damaged 1.5–3.5 times more leaves, while feeding on infected vs. uninfected plants. Survival of Sparganothis fruitworm larvae was also ~1.5 times higher on infected plants, while spotted fireworm and gypsy moth larval survival was not affected. In a long-term (5-week) assay, gypsy moth larval

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survival and mass were enhanced when feeding on phytoplasma-infected leaves. Levels of important plant nutrients (e.g., N, P, K, Ca, S, Mn, Fe, B, Al, and Na) were higher in infected plants, while levels of defensive proanthocyanidins were reduced by 20–40% compared to uninfected plants. In contrast, levels of Mg were lower in infected plants, while concentrations of Cu, Zn, and defensive flavonols were not affected. Taken together, these findings suggest that phytoplasma infection enhances plant nutritional quality, while reducing plant defenses in cranberries. These effects, in turn, may explain the observed enhancement of non-vector herbivore performance, as well as the higher number of damaged leaves, on infected plants. Improved understanding of the ecology of pathogen-plant-herbivore interactions could aid efforts to enhance plant resistance and suppress disease transmission in agricultural settings.

INTRODUCTION

Insect-borne plant pathogens are common in both natural and agricultural ecosystems (Eigenbrode et al., 2018). These pathogens, which include viruses, bacteria, and fungi, often depend on insect herbivores as vectors for their transfer from infected to healthy, uninfected plants (Eigenbrode et al., 2018). Consequently, the epidemiology of these pathogens can be strongly influenced by the host selection and feeding behaviors of vector insects, which, in turn, are influenced by the levels of primary and secondary metabolites in plants (Gandon, 2018). Over the past two decades, many studies have demonstrated the ability of pathogens to affect vector behavior by altering features of host-plant chemistry (Eigenbrode & Bosque-Pérez, 2016; Ingwell et al., 2012; Mauck et al., 2012; Mauck, 2016 Stafford et al., 2011), including plant defense signals (Zhang et al., 2016; Carr et al., 2018), volatile emissions (Eigenbrode et al., 2002; Jiménez-Martínez et al., 2004; Mauck et al., 2010) and nutrition (e.g., leaf and/or

phloem amino acid content) (Blua et al., 1994; Mauck et al., 2014; McMenemy et al, 2012). In addition to influencing host-plant interactions with vectors, such effects are likely to influence interactions with non-vector organisms, including other herbivores (e.g. Kersch-Becker & Thaler, 2014). Such effects on host-plant interactions with non-vectors may likely have broader implications for the ecology of biological communities and ecosystems, given increasing appreciation for the ecological significance of parasite effects on host phenotypes (Lefèvre et al., 2009). Yet, to date, relatively few studies have explored interactions among plant-pathogens, host plants, and non-vector organisms (but see Mauck et al. 2015).

Phytoplasmas are economically important bacterial plant pathogens, transmitted exclusively by insects in the order Hemiptera, that cause severe economic losses to agricultural crops worldwide (Bai et al., 2006; Weintraub & Jones, 2009). Common symptoms caused by phytoplasmas include stem proliferation referred to as witch's broom, changes of the flower structures to leaf-like structures (known as phyllody), yellowing (chlorosis), and stunting (Christensen et al., 2005; Lee et al., 2000). Phytoplasma infection can also alter diverse aspects of host plant chemistry (Oliveira et al., 2005; Musetti, 2009; Sugio et al., 2011a). For example, infection by phytoplasmas can alter carbohydrate and amino acid levels (Lepka et al., 1999; Tan & Whitlow, 2001), induce changes in volatile emissions (Mayer et al., 2008a, 2008b; Orlovskis & Hogenhout, 2016), and affect defense signaling (Sugio et al., 2011b) in plants. Phytoplasma infection has also been shown to increase levels of plant secondary metabolites, including phenolic compounds and hydrogen peroxide (Junqueira et al., 2004; Musetti et al., 2004; Musetti, 2009). Previous work has shown that these changes in the phytochemistry of phytoplasma-infected plants can influence the behavior of insect vectors (e.g., leafhoppers and psyllids) (Kaul et al., 2009; Maixner et al., 2014;

Mayer et al., 2008a, 2008b, 2011; Weintraub & Beanland, 2005). For example, Beanland et al. (2000) showed that aster leafhoppers, *Macrosteles quadrilineatus* Forbes, live longer and have higher fecundity on asters (*Callistephus chinensis* (L.) Nees) infected by the aster yellows phytoplasma than on uninfected plants. Changes in host plant chemistry induced by phytoplasma infection may also have effects on non-vector herbivores, as host plants are usually shared by a community of insect herbivores that may be differentially influenced by pathogen infection (Barbosa, 1991). However, to our knowledge no previous study has investigated whether changes in plant chemistry due to phytoplasma infection affects the performance of non-vector herbivores.

In cranberries (*Vaccinium macrocarpon* Ait.), a crop native to North America, a phytoplasma pathogen causes false blossom, an economically-important disease that decreases crop productivity by sterilizing flowers (Chen, 1971; Polashock et al., 2017). This pathogen is transmitted exclusively by the blunt-nosed leafhopper (*Limotettix vaccinii* Van Duzee; Hemiptera: Cicadellidae) (Beckwith & Hutton, 1929; De Lange & Rodriguez-Saona, 2015; Dobroscky, 1931); however, many other herbivorous insects that do not transmit false blossom also feed on cranberries in the northeastern United States, including many Lepidopteran species such as the spotted fireworm (*Choristoneura parallela* Robinson; Tortricidae), Sparganothis fruitworm (*Sparganothis sulfureana* Clemens; Tortricidae), and gypsy moth (*Lymantria dispar* L.; Erebidae) (Averill & Sylvia, 1998). All of these species feed on cranberry leaves, and spotted fireworm and Sparganothis fruitworm can also damage fruits (Averill & Sylvia, 1998).

The current study tested the hypothesis that changes in plant chemistry due to phytoplasma infection affects the performance of, and the amount of damaged leaves

by, non-vector phytophagous insects in cranberries. Specifically, we asked the following questions: (1) does performance (i.e., mass and survival) of non-vector insects (spotted fireworm, Sparganothis fruitworm, and gypsy moth), and the number of damaged leaves, differ between phytoplasma-infected and uninfected plants?; and (2) does phytoplasma infection alter features of plant chemistry that affect the plant's suitability for herbivores, including nutrient levels and chemical defenses?

MATERIALS AND METHODS

Plant Preparation

Phytoplasma-infected and uninfected cranberries (*V. macrocarpon* cv. 'Crimson Queen') were collected in November 2016 (at the dormant stage) and maintained at 10°C for about three months. Uninfected plants were taken from stolons provided by Integrity Propagation (http://integritypropagation.com/; Chatsworth, NJ, USA; this nursery regularly tests its plants to ensure they are free of any common cranberry viruses and phytoplasmas), while phytoplasma-infected plants were taken from a commercial cranberry field in Chatsworth, NJ, that was originally planted with material originated from Integrity Propagation. In February 2017, infected and uninfected plants were propagated clonally by stem cuttings (~7 cm each), that were transferred to individual 4 × 4-cm cells and placed in a greenhouse (20 ± 2°C, 70 ± 10% relative humidity [RH], and 15:9 light:dark [L:D]) for rooting. Plants were grown in a 50:50 v/v peat:sand mix, fertilized once a month from March till May with PRO-SOL 20-20-20 of nitrogen (N)-phosphorus (P)-potassium (K) All Purpose Plant Food (Pro Sol, Inc., Ozark, AL, USA) at a rate of 165 ppm N and were watered daily. After the cuttings developed roots, groups of five cuttings were transplanted into single pots (7 × 7 cm^2). Plants were allowed to grow in the greenhouse until August 2017 when they

were used in experiments. Prior to conducting the experiments, 10 plants (five plants from infected and uninfected plants) were randomly tested by DNA fingerprinting, using sequence characterized amplified region markers (Polashock & Vorsa, 2002), to verify that all plants were genetically the same. Another 10 plants (five plants from infected and uninfected plants) were randomly selected to test for phytoplasma infection by using a nested PCR assay (Lee et al., 2014). These tests showed that all plants belonged to the same genotype (Crimson Queen) and that only infected plants were positive for the presence of phytoplasma (data not shown). Visually, phytoplasma-infected plants did not show symptoms of any other cranberry disease except for those associated with false-blossom disease (e.g. bushy characters, short and straight uprights; Fig. 3.1). Therefore, our methods ensured that the plants were genetically similar, that uninfected plants were free from phytoplasma, and that growing conditions, propagation methods, and handling were uniform for uninfected and infected plants.

For insect assays (see below), all five plants from each pot were used to study the performance of a single herbivore species. A total of 5, 6, and 9 pots of infected plants and the same number of pots of uninfected plants (N = 25, 30, 45 total plants each) were used to study the performance of spotted fireworm, Sparganothis fruitworm, and gypsy moth larvae, respectively. For plant chemistry analyses (see below), one plant was selected from each pot and a total of 10 and 15 uninfected plants and the same number of infected plants were used for nutrient and phenolic analyses, respectively. Different plants were used for the insect performance and chemical assays, and all material for plant chemistry analyses was harvested at the end of performance assays (August 2017). All plants were at the vegetative stage when used.

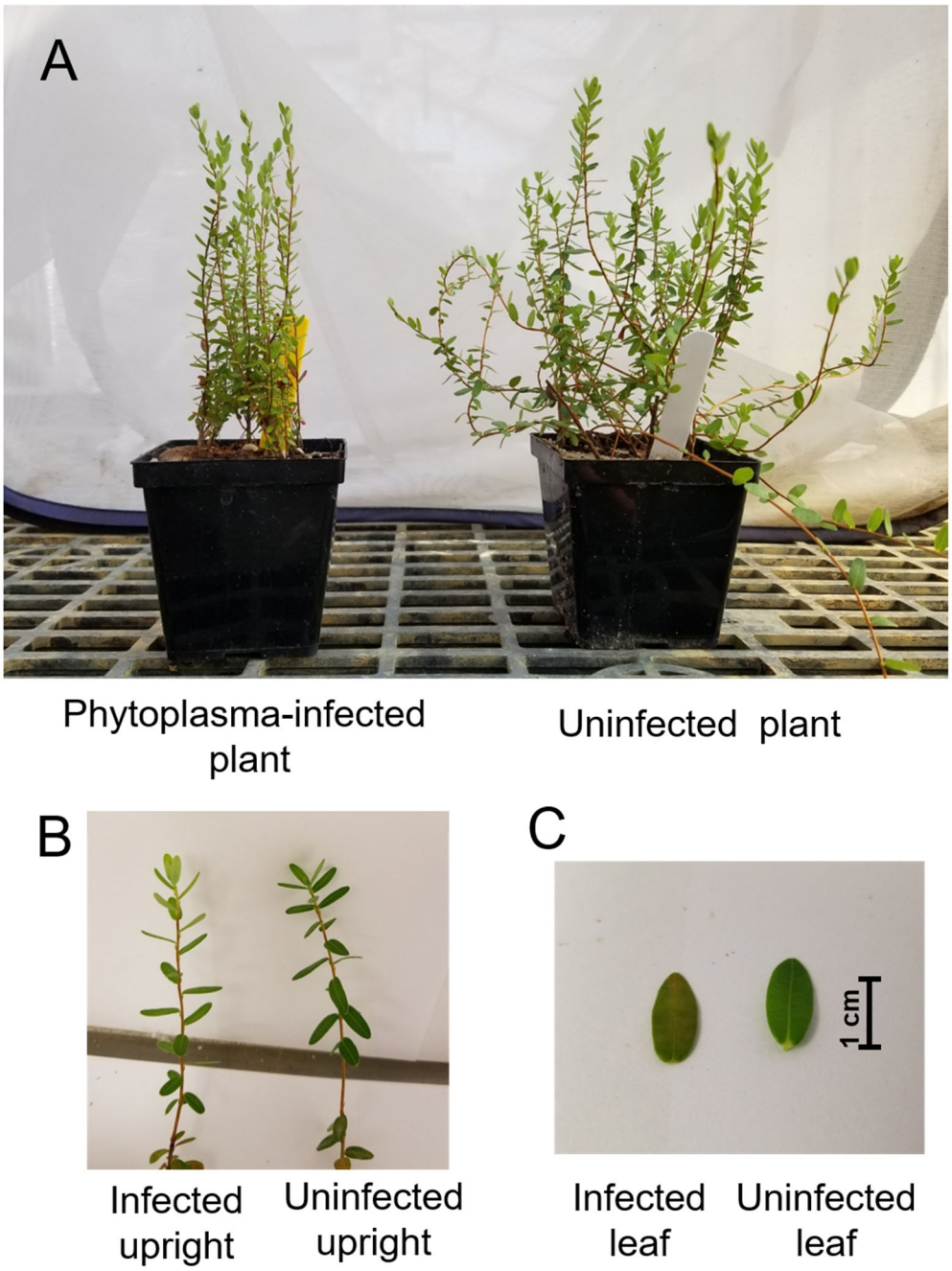

Figure 3.1 Morphological differences (A) and close-ups of uprights (B) and leaves (C) of phytoplasma-infected and uninfected cranberry (*Vaccinium macrocarpon*) plants.

Insects

Colonies of spotted fireworm, Sparganothis fruitworm, and gypsy moth were maintained at the Philip E. Marucci Entomology Laboratory (Chatsworth, NJ, USA) (24 ± 1°C, 65% RH, and 14:10 L:D). The spotted fireworm and Sparganothis fruitworm colonies originated from larvae collected from commercial cranberry bogs in Chatsworth, NJ (USA), and larvae were reared on the Stonefly Heliothis Diet (Ward's Scientific, Rochester, NY, USA). Gypsy moth eggs were obtained from the USDA APHIS (Massachusetts, USA), and larvae were reared on a wheat germ diet (Bell et al., 1981). Colonies were supplemented yearly with new individuals to reduce inbreeding depression. Neonates were used for all experiments.

Insect Performance and Leaf Damage

To assess larval performance and amount of leaf damage on phytoplasma-infected and uninfected cranberry leaves, feeding experiments were conducted in a greenhouse at 22 ± 2°C, 70 ± 10% RH, and 15:9 L:D. One hundred infected and uninfected plants (total of 20 pots each; $N = 200$ plants total) were individually covered with 18 × 42 × 48-cm gauze bags (Temkin International; Springville, UT, USA). Each plant then received either three spotted fireworm neonates, three Sparganothis fruitworm neonates, or one gypsy moth neonate [$N = 25$ (5 pots), 30 (6 pots), and 45 (9 pots), respectively]. Plants were assigned randomly to treatments, and each plant was considered a replicate. Larval mortality and mass were assessed after 7 days (for gypsy moth) or 14 days (for spotted fireworm and Sparganothis fruitworm, whose larvae are smaller than gypsy moth larvae). The number of damaged leaves was estimated by counting the number of leaves with visible signs of larval feeding; note that leaves of

phytoplasma-infected and uninfected cranberries have similar surface area (~0.4 cm^2; Fig 3.1C).

An additional laboratory study was conducted to assess the long-term effects of feeding on phytoplasma-infected plants on gypsy moth larval performance (at 24 ± 1°C, 65% RH, and 14:10 L:D). Gypsy moth neonates were placed individually in 30 1-oz (29.57 ml) plastic cups (Maryland Plastic, Inc., Federalsburg, MD, USA) (i.e., one larva per cup). Fifteen larvae were fed uninfected cranberry leaves, while the other 15 larvae were fed phytoplasma-infected cranberry leaves (N = 15 replicates per plant type). Leaves (0.1 g) were replaced with new ones every 3 days; larval mortality and mass were recorded weekly for a total of 5 weeks.

Plant Nutrient Analysis

To explore the effects of phytoplasma infection on plant nutrients, leaves were taken from ten randomly selected infected and uninfected plants (N = 10 replicates per plant type; 1 plant per pot), kept separately in paper bags, and allowed to dry. For each sample, leaves were randomly collected from different positions within the plant. Dried samples (1.5 g) were sent for nutrient analyses to the Penn State University Agricultural Analytical Service Laboratory (http://agsci.psu.edu/aasl). Total N was analyzed by combustion with an Elementar Vario Max N/C analyzer (Horneck & Miller, 1998), whereas P, K, calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe), copper (Cu), boron (B), aluminum (Al), zinc (Zn), sodium (Na), and sulfur (S) were analyzed by inductively coupled plasma emission spectroscopy (Huang & Schulte, 1985). For total N concentration, ground dried plant samples (at least 0.1 g) were combust at a high temperature. The gas from combustion was oxidized by copper oxide, then tungsten and Cu turned nitrogen oxide to nitrogen (N) inside the Elementar Vario Max N/C

analyzer. Total N concentrations are estimates from the proportion of electrical signal produced by thermal conductivity detector. For the other chemical elements, ground dried plant samples (0.25 g) were predigested in concentrated HNO_3 from room temperature to 60°C for 30 min, followed by digestion with H_2O_2 at 90°C for 90 min. The sample solution was introduced into the spectrometer that detected the element emission and calculated its concentration.

Phenolic Analysis

To explore the effects of phytoplasma infection on plant defenses, we measured flavonoid levels (i.e., proanthocyanidins and flavonols)—important secondary metabolites involved in plant defense against herbivores (Bernays & Chapman, 1994; Simmonds, 2001)—in phytoplasma-infected and uninfected plants. Leaves were randomly selected from each of 15 infected and 15 uninfected plants (1 plant per pot) and stored at −20°C before extraction ($N = 15$ replicates per plant type). Frozen samples were ground in liquid nitrogen, the ground material (approximately 30 mg) was then placed in 2 ml Eppendorf tubes, and 0.6 ml of the extraction solution (80% acetone: 19.9% distilled water: 0.1% acetic acid) was added to each tube. Samples were vortexed for 5 min, followed by sonication for 10 min. After sonication, samples were centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a new Eppendorf tube, and the same procedure was repeated twice with the remaining sample by adding 0.6 ml of the extraction solution each time. The supernatants from these three extractions (approximately 1.8 ml) were transferred to a 2 ml microcentrifuge tube and dried in a centrifugal vacuum for 24 h. The dried extracts were dissolved in 0.5 ml of 100% methanol and analyzed for quantification of flavonols and proanthocyanidins in

a Waters Alliance high-pressure liquid chromatography (HPLC) system. HPLC conditions followed those described in (Wang et al., 2017).

For flavonol analysis, a Gemini® 150 × 4.6 mm C18 110 Å, 5 µm LC column was used with water + 0.1% formic acid as solvent A and acetonitrile + 0.1% formic acid as solvent B. the elution gradient was 0%-15% B from 0-1 min; 15%-16% B from 1-5 min; 16% B from 5-10 min; 16%-17% B from 10-25 min; 17% B from 25-28 min; 17%-30% B from 28-30 min; 30%-45% B from 30-38 min; 45%-80% B from 38-40 min; 80%-0% B from 40-43 min and 0% B from 43-50 min. Flow rate was 1 ml/min and injection volume was 10 µl. Compounds were detected in a photodiode array (PDA) detector at 366 nm. For proanthocyanidins, a Develosil® 250 × 4.6 mm 100Diol-5, 5 µm LC column was used with 98% acetronitrile + 2% acetic acid as solvent A and 95% methanol + 3% water + 2% acetic acid as solvent B. The elution gradient was 0%-10% B from 0-5 min; 10%-12% B from 5-7 min; 12% B from 7-8 min; 12%-13% B from 8-10 min; 13%-20% B from 10-15 min; 20%-40% B from 15-35 min; 40%-0% B from 35-45 min and 0% B from 45-50 min. Flow rate was 1 ml/min and injection volume was 10 µl. Compounds were detected in fluorescence detector with excitation/emission wavelengths at 280/308 nm.

Statistical Analysis

Prior to analysis, all data were checked for normality and homoscedasticity. If needed, data were square root or $\log_{10}$ transformed to meet the assumptions for analysis of variance (ANOVA); otherwise, non-parametric Mann-Whitney U-tests were used. All parametric and non-parametric tests were performed using IBM® SPSS® version 24. Because each cranberry plant was considered a replicate, the mass and survival of spotted fireworm and Sparganothis fruitworm larvae from the same plants were

averaged prior to statistical analysis. Differences in the masses of the spotted fireworm and gypsy moth larvae between uninfected and phytoplasma-infected plants were tested using Mann-Whitney U-tests, whereas differences in Sparganothis fruitworm larval mass were tested using a mixed model that included infection as a fixed factor and pot as a random factor. Larval survival of the spotted fireworm and Sparganothis fruitworm on uninfected and phytoplasma-infected plants was compared by Mann-Whitney U-tests, while gypsy moth larval survival was compared by a chi-square test. Differences in the number of leaves damaged by the spotted fireworm and gypsy moth between uninfected and phytoplasma-infected plants were compared by Mann-Whitney U-tests, whereas we used a mixed model to test for differences in the number of leaves damaged by Sparganothis fruitworm. For the long-term performance assay, we analyzed gypsy moth weekly survival using the Kaplan-Meyer survivorship curve and compared the weekly larval mass gained between infected versus uninfected plants using one-way ANOVA.

Principal component analysis (PCA) was used to visualize differences in nutrient content and levels of proanthocyanidins and flavonols between uninfected and phytoplasma-infected plants (Minitab® version 18). Differences in the amounts of individual nutrients between uninfected and phytoplasma-infected plants were compared by one-way ANOVA, except for Mg, S, Cu, and Zn, which were analyzed using Mann-Whitney U-tests. Differences in total amounts and amounts of individual proanthocyanidins and flavonols between uninfected and phytoplasma-infected plants were compared by one-way ANOVA.

RESULTS

Phytoplasma Infection Improves Larval Performance and Leaf Damage

Larval mass was consistently enhanced when feeding on phytoplasma-infected plants as compared to uninfected plants (Fig. 3.2). Spotted fireworm ($U = 118.5$, $p = 0.004$; Fig. 3.2A), Sparganothis fruitworm ($F_{1,5.54} = 81.027$, $p < 0.001$; Fig. 3.2B), and gypsy moth ($U = 62$, $p < 0.001$; Fig. 3.2C) larvae feeding on infected plants were 2, 1.9, and 3 times bigger, respectively, than those feeding on uninfected plants. Survival of Sparganothis fruitworm larvae was also ~1.5 times higher when feeding on infected plants ($U = 239$, $p = 0.001$; Fig. 3.2A), whereas larval survival of spotted fireworm ($U = 300$, $p = 0.797$; Fig. 3.2B) and gypsy moth ($\chi^2 = 1.029$, $p = 0.31$; Fig. 3.2C) showed no significant effects.

The number of damaged leaves was also consistently higher on phytoplasma-infected plants compared to uninfected plants (Fig. 3.2). Spotted fireworm ($U = 162$, $p = 0.039$; Fig. 3.2A) and Sparganothis fruitworm ($F_{1,5.09} = 18.898$, $p = 0.007$; Fig. 3.2B) larvae damaged approx. 1.6–1.7 times more infected than uninfected leaves. Similarly, gypsy moth ($U = 12.5$, $p = 0.028$; Fig. 3.2C) larvae damaged 3.4 times more infected than uninfected leaves.

In the long-term performance assay, survival of gypsy moth larvae on phytoplasma-infected plants was significantly higher than on uninfected plant ($\chi^2 = 7.995$, $p = 0.005$; Fig. 3.3A). Larvae fed on phytoplasma-infected leaves had higher mass than larvae fed on uninfected leaves (week 1: $F_{1,16} = 12.308$, $p = 0.003$; week 2: $F_{1,13} = 10.305$, $p = 0.007$; week 3: $F_{1,12} = 6.714$, $p = 0.024$; week 4: $F_{1,11} = 22.190$, $p = 0.001$) (Fig. 3.3B). After 5 weeks, the mean mass of gypsy moth feeding on phytoplasma-infected leaves was 3.6 times that of larvae feeding on uninfected leaves

(mean larval mass ± SE on infected plants = 137.07 ± 21.19 mg and on uninfected plants = 38.03 ± 17.36 mg; $F_{1,11} = 10.694$, p = 0.007).

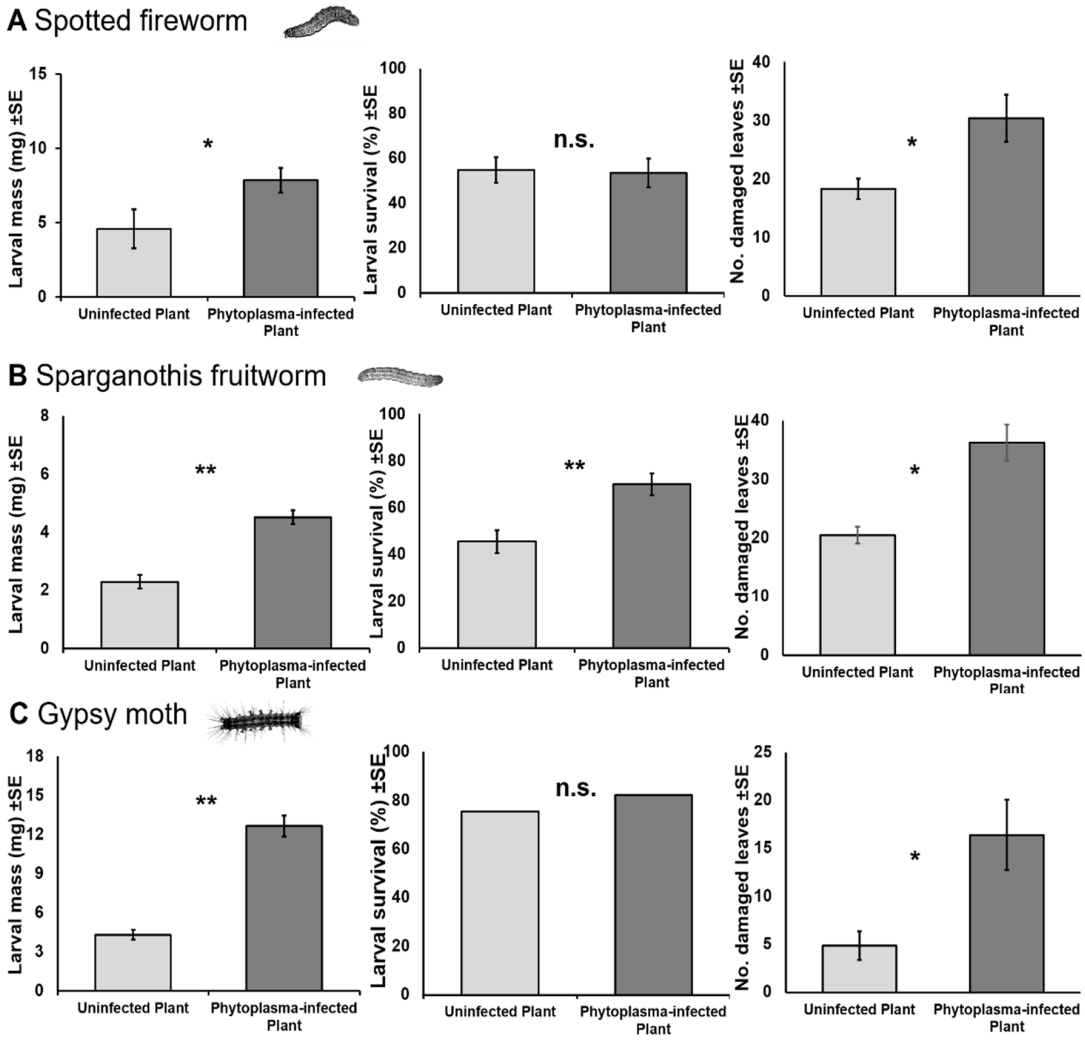

Figure 3.2 Mean (±SE) larval mass and survival of spotted fireworm (*Choristoneura parallela*) (A), Sparganothis fruitworm (*Sparganothis sulfureana*) (B), and gypsy moth (*Lymantria dispar*) (C), and number of damaged leaves, when fed on uninfected and phytoplasma-infected cranberry plants after 7 or 14 days. Asterisks indicate statistically significant differences (* = p ≤ 0.05, ** = p ≤ 0.001). n.s. = not significant (p > 0.05). *N* = 25, 30, and 35 for spotted fireworm, Sparganothis fruitworm, and gypsy moth, respectively.

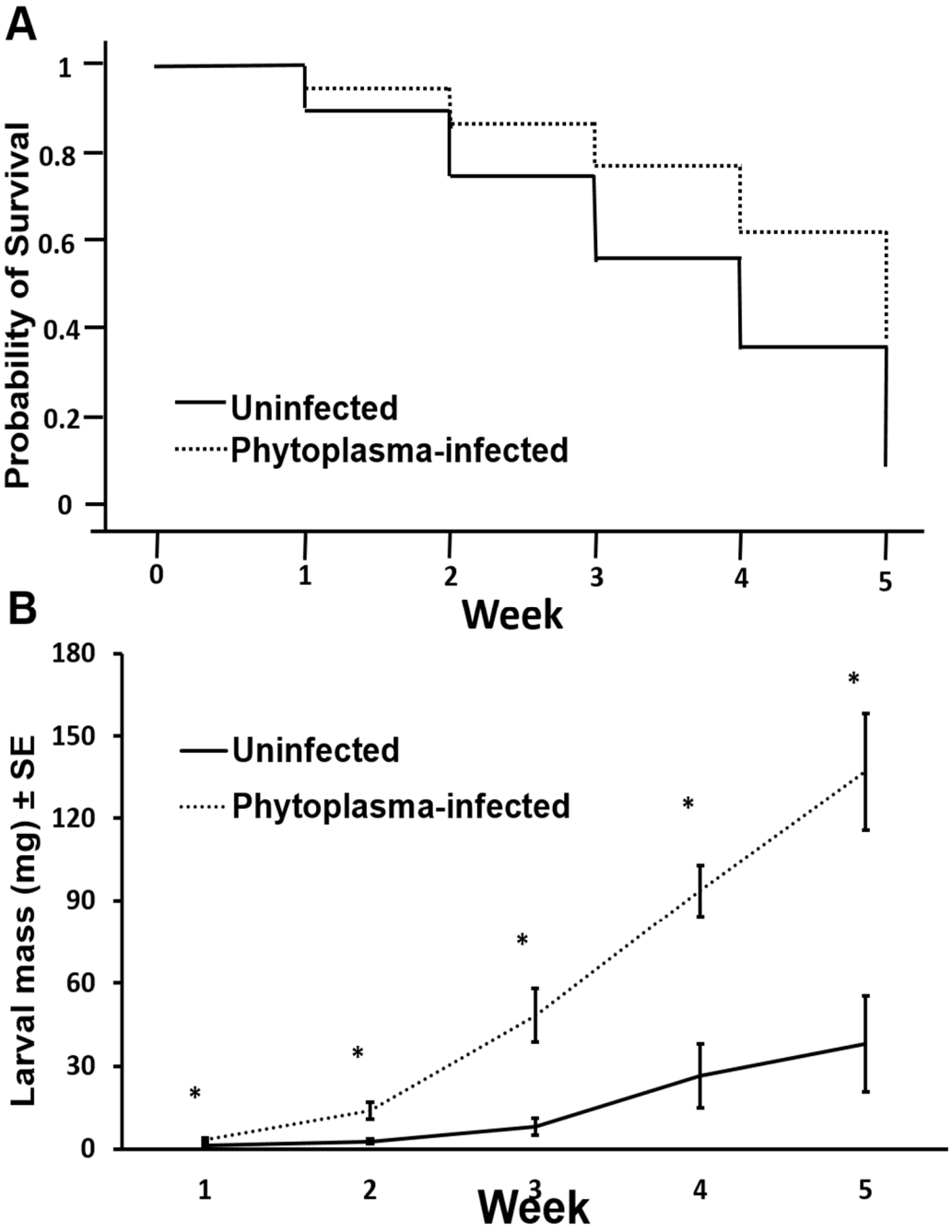

Figure 3.3 Survival (Kaplan-Meyer curve) (A) and mass (B) of gypsy moth (*Lymantria dispar*) larvae fed on uninfected and phytoplasma-infected cranberry leaves for 5 weeks. Asterisks indicate statistically significant differences (* = $p \leq 0.05$). $N = 15$.

Phytoplasma Infection Increases Plant Nutrients

The PCA revealed clear separation in nutrient composition between phytoplasma-infected and uninfected plants (Fig. 3.4A). The first two components explained 74.7% of the total variation (53.1% and 21.5% for 1st and 2nd components, respectively). Additionally, levels of 10 (out of 13) individual nutrients were significantly higher in phytoplasma-infected than uninfected plants (Table 3.1). Levels of Mg were lower in infected than uninfected plants, whereas levels of Cu and Zn were not significantly affected by phytoplasma infection (Table 3.1).

Table 3.1 Effects of phytoplasma infection on the amounts of nutrients in cranberry leaves.

Nutrients	Mean ± SE		Statistical value[1]	p value
	Uninfected plant	Phytoplasma-infected plant		
Nitrogen (N)[2]	1.31 ± 0.01	1.62 ± 0.08	$F_{1,18} = 6.312$	0.022
Phosphorus (P)[2]	0.18 ± 0.01	0.22 ± 0.01	$F_{1,18} = 6.278$	0.022
Potassium (K)[2]	0.97 ± 0.04	1.12 ± 0.03	$F_{1,18} = 7.550$	0.013
Calcium (Ca)[2]	0.49 ± 0.02	0.59 ± 0.01	$U = 6$	0.001
Magnesium (Mg)[2]	0.19 ± 0.01	0.17 ± 0.003	$U = 18$	0.012
Sulfur (S)[3]	0.054 ± 0.003	0.067 ± 0.004	$U = 221$	0.010
Manganese (Mn)[3]	287.5 ± 14.91	347.4 ± 10.41	$F_{1,18} = 10.855$	0.004
Iron (Fe)[3]	34.9 ± 3.83	66.8 ± 3.75	$F_{1,18} = 35.436$	<0.001
Copper (Cu)[3]	6.5 ± 0.31	7.5 ± 0.48	$U = 27.5$	0.067
Boron (B)[3]	22.2 ± 1.26	32.7 ± 0.82	$U = 0$	<0.001
Aluminium (Al)[3]	58.8 ± 3.48	91.7 ± 5.18	$U = 6$	0.001
Zinc (Zn)[3]	15.5 ± 0.76	18.7 ± 1.56	$U = 35.5$	0.263
Sodium (Na)[3]	115.4 ± 6.16	145.7 ± 8.71	$U = 16$	0.010

[1] Data were analyzed using one-way ANOVA (*F* values, df) or Mann Whitney U tests.
[2] Amounts are in percentages.
[3] Amounts are in mg/kg.

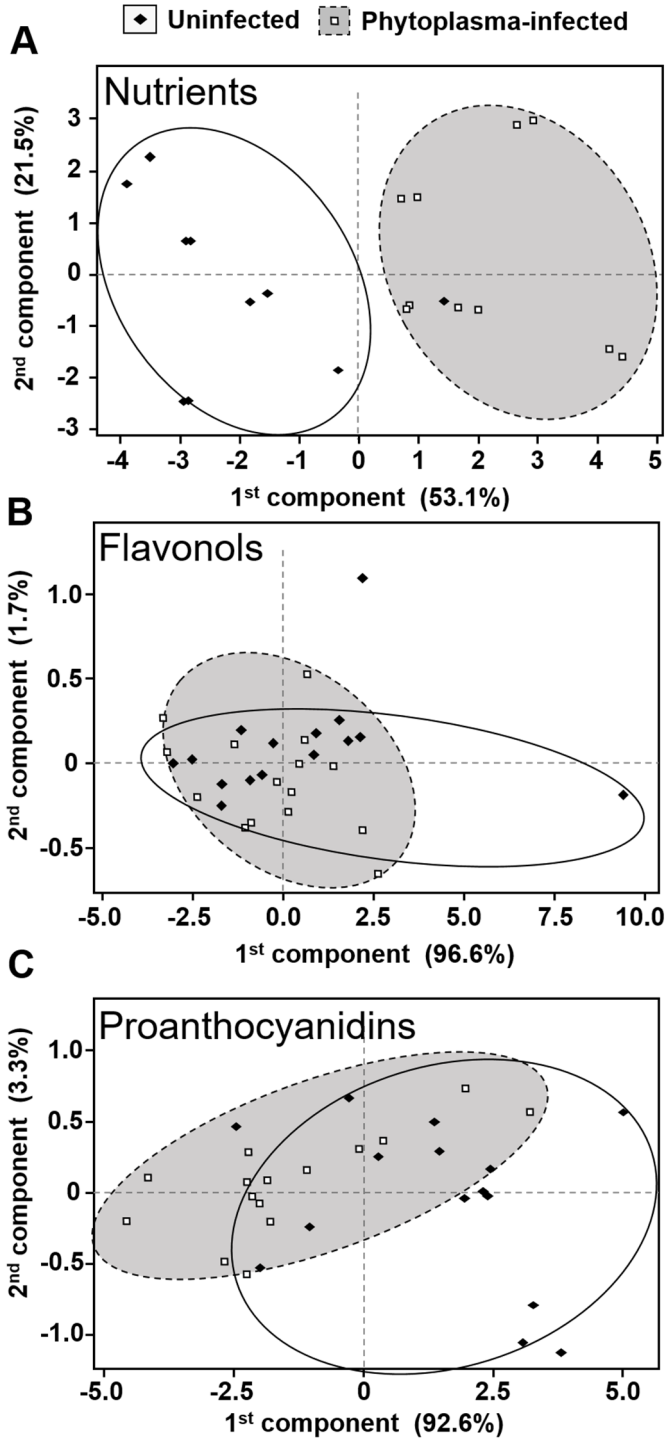

Figure 3.4 Score plots for principal components 1 and 2 from Principal Component Analysis (PCA) for differences in nutrient (A), flavonol (B), and proanthocyanidin (C) composition between uninfected and phytoplasma-infected cranberry leaves. Data are grouped into clusters, each cluster enclosing 80-100% of the data for a particular group.

Phytoplasma Infection Lowers Proanthocyanidin Content

Six flavonols were identified and quantified from cranberry leaves: quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xyloside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside, and quercetin-3-rhamnoside. The PCA revealed a high degree of overlap in flavonol composition between uninfected and phytoplasma-infected cranberry plants, with the first two components explaining 98.3% of total variation (Fig. 3.4B). There were also no significant difference in the levels of total amounts and of all individual flavonols between uninfected and phytoplasma-infected plants (all p values > 0.05) (Table 3.2).

Table 3.2 Concentration (±SE) of flavonols in uninfected and phytoplasma-infected cranberry plants.

Flavonols	Mean (mg/100 g) ± SE		Statistical value[1]	p value
	Uninfected plant	Phytoplasma-infected plant		
Quercetin-3-galactoside	169.53 ± 22.79	137.42 ±12.24	1.100	0.303
Quercetin-3-glucoside	2.38 ± 0.36	2.29 ± 0.22	0.573	0.455
Quercetin-3-xyloside	28.01 ± 3.09	24.45 ± 2.17	0.874	0.358
Quercetin-3-arabinopyranoside	17.35 ± 2.36	13.94 ± 1.32	1.591	0.218
Quercetin-3-arabinofuranoside	152.28 ± 16.07	138.05 ± 11.42	0.573	0.455
Quercetin-3-rhamnoside	201.39 ± 32.36	181.35 ± 17.01	0.033	0.857
Total	570.94 ± 76.41	497.49 ± 43.48	0.446	0.510

[1] Data were analyzed using one-way ANOVA (*F* values, df = 1,18).

Monomeric and oligomeric proanthocyanidins from cranberry leaves were separated by their degree of polymerization (DP) into DP1, DP2, DP3, DP4, DP5, DP6, and DP7+ (polymeric size more than 6). The PCA for the proanthocyanidin polymers shows the first two components explaining 95.9% of the variation, with the first component explaining 92.6% of the variation and separating most of the data into two distinct groups (i.e., phytoplasma-infected and uninfected plants) (Fig. 3.4C). Phytoplasma infection reduced the concentrations of all the above-mentioned polymers by 20–40% (DP1: $F_{1, 28} = 13.523$, $p = 0.001$; DP2: $F_{1, 28} = 9.404$, $p = 0.005$; DP3: $F_{1, 28} = 9.463$, $p = 0.005$; DP4: $F_{1, 28} = 13.596$, $p = 0.001$; DP5: $F_{1, 28} = 8.345$, $p = 0.007$; DP6: $F_{1, 28} = 12.266$, $p = 0.002$; DP7+: $F_{1, 28} = 25.165$, $p < 0.001$; Total: $F_{1, 28} = 17.627$, $p < 0.001$) compared with uninfected plants (Fig. 3.5).

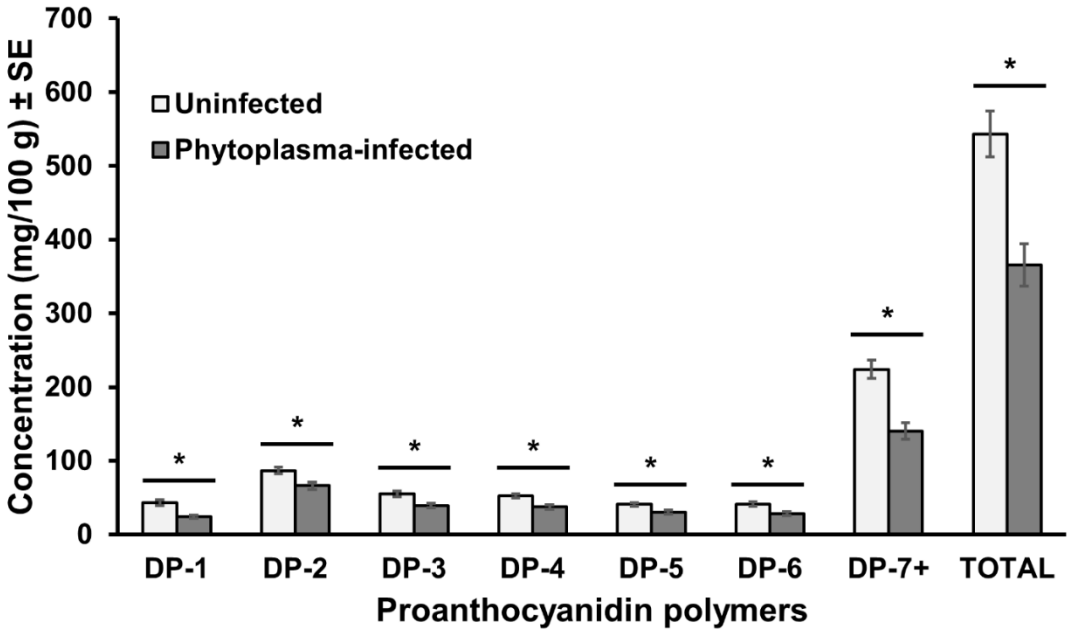

Figure 3.5 Mean (±SE) amounts of proanthocyanidin polymers in uninfected and phytoplasma-infected cranberry leaves. DP = degree of polymerization. An asterisk indicates statistically significant differences (* = $p \leq 0.05$). $N = 20$.

DISCUSSION

The performance of larvae from three lepidopteran species was strongly enhanced by phytoplasma infection relative to uninfected plants. Spotted fireworm, Sparganothis fruitworm, and gypsy moth larvae all grew significantly bigger and also damaged significantly more leaves when fed on infected plants. This increase in the number of damaged leaves may be reflection of greater larval consumption and/or more frequent larval movement from leaf to leaf due to phytoplasma infection. Though not recorded, a higher biomass in the phytoplasma treatment could also mean that the caterpillars were already in another (later) instar than the ones in the uninfected treatment. Faster growth on phytoplasma-infected plants could have a fitness advantage as the insects are then not exposed as long to predators and entomopathogens. Previous studies on phytoplasma infection have reported positive (Maixner et al., 2014), negative (Mayer et al., 2011), and neutral effects (Vega et al. 1995) on the performance of insect vectors. There are, however, fewer data on the impact of plant bacterial infections on non-vector species. One study found that the non-vector *Peregrinus maidis* (Ashmead) (Homoptera: Delphacidae) feeding on corn infected with corn stunt spiroplasma had decreased body mass but increased fecundity (Vega et al. 2015). To our knowledge, this study is the first to document the (positive) effects of phytoplasma infection on non-vector insect herbivores in a cropping system.

The enhancement of non-vector herbivore performance observed in the present study may reflect a pathogen manipulation of the host plant to enhance vector transmission. Because phytoplasmas persist and replicate inside the vector (Hogenhout et al., 2008; Maejima et al., 2014), they may benefit from prolonged vector feeding on infected plants, which in turn may increase the likelihood that vectors will acquire the pathogen. In the current system, we have previously observed that the performance of

blunt-nosed leafhoppers, which serve as phytoplasma vectors, was enhanced on infected, relative to uninfected, cranberries (NP, unpublished data). This is consistent with previous findings that persistently transmitted plant viruses tend to enhance host-plant quality for aphid vectors (e.g. Mauck et al., 2012). The current data reveal similar effects on the performance of non-vector herbivores. Although is unknown whether the phytoplasma itself affects, or persists inside, non-vectors, the benefits observed in our study on herbivore performance are most likely mediated by the plant.

To explore the effects of false blossom disease on cranberry chemistry, we examined the effects of infection on plant nutrient status and chemical defenses. With respect to nutrition status, we found that levels of most of the plant mineral concentrations examined were increased in phytoplasma-infected cranberry plants compared to uninfected plants. Previous studies have shown that plant nutrient modulations induced by phytoplasma can vary greatly (e.g., Zhao and Liu, 2009; Al-Ghaithi et al., 2016). In our study, N levels were elevated in phytoplasma-infected cranberry plants. Higher N levels have not only been reported to enhance pathogen infection, growth, and reproduction (Mitchell et al., 2003), but also the growth, development, and fecundity of herbivorous insects (Awmack and Leather, 2002; Chen et al., 2004; Mattson, 1980). In fact, higher plant N concentrations have previously been shown to enhance the growth rates of many Lepidopteran larvae (Chen et al., 2004; Coley et al., 2006). Some of the other nutrients we found to be elevated in phytoplasms-infected plants, including K, are also important for both pathogens and insects (Amtmann et al., 2008). These nutrient changes in cranberries due to phytoplasma infection may thus benefit both pathogens and herbivores.

We also compared levels of several plant defense compounds between phytoplasma-infected and uninfected cranberry plants. Although phytoplasma infection

did not affect flavonol levels, it significantly reduced concentrations of proanthocyanidins; these are polyphenolic compounds found in many vascular plants that play an important role in anti-microbial defense but can also act as herbivore deterrents and thus reduce insect feeding (Fisk, 1980; Bernays, 1981; Van Huynh and Bevington, 2014). This result is consistent with previous studies reporting that plant chemical defenses are compromised by pathogen infection (Junqueira et al., 2004; Rusjan et al., 2012). Thus, the increased performance and feeding of non-vector insects on phytoplasma-infected cranberry plants may be due in part to this reduction in plant defenses. In addition to providing plants with protection against pathogens and insect herbivores (Koskimäki et al., 2009), the presence of these and other phenolic compounds have been suggested to have benefits for human health (Côté et al., 2010), making higher quantities of these compounds in cultivated fruits desirable (Gallardo et al., 2018); however, it was not possible to assess effects on fruit chemistry in our study system as false-blossom plants are sterile. Furthermore, since we measured only proanthocyanidin and flavonol levels, further investigation is needed to determine whether other secondary metabolites are differentially affected by phytoplasma infection in cranberry.

There are at least two possible conflicting scenarios via which phenotypic changes in plant chemistry due to phytoplasma infection might arise as the result of a plant-pathogen arms-race. First, phytoplasmas could trigger a defense response (i.e., systemic acquired resistance; Sticher et al., 1997) in plants to suppress the infection: the “induced plant defense” hypothesis. Under this scenario we would expect an increase in levels of secondary metabolites and/or increased resistance against herbivores. However, our data for false blossom disease in cranberries does not support this hypothesis. Alternatively, phytoplasmas could manipulate the plant defense

responses for its own benefit and the benefit of the vector: the “vector manipulation” hypothesis (Ingwell et al. 2012). Under this scenario the positive effects on vectors could also conceivably be a side effect of manipulation to enhance pathogen performance. In this case, we would expect reduced secondary metabolites and/or increased nutrient content. These predictions are more consistent with our observations in cranberry. Furthermore, as the current results demonstrate, these effects may enhance host plant quality not only for the leafhopper vector (NP, unpublished data), but could also for other herbivores, an observation with potential implications for pest management such as an increase in chemical control measures to manage these pests.

In conclusion, our study demonstrates that phytoplasma-induced changes in cranberry, including increasing mineral nutrient status and lowering defenses, facilitate non-vector herbivore performance and leaf damage. However, many additional questions remain about the relationship between phytoplasma, the host plant, and herbivorous insects. For instance, our study assessed herbivore performance on relatively young (i.e., <6 months old rooted clones) tissues; additional research is needed to evaluate the effects of phytoplasma infection on herbivore population and community dynamics in cranberry with varying tissue maturities and determine whether phytochemistry levels change with the plant’s ontogeny such as at the reproductive (flowering) stage. In our study, changes in phytochemistry due to phytoplasma infection were only investigated before the insects fed on the plants, whether the levels of primary and secondary metabolites are affected by herbivore feeding needs investigation. However, at least in the short term, we document a positive effect of phytoplasma infection on herbivore performance that was seen through most of the immature development in the gypsy moth. Future transcriptomic and gene expression studies may also provide us with more details on the mechanisms that

underlie host plant manipulation by phytoplasmas. Indeed, the mechanisms by which phytoplasmas and other pathogens with small genomes (Marcone et al., 1999) are able to manipulate their hosts to influence interactions with insect vectors is a topic of emerging interest (Musetti, 2009). Addressing these gaps in our existing knowledge will not only provide information to control the spread of important agricultural pathogens (by inducing defenses that could suppress them), but also give us a clearer view of this complex tripartite host plant-herbivore-pathogen relationship in the ecosystem.

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CHAPTER FOUR

PHYTOPLASMA INECTION INFLUENCES GENE EXPRESSION IN THE AMERICAN CRANBERRY, *VACCINIUM MACROCARPON*

ABSTRACT

Cranberry false blossom disease (CFBD) is caused by a leafhopper-vectored phytoplasma infection. CFBD results in distinctive branching of the upright shoots (witches' broom) and the formation of deformed flowers that fail to produce fruit. This disease is reemerging and poses a serious threat to the cranberry industry. To determine the impact of the disease on host gene expression, we compared the transcriptome profiles between plants with CFBD and uninfected cranberry plants. We found that phytoplasma infection induced the expression of 132 genes, while suppressing 225 genes, compared to uninfected cranberry plants. Differentially expressed genes between uninfected and infected plants were largely associated with primary and secondary metabolic, defensive, and developmental pathways. Phytoplasma infection increased the expression of genes associated with nutrient metabolism, while suppressing genes associated with defensive pathways. This expression profile change supports the "host manipulation hypothesis," whereby CFBD enhances host quality for insect vectors, thus promoting phytoplasma transmission.

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INTRODUCTION

Phytoplasmas, class Mollicutes, phylum Tenericutes (Hogenhout et al., 2008), are obligate vector-borne bacteria that colonize the phloem tissue of infected plants (Chen, 1971). Collectively, phytoplasmas cause over 700 plant diseases worldwide (Maejima et al., 2014), frequently resulting in devastating losses to agricultural productivity (Bertaccini, 2007; Lee et al., 2000). Due in part to a lack of a rigid cell wall and a minimal genome devoid of coding genes for ATP synthases and sugar uptake, phytoplasmas are highly dependent on their plant hosts (Christensen et al., 2005; Kube et al., 2012). Phytoplasmas are almost exclusively vectored by insects in the order Hemiptera, such as leafhoppers, planthoppers, and psyllids (Hogenhout et al., 2008; Weintraub & Beanland, 2005). When inhabiting the host plant, phytoplasmas rely on the nutrient-rich phloem tissue (Chen, 1971); while in the insect vector, the phytoplasmas rely on the nutrient-rich food sources in the hemolymph (Hogenhout et al., 2008). Phytoplasma-infected plants develop diverse symptoms, such as witches' broom, leaf yellowing or reddening, growth aberrations (proliferations, internode shortening, and stunting), and flower malformations (size reduction, virescence, and phyllody) (Chang, 1998; Namba, 2002). Phytoplasma infection can also cause phloem tissue aberrations, such as extensive phloem necrosis and excess formation of phloem, resulting in swollen veins (Lee et al., 2000).

The physiological, biochemical, and molecular mechanisms of phytoplasma infection are still unclear. Furthermore, the interactions between phytoplasmas, their plant host, and their insect vector are poorly understood. This is partly because phytoplasmas currently cannot be cultured in the laboratory (Mou et al., 2013). Despite the challenges this presents, recent research has demonstrated that phytoplasmas produce effector proteins that interact with plant transcription factors. The phytoplasma

can thereby manipulate infected plants developmental processes, phytohormone biosynthesis, and defense responses (Sugio & Hogenhout, 2012). Moreover, some phytoplasma-produced effector proteins were shown to induce phytoplasma-associated disease symptoms in *Arabidopsis thaliana* L. (Heynh.), such as phyllody, witches' broom, and dwarfism (Hoshi et al., 2009; Maejima et al., 2014; Sugio & Hogenhout, 2012), while an effector protein of aster yellows phytoplasma strain witches' broom was shown to suppress salicylic acid (SA)-mediated defense responses (Lu et al., 2014). Some phytoplasmas harbor plasmids (Lee et al., 2004; Bai et al., 2006; Ishii et al., 2009). The causal agent of *Paulownia* witches' broom was shown to harbor two plasmids that encode several expressed proteins, some of which were predicted to encode secreted or membrane-localized proteins that may act as effectors (Lin et al., 2009). *Candidatus Phytoplasma asteris*, onion yellows strain (OY-M), was shown to harbor a plasmid in which ORF3 encodes a membrane protein that is important in adaptation to its insect host (Ishii et al., 2009).

Phytoplasma infection can lead to the production of defense proteins, increased phenolic compounds, and overproduction of hydrogen peroxide in host plants, such as apple and corn (Junqueira et al., 2004; Musetti et al., 2004). The phytoplasma that causes Bois noir disease of grapevine was found to affect host carbohydrate metabolism (Hren et al., 2009). Although the previously mentioned effector protein of aster yellows phytoplasma strain witches' broom suppresses SA-mediated defense responses in *Arabidopsis* (Lu et al., 2014), phytoplasma infection in apple induced SA-mediated defense responses and suppression of the jasmonic acid (JA) biosynthetic pathway (Musetti et al., 2013). Normally, plants induce SA against biotrophic and hemibiotrophic pathogens (Thomma et al., 1998) and against piercing-sucking insects (War et al., 2012), whereas JA is primarily induced as defense against leaf-chewing

insects and necrotrophic pathogens (Liu et al., 2016). The suppression of SA- and/or JA-mediated responses in the phytoplasma-infected plants may facilitate feeding by herbivorous insects. In addition, phytoplasma-encoded effector proteins can increase volatiles that attract the insect vectors (Orlovskis & Hogenhout, 2016). Thus, modification of host plant phytochemical processes (such as nutrient translocation, chemical defense production, and volatile production) not only affects the host plant growth and development but also influences the host plant-insect vector interaction.

A phytoplasma in the subgroup 16SrIII-Y causes cranberry false blossom disease (CFBD) in the American cranberry (*Vaccinium macrocarpon* Aiton) (Lee et al., 2014, Polashock et al., 2017). CFBD relies specifically on the blunt-nosed leafhopper (*Limotettix vaccinii* Van Duzee) to vector the phytoplasma that causes disease (Beckwith & Hutton, 1929). CFBD-affected plants produce bunched upright shoots forming a witches' broom, leaves turn reddish earlier than uninfected plants in the fall (De Lange & Rodriguez-Saona, 2015), and flowers are malformed, exhibiting enlarged calyx and shortened, discolored, and streaked petals (Dobroscky, 1931); affected flowers fail to set fruit. Between 1920 and 1940, this disease was a major threat to cranberry production and almost wiped out the industry in New Jersey, USA. However, CFBD was subsequently controlled with the use of insecticides, removal of infected plants from the fields, and the release of improved cultivars that are less attractive to the insect vector (Chandler et al., 1947). After several years, disease incidence declined, and the disease was only sporadically reported. Recently, the disease has reappeared on many cranberry farms with increasing incidence being reported (Lee et al., 2014).

As a first step to broaden our understanding of the physiological, biochemical, and molecular mechanisms underlying CFBD, we performed transcriptome analysis to determine the phytoplasma-induced alterations in gene expression in infected

cranberry. This information will help to further elucidate how changes in gene expression, induced in the host, contribute to vector attraction, potentially promoting transmission of this important reemerging disease.

MATERIALS AND METHODS

Plant materials

Healthy (uninfected) runners (*V. macrocarpon* cv. Crimson Queen) were kindly provided by Integrity Propagation (Chatsworth, NJ, USA), while phytoplasma-infected plants of the same cultivar were collected from a commercial cranberry farm in Chatsworth, NJ. The runners were obtained in November 2016 and 2017 and stored at 10 °C until used for propagation. The plants collected in November 2016 were clonally propagated in February 2017 and used for the transcriptional profiling study, whereas the plants collected in 2017 were propagated in February 2018 and were used for the real-time quantitative PCR (RT-qPCR) analysis of key selected genes.

For propagation, stem cuttings (~7 cm) were rooted in 50:50 v/v peat:sand mix in 4 × 4-cm cells. All cuttings were placed in a greenhouse (20 ± 2 °C; 70 ± 10% relative humidity; 15:9 light:dark) and fertilized every three weeks, with PRO-SOL 20-20-20 N-P-K All Purpose Plant Food (Pro Sol Inc., Ozark, AL, USA) at a rate of 165 ppm N, and watered daily. After rooting, plants of each type (infected and uninfected) were transplanted into 7 × 7-cm pots. To ensure a sufficient number of uniform plants for subsequent experiments, five rooted cuttings were transplanted per pot. Plants were grown in the greenhouse under ambient conditions until they were used in experiments in June.

Prior to experiments, 10 plants (5 plants from infected and uninfected plants) from 10 randomly selected pots were DNA fingerprinted using sequence characterized

amplified region (SCAR) markers (Polashock & Vorsa, 2002) to verify that all plants were genetically identical (cv. Crimson Queen). Another 10 plants (5 plants from infected and uninfected plants) from 10 pots were randomly selected and tested for phytoplasma infection by using a nested PCR assay (Lee et al., 2014) to verify that only infected plants were positive for the presence of phytoplasma.

Total RNA extraction

For the transcriptional profiling study, total RNA was extracted from leaves of three uninfected plants and three infected plants from six different pots. Total RNA was also extracted from five plants from each group (uninfected and infected) for RT-qPCR analysis of selected genes. Briefly, fresh cranberry leaves, approximately 50–75 mg, and two 5-mm stainless steel beads were added to 2-mL Safe-Lock tubes (Eppendorf North America, Hauppauge, NY) filled with 800 µL cetyltrimethylammonium bromide buffer. Samples were ground for 1 min at 30 Hz with a TissueLyser II instrument (Qiagen, Germantown, MD). The sample suspensions were extracted with 700 µL chloroform and then centrifuged at 11,000 *g* for 5 min. Supernatants were transferred to new tubes. For nucleic acid precipitation, 0.7 volumes of isopropanol were added to each tube, incubated on ice for 10 min, and centrifuged at 13,000 *g* for 5 min. Pellets were washed in 500 µL of 70% ethanol and centrifuged at maximum speed for 1 min and then all remaining ethanol was removed from the tubes by using a micropipette. Remaining pellets were resuspended in 400 µL of RNase-free water, and the RNA was precipitated by the addition of 100 µL of 10 M lithium chloride and an overnight incubation on ice. After the overnight incubation, the samples were centrifuged at 13,000 *g* for 5 min. The pellets were resuspended in 400 µL RNase-free water. Forty microliters of 3 M sodium acetate and 880 µL of 100% ethanol were added for RNA

reprecipitation. After a 10-min incubation on ice, the samples were centrifuged at 13,000 *g* for 10 min. Pellets were washed with 70% ethanol and resuspended in 50 µL RNase-free water. The RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) with absorbance ratios of A260/280 and A260/230 nm.

cDNA library construction

RNA was sent to the Novogene Corporation (Sacramento, CA) for library construction and sequencing. Three independent cDNA libraries were constructed for each treatment (uninfected and infected). Prior to library construction, all samples were tested for RNA integrity and determined to be of sufficient quality, as determined by Novogene by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After passing the quality-control procedures, mRNA was enriched using oligo(dT) beads. The mRNA was randomly fragmented, and the first-strand cDNA was synthesized using random hexamers and reverse transcriptase. Then, the second strand was synthesized with a custom Illumina second-strand synthesis buffer (Illumina Inc, San Diego, CA) with DNA polymerase I, dNTPs, and RNase H. The double-stranded cDNA fragments were purified, end repaired, poly-A tailed, adapter ligated, size selected, and PCR enriched. Library concentration was quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA), then diluted to 1 ng/µL before checking insert size on an Agilent 2100, and quantified to greater accuracy by quantitative PCR. Libraries were sequenced using the Illumina HiSeq platform. Clean reads were obtained for sequence assembly after removing adaptors and reads of poor quality. Transcriptome de novo assembly was carried out with Trinity (Grabherr et al., 2011). The assembled transcriptome was annotated using BLAST, applied by Novogene

(Sacramento, CA, USA), using seven databases, namely, NCBI nonredundant protein sequences, NCBI nucleotide sequences, Protein family, Eukaryotic Orthologous Groups/Cluster of Orthologous Groups of protein, SwissProt, Kyoto Encyclopedia of Genes and Genome (KEGG), and Gene Ontology (GO). The evaluation threshold was 1e-5 for NCBI nonredundant protein sequences, NCBI nucleotide sequences, and SwissProt databases; 1e-3 for Eukaryotic Orthologous Groups; 0.01 for Protein family; 1e-6 for GO; and 1e-10 for KEGG. Corset (Davidson & Oshlack, 2014) was used for categorical clustering of de novo-assembled contigs, while individual reads were aligned with RSEM (Li & Dewey, 2011). Differentially expressed genes (DEGs) between uninfected and infected cranberry plants were identified using DESeq (Anders & Huber, 2010).

Gene expression

In 2018, five plants from each treatment (uninfected and infected) were selected for RT-qPCR. Total RNA was extracted from each sample by using the method described above. The total RNA was treated with Optizyme DNaseI (Fisher Scientific, Hampton, New Hampshire, USA) to remove any residual DNA. The cDNA was synthesized using 1 µg of RNA and the Superscript Vilo cDNA synthesis kit as per the manufacturer's (Invitrogen) protocol.

Based on the transcriptome data and de novo annotation, six candidate target genes were selected for RT-qPCR. The genes and the primers for the selected genes are listed in Table 4.1. The expression of actin and RNA helicase 8 genes were used as the endogenous controls (Rodriguez-Saona et al., 2013), while the dehydrodolichyl diphosphate synthase 6 gene was used as the interplate calibrator. The primers of all

candidate genes and endogenous controls were designed using PrimerQuest (Integrated DNA Technologies Inc., Skokie, IL, USA).

RT-qPCR reactions were performed using the Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) according to manufacturer's directions. The reactions were run on a QuantStudio 5 RT-qPCR System (Applied Biosystems) with the following conditions: 50°C for 2 min; 95°C for 10 min; and 40 cycles at 95°C for 15 sec and 58°C for 1 min, with melting curve set at 95°C for 15 sec, 60°C for 1 min, and 95°C for 1 sec. There were three technical replicates for each sample. Relative expression levels were calculated using the ΔΔCT method using the QuantStudio Design & Analysis Software version 1.4.3 (Applied Biosystems). The relative expression data were tested for normality and for homogeneity of variance to check the parametric assumption requirement. If needed, data were $\log_{10}$ transformed before using analysis of variance (ANOVA); otherwise, nonparametric tests such as the Mann-Whitney U test were used. All parametric and nonparametric tests were performed using IBM SPSS Statistics package version 24.

Table 4.1 Target genes and primer sequence for real-time qPCR

Target gene (Aberration)	Primer	
	F Primer	R Primer
Fructokinase-6	CTTGTGGTTTGCTTTGGTGAG	ACCAAGACGAGCAATACCAAC
Calcium-binding protein (CML)	TGAAGCTCCTCGCATGAAATA	CAAAGAAGGCGAGGAAAGTAATG
Phenylalanine ammonia-lyase (PAL)	TGGCTTCTATGGTCCTTTTCG	GGATGGTGCTTGAGTTTGTG
AP2-like factor (AP2)	GCAGTGGGAGTGATCAGATG	GAGATTCCCATCGACCAGTTC
Wheat blue dwarf phytoplasma plasmid (pWBD2)	TGTAGGAGAATTTATATTAGGAGCAGG	CCCGCTATTGCTCCAATTTTC
Unknown, Possible phytoplasma protein (Unknown Phyt)	GCAAGAACGCTTTCTGAACTAAA	CCTGCCTTATGAGGATACAACTC

RESULTS

Cranberry identity and false blossom phytoplasma detection

All cranberry plants were of the same cultivar (Crimson Queen), as determined by SCAR markers (data not shown). The false blossom-infected plants used for these experiments tested positive for phytoplasma by using the nested PCR assay described above. Uninfected plants were all negative (data not shown).

Transcriptome assembly and annotation

An average of 58,605,833 reads were generated for uninfected plants, while infected plants averaged 59,361,545 reads. The raw reads were filtered to remove reads of low quality, leaving an average of 55,074,020 (Q20: 97.7%, GC content: 46.08%) and 55,502,498 clean reads (Q20: 97.82%, GC content: 45.88%) for the uninfected and infected transcriptomes, respectively. The clean reads were de novo assembled using Trinity, resulting in 131,404 total unigenes with a size distribution consisting of 32,050 unigenes of 200–500 bp, 36,313 unigenes of 500–1000 bp, 34,553 unigenes of 1000–2000 bp, and 28,488 unigenes of ≥2000 bp. The average length of unigenes was 1,346 bp with an N_{50} of 2,024 bp. Contigs were BLASTed against seven databases, applied by Novogene, to achieve comprehensive gene functional annotation. Of the 131,404 unigenes, 63.59% (83,564 unigenes) were able to be annotated using at least one database.

Of the total unigenes, 43.83% (57,602 unigenes) were annotated with Blast2GO version 2.5 (Götz et al., 2008), based on the protein annotation results of NCBI nonredundant protein sequences and Protein family databases. After annotation, unigenes were grouped into three main GO domains namely, Biological Process, Cellular Component, and Molecular Function (Fig 4.1). The predominant GO terms

were cellular process, metabolic process, binding, catalytic activity, and single-organism process.

A large proportion of genes in the GO category biological process were associated with “cellular process,” “metabolic process,” and “single-organism metabolic process.” In the molecular function category, “catalytic activity,” “binding,” and “transporter activity” were of the highest proportion. Of the genes categorized as cellular components, “cell part,” “membrane part,” and “organelle part” were the most enriched terms (Fig 4.1).

For Eukaryotic Orthologous Groups (KOG) classification, 26,863 unigenes were annotated using the NCBI gene orthologous relationships. Of the KOG categories “general function prediction only” is the largest category in which most genes were categorized, followed by “post-translational modification, protein turnover, and chaperones” and the group “translation, ribosomal structure, and biogenesis.”

The 30,744 unigenes were annotated through the KEGG Orthology database and were placed into 129 KEGG pathways. The most represented pathways were “carbohydrate metabolism,” “translation,” and “folding, sorting and degradation.”

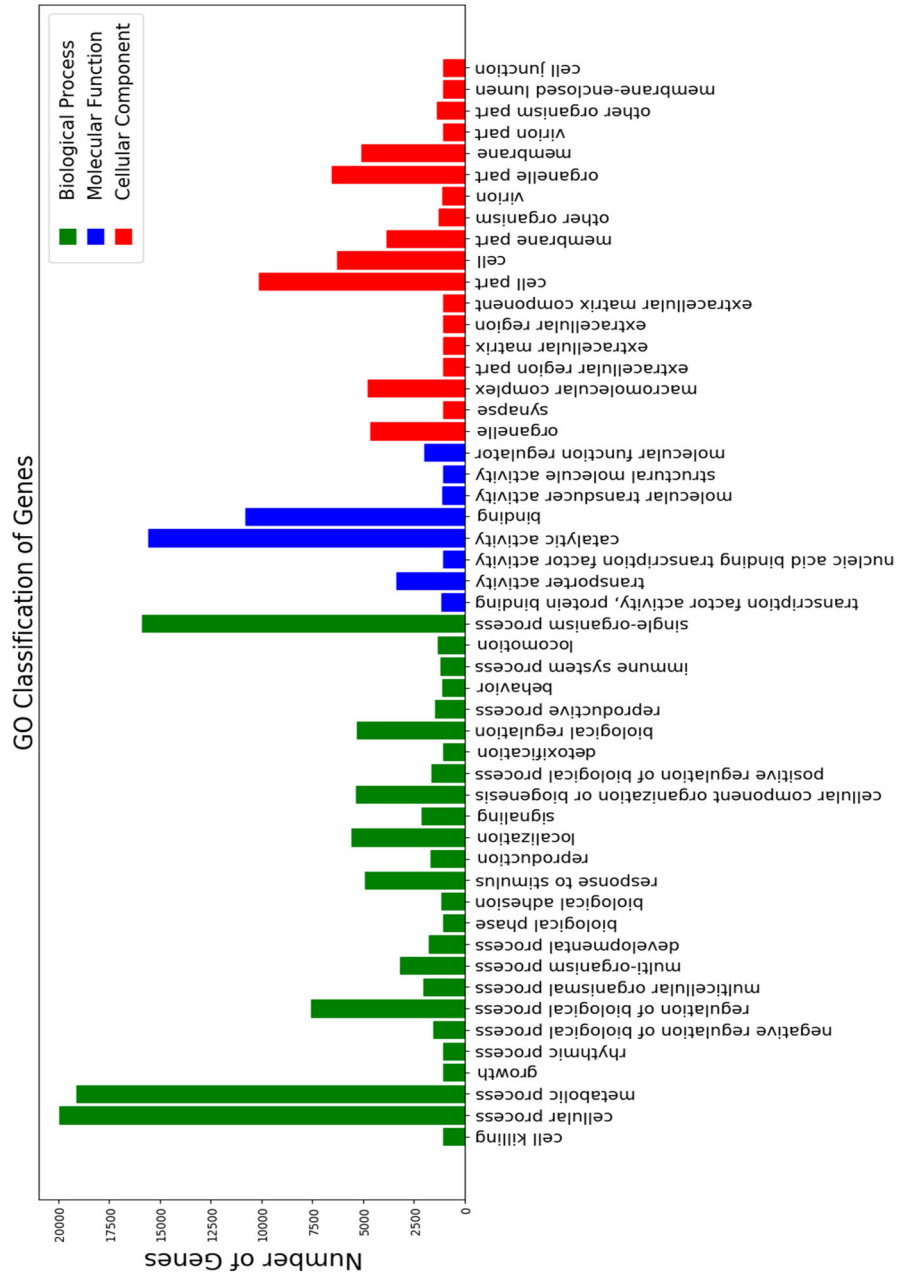

Figure 4.1. GO Classification of Genes. Total counts of GO terms associated with all annotated unigenes in 3 categories; Biological Process (Green), Molecular Function (Blue), and Cellular Component (Red).

Differential expression of genes

Differential expression analysis between uninfected and infected plants showed 356 DEGs. Infected plants showed an upregulation of 131 genes and downregulation of 225 genes, as compared with uninfected plants. Two genes expressed only in infected plants appear to be derived from the phytoplasma, as determined by BLAST. As noted in Table 1, one was similar to wheat blue dwarf phytoplasma plasmid (pWBD2), suggesting that the phytoplasma that caused CBFD in this experiment might harbour a plasmid. The other gene was identified as encoding an unknown phytoplasma protein.

The DEGs mapped in the KEGG database showed that the pathways most affected (q-value, ≤0.1) by CFBD were "cutin, suberin, wax biosynthesis," "carotenoid biosynthesis," and "fatty acid elongation" (Fig 4.2). Specifically, the genes related to cutin, suberin, wax biosynthesis, fatty acid omega-hydrolase gene (CYP86A1), and alcohol-forming fatty acyl-CoA reductase gene (FAR) were downregulated. The carotenoid biosynthesis pathway showed two different forms (sequences) of the (+) -abscisic acid 8'-hydroxylase, one of which was upregulated while the other was downregulated. For fatty acid elongation, the very-long-chain (3R)-3-hydroxyacyl-CoA dehydrogenase gene (PAS2) was downregulated, as well as the 3-ketoacyl-CoA synthase gene.

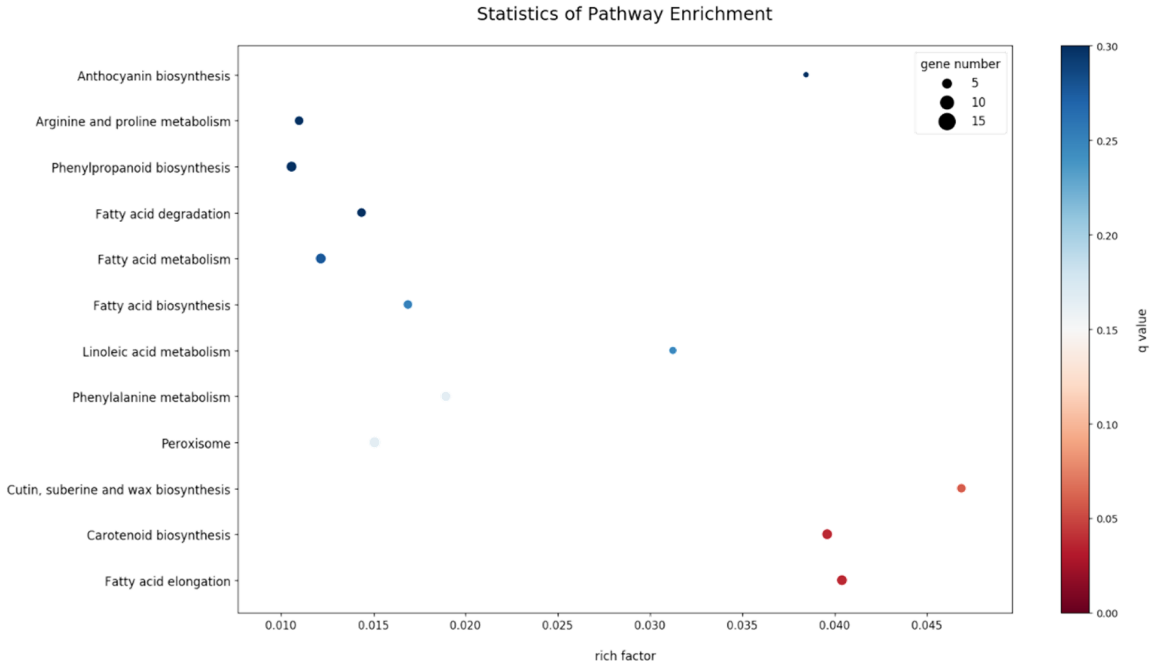

Figure 4.2. KEGG Enrichment Scatter Plot of the 15 most enriched pathways. The 15 most significantly DEG-enriched pathways (padj < 0.05) are displayed on the y-axis and their associated level of enrichment is displayed along the x-axis. The degree of KEGG enrichment is measured by rich factor; described as the ratio of the number of DEGs in the pathway compared to the total number of genes found in the pathway. The greater the rich factor, the greater the degree of gene enrichment. The q-value is the p-value adjusted to sample distribution (also referred to as padj) and indicates significance of pathway enrichment. Smaller q-values indicate more significant levels of enrichment. Dot size represents the number of different genes and the color indicates the q-value.

1. Defense-related genes

Plant defenses, in part, rely on changes in gene expression in a myriad of biological pathways. The list of genes potentially involved in defense can include those that relate to molecular signaling, physical defenses (such as cell wall strengthening), and chemical defense (secondary metabolite biosynthesis). In this study, many of the genes related to plant defense were downregulated in phytoplasma-infected plants relative to uninfected plants (Fig 4.3). For example, two members of the ATP-binding cassette transporter (ABC) family (ABCC and ABCG) and a predicted endochitinase A were expressed only in uninfected plants (i.e., they were downregulated in infected plants). In contrast, some genes such as those related to mismatch repair (MSH1 and RAD7), a gene in the glycosyl hydrolase family, and a GTP pyrophosphokinase were expressed primarily in infected plants.

Molecular signaling genes related to plant defense include calcium-binding protein calmodulin-like protein 44 (CML), ABC in various families and members, and cysteinyl-tRNA synthase. CML is upregulated in phytoplasma-infected plants relative to uninfected plants, whereas those in the ABC family show downregulation in phytoplasma-infected plants. Cysteinyl-tRNA synthase is slightly downregulated in infected plants. Genes related to cell wall and wax synthesis include xyloglucosyl transferase, beta-D-xylosidase7, and 3-ketoacyl-CoA synthase. These genes were all downregulated in infected plants. The genes associated with plant secondary compound biosynthesis include enzymes such as anthocyanidin 3-O-glucoside 5-O-glucosyltransferase, UDP-glucosyl transferase 73C, phenylalanine ammonia-lyase (PAL), ent-copalyl diphosphate synthase, and peroxidase. Anthocyanidin 3-O-glucoside 5-O-glucosyltransferase and UDP-glucosyltransferase 73C were slightly upregulated in phytoplasma-infected plants relative to uninfected plants, whereas PAL,

ent-copalyl diphosphate synthase, and peroxidase were slightly downregulated in infected plants.

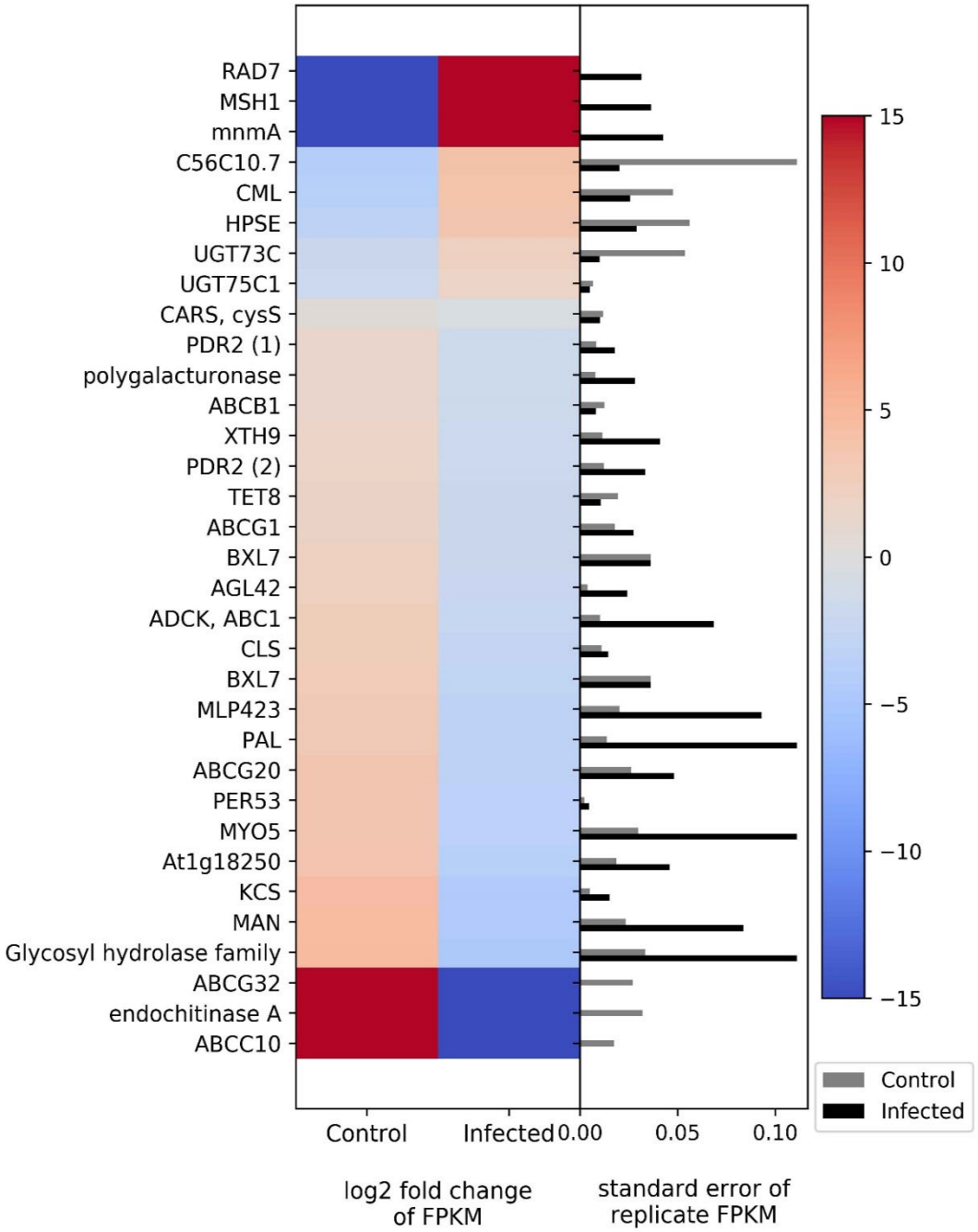

Figure 4.3. Heatmap of DEGs related to defense. The left side of the plot is the heatmap of log2 fold change of FPKM between all infected and uninfected groups. The right side of the plot is the standard error for all replicates divided by the average FPKM for the uninfected and infected groups.

2. Photosynthesis and carbohydrate metabolism

The transcriptome results show differential expression of two genes involved in photosynthesis; cytochrome b599 was upregulated in infected plants, whereas photosystem II reaction center I protein was downregulated (Fig 4.4). Genes related to carbohydrate metabolism were also differentially regulated (Fig 4.4). The genes that show upregulation in infected plants include fructokinase, raffinose synthase, and malate dehydrogenase, whereas the genes that show downregulation relative to uninfected plants include polygalacturonase, xyloglucosyl transferase, laccase, galacturonosyltransferase, mannan endo-1,4-beta-mannosidase, and glycosyltransferase.

3. Flowering and development

The genes AP2-like factor (AP2) and (+)-abscisic acid 8'-hydroxylase (E1.14.13.93) are related to the plant hormones ethylene and abscisic acid, respectively. Both genes show upregulation in infected plants (Fig 4.5). Other genes related to flowering and development, such as FAR, suppressor of overexpression of CONSTANS 1, circadian clock coupling factor ZGT, phytochrome A, and transcription factor TGA, show downregulation (Fig 4.5).

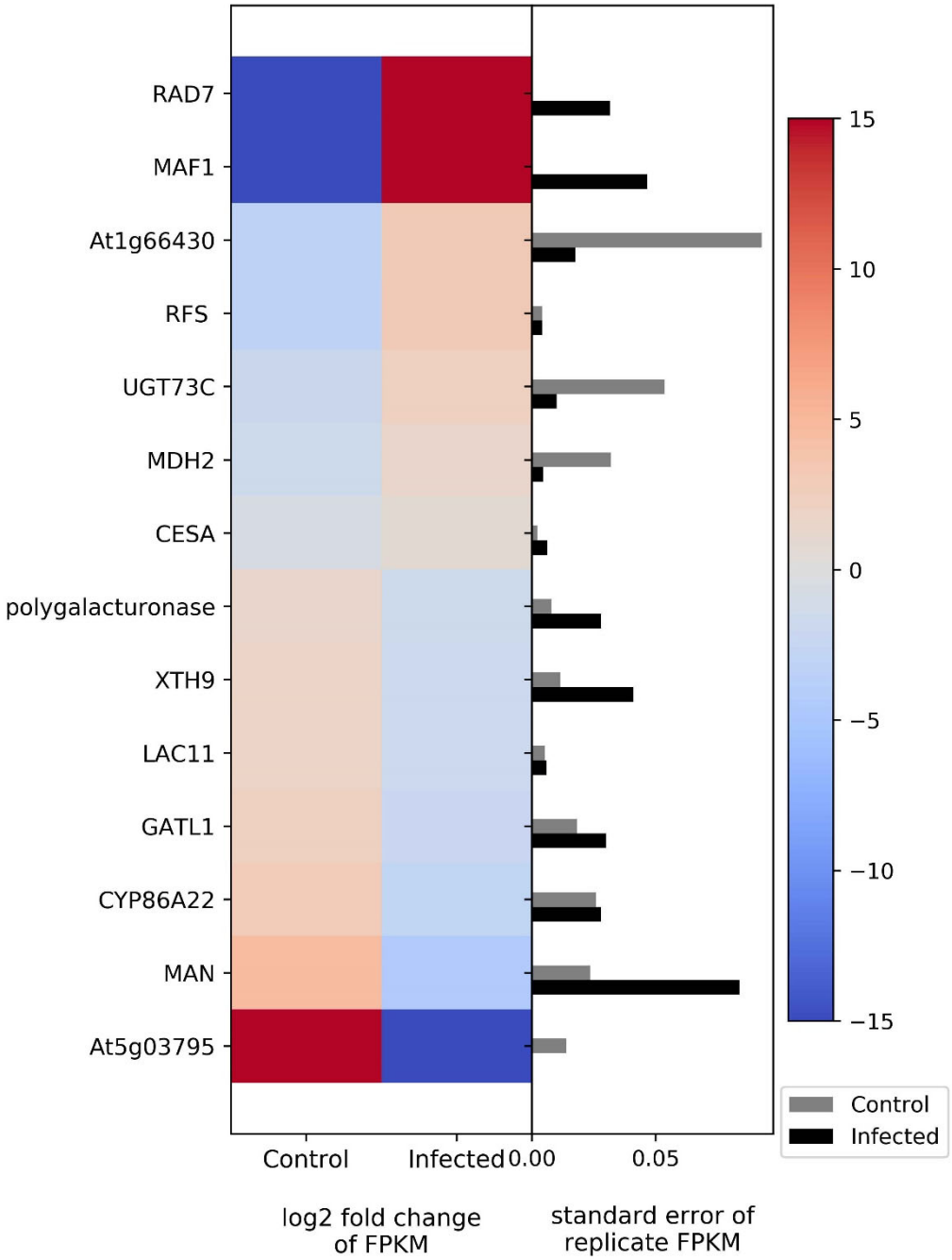

Figure 4.4. Heatmap of DEGs related to photosynthesis and carbohydrate metabolism. The left side of the plot is the heatmap of log2 fold change of FPKM between all infected and uninfected groups. The right side of the plot is the standard error for all replicates divided by the average FPKM for the uninfected and infected groups.

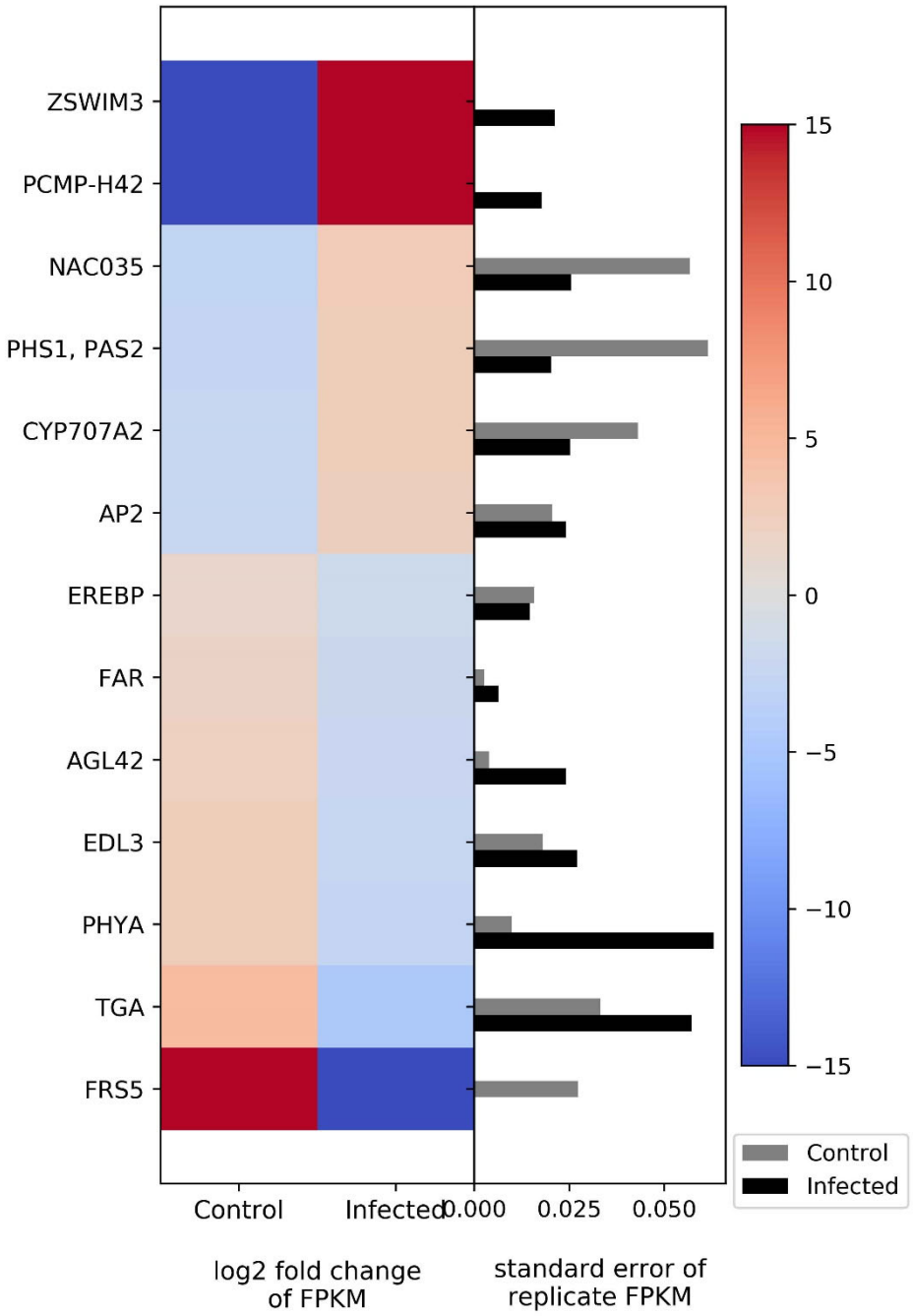

Figure 4.5. Heatmap of DEGs related to flowering and development. The left side of the plot is the heatmap of log2 fold change of FPKM between all infected and uninfected groups. The right side of the plot is the standard error for all replicates divided by the average FPKM for the uninfected and infected groups.

Expression of selected target genes (RT-qPCR)

The target genes (Table 4.1) were selected to verify differences in the expression of phytoplasma-infected plants, relative to uninfected plants, by RT-qPCR. The genes were selected to be representative of the three functional groups described above (defense, photosynthesis and carbohydrate metabolism, and flowering and development). The genes selected were fructokinase-6 (FX), CML, PAL, and AP2. The expression of FX, which is related to carbohydrate metabolism, was significantly ($p \leq 0.01$) increased in infected plants (Fig 4.6). The CML-encoding gene, which is involved in defense signaling, was significantly ($p \leq 0.05$) upregulated in infected plants. The expression of the PAL-encoding gene, which is involved in the early stages of secondary chemical defense synthesis, was lower in the infected plants in the transcriptome data (Fig. 4.3), but the difference in expression by RT-qPCR was not significant ($p = 0.897$) (Fig 4.6). The expression of the AP2-encoding gene, which is involved in floral meristem development, was significantly ($p \leq 0.01$) increased in infected plants (Fig 4.6).

We also selected two genes that appear to be encoded by the phytoplasma. One has similarity to wheat blue dwarf phytoplasma plasmid (pWBD2), and the other (Unknown Phyt) encodes a protein with similarity to "hypothetical protein CPX 001471" (from *Candidatus Phytoplasma pruni*, accession number KOR75512.1) and "hypothetical protein" from *Vaccinium* witches' broom phytoplasma (accession number WP 017193546.1). These two genes were shown in the transcriptome data to be expressed only in false blossom-infected plants, and RT-qPCR data confirmed their expression only in infected plants (data not shown). Either one or both of these genes may encode an effector and this possibility should be further explored.

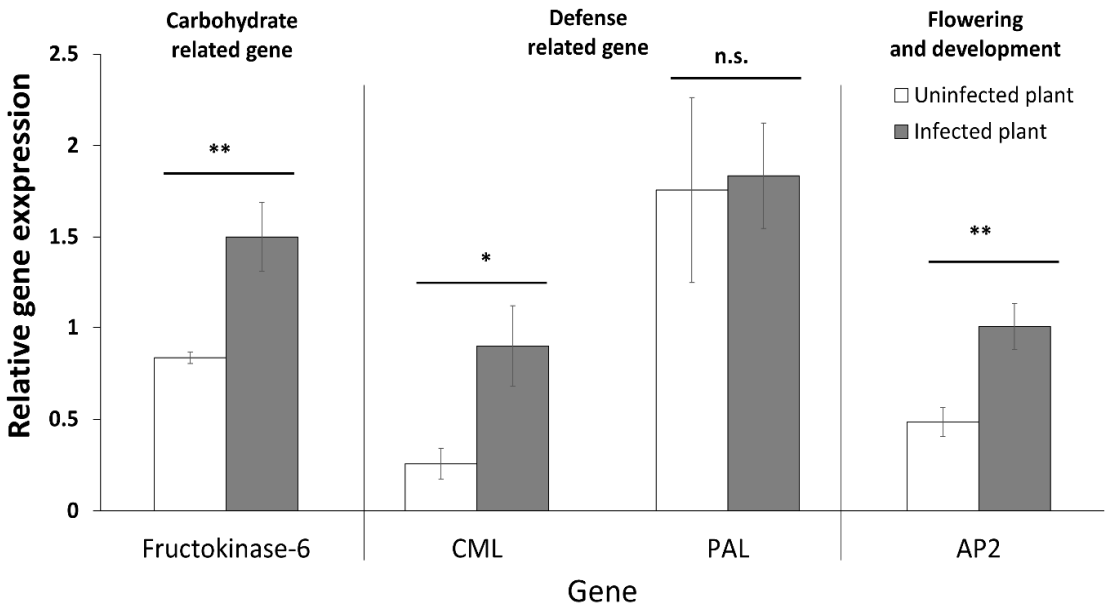

Figure 4.6. Relative gene expression (Mean, ±SE) of selected genes. Asterisk indicates statistically significant differences (* = $p \leq 0.05$, ** = $p \leq 0.01$). n.s. = not significant ($p > 0.05$).

DISCUSSION

Cranberry false blossom disease (CFBD), caused by a phytoplasma, is a serious threat to the cranberry industry. This disease causes physiological and biochemical changes in cranberry that influence the host-vector (blunt-nosed leafhopper) interaction. Here, we show the changes in the cranberry transcriptome associated with CFBD. Surprisingly, of the more than 131,000 unigenes in the transcriptome, the expression of only 356 genes were influenced by phytoplasma infection. Phytoplasma infection induced the expression of 132 genes and suppressed the expression of 225 genes, relative to the uninfected, healthy plants. Other studies found phytoplasma infection influences the expression of more than 2,000 genes in grapevine (1,326 upregulated genes and 1,130 downregulated genes), Mexican lime tree (1,943 upregulated genes and 862 downregulated genes), and Chinese jujube (2,070 upregulated and 2,196 downregulated genes) (Fan et al., 2017).

The stage of infection by phytoplasma, as well as the plant tissue collected and timing of collection of those tissues, can influence the differences in quality and quantity of gene expression detected. In Jujube witches' broom, the stage wherein the plant showed weak symptoms had the greatest number of DEGs, whereas the primary and late stage of phytoplasma infection had a fewer number of DEGs (Wang et al., 2018). In this study, the plants were around 4 months old when the leaf tissue was collected for the experiments. At the time tissue was collected, the infected plants were smaller in size and bushy (shorter internodes) relative to the uninfected plants. The plants were considered to be young and, therefore, at the primary stage of symptom development. In the field, severe symptom development (witches' broom) occurs in plants that have been infected for many years. Moreover, the titer of the bacteria in the plant can also play a role in the gene expression. Early in the growing season, jujube

trees with Jujube witches' broom have high bacterial density in the root but very low in the petiole, whereas when they are actively growing during the season, a high density of phytoplasma is found in the petiole (Yi et al., 2001). Here, we considered our plants to be early in the growing season, and this may be one reason why the number of DEGs we detected seems relatively low. Furthermore, the most dramatic morphological change in cranberry with CFBD is the formation of deformed flowers. Thus, the transcriptome of developing flower tissues is likely to exhibit a high number of DEGs. It is also possible that the DEGs detected here might differ in number and/or the magnitude of differential expression in other cranberry cultivars.

We considered three general groups when examining the DEGs, namely, defense related, photosynthesis and carbohydrate metabolism related, and flowering and development related, as changes in these groups are likely to affect plant-vector interactions and possibly disease spread .

1. Defense-related genes

The defense mechanisms of plants can be separated into physical and chemical defenses (Bennett & Wallsgrove, 1994; Will & van Bel, 2006). The physical defenses induce changes in the physical structure of the plant, whereas the chemical defenses range from changing phytohormone signaling, such as JA or SA, to secondary plant chemical production and accumulation (Freeman & Beattie, 2008).

The first physical barrier for the plant pathogen is the plant cell wall, a rigid cellulose-based support. Cell walls are important for maintaining cell structure, intercellular communication (Clarke et al., 1985), and defense against pathogens (Malinovsky et al., 2014; Underwood, 2012). High-molecular-weight polysaccharides, including cellulose, hemicellulose (typically xyloglucan or xylan), and pectin, are the main components of plant cell walls. Phytoplasma infection results in the structural

modification of host plants, such as cell wall enhancement (Rudzińska-Langwald & Kamińska, 2001). The genes that are related to cell wall modification in this study, such as xyloglucan:xyloglucosyl transferase and beta-D-xylosidase 7, tended to be suppressed in the infected plant (Fig 4.3). Xyloglucan is a structural polysaccharide of the primary cell wall (Levy et al., 1991). The xyloglucan:xyloglucosyl transferase, also known as xyloglucan endotransglycosylase/hydrolase, is an enzyme involved in cell wall elongation and reconstruction. Increased xyloglucan endotransglycosylase/ hydrolase activity at the infection site and adjacent tissue were observed in tomato when parasitized by dodder (Albert et al., 2004). The beta-D-xylosidase releases xylose from xylan-containing oligosaccharides (Rahman et al., 2003) and is potentially involved in secondary cell wall hemicellulose metabolism. The suppression of this gene in plants can facilitate the invasion of disease into the cell of the host plant (Minic, 2007; Lippmann et al., 2009).

Phytoplasmas can be detected in most organs of phytoplasma-infected plants. These bacteria colonize the sieve tubes of phloem and seem to be in close contact with the plasma membrane (Marcone, 2009). Membrane proteins of the phytoplasma are considered a factor that plays a role in colonization and infection of the host cell and triggers changes in the chemical metabolism of the host plant (Malembic-Maher et al., 2005). Moreover, phytoplasmas express effector proteins, specific molecules that alter the host, to facilitate successful invasion of the plant (Sugio et al., 2011). Our transcriptome and RT-qPCR results show expression of at least one gene that may be on a plasmid of the pathogen and one “hypothetical protein” that may encode effectors.

Genes involved in signal transduction pathways, such as CML and ABC in various families and members, can be important in sensing and responding to phytoplasma infection. CML is an important Ca^{2+} sensor, which plays a significant role

in plant tolerance to several biotic and abiotic stress (Ali et al., 2003). Specifically, calcium/calmodulin-mediated regulation plays a role in plant phytohormones, including SA and glucosinolate (Cheval et al., 2013). In this study, phytoplasma infection increased CML expression (log_2Fold change = 3.768, Fig 4.3), suggesting an induction of phytohormone-mediated signaling.

Functional ABC transporters are located in cell membranes and act as ATP-driven pumps. ABC transporters move various chemical substrates, such as lipids, phytohormones, and heavy metals, through the plasma membrane (Kretzschmar et al., 2011). ABC transporters are known to respond to abiotic and biotic stresses and play important roles in detoxification (Lu et al., 1998), hormone transport (Geisler et al., 2005; Kuromori et al., 2010), and chemical defense (Badri et al., 2009). In rice, nearly half of the ABCG members have a positive response to JA and SA, the phytohormones associated with herbivore and pathogen defense (Moons, 2008). NpPDR1, an ABCG protein of Tex-Mex tobacco, was implicated in pathogen defense and was shown to be involved in active terpenoid transport in plants (Jasiński et al., 2001). Both JA and SA promote NpPDR1 expression, supporting the association with defense signaling pathways (Jasiński et al., 2001). In this study, the genes encoding ABC transporters tended to be downregulated in phytoplasma-infected plants, whereas in coconut palm, phytoplasma infection caused upregulation of ABC transporter genes (Nejat et al., 2015). It is possible that the dampening of plant defenses by some phytoplasmas, such as the cause of CFBD, suppresses ABC transporter expression.

In this study, we hypothesized that phytoplasma infection would influence the expression of genes related to phytohormone signaling because ethylene, JA, and SA play a central role in the regulation of defense responses. SA is generally stimulated by pathogens (Thomma et al., 1998) and feeding by piercing-sucking insects (War et al.,

2012). In this study, genes directly related to phytohormones, such as JA, SA, and cytokinin, were not influenced by phytoplasma infection. These results are consistent with our measurement of the phytohormone levels in CFBD-infected plants vs. uninfected plants that show no significant differences (Chapter 2). The effect of phytoplasma infection on the expression of genes related to JA and SA signaling in plants is quite variable. Some studies show upregulation in both hormones (Mardi et al., 2015; Mou et al., 2013; Wang et al., 2018), whereas others show upregulation in SA and downregulation in JA (Musetti et al., 2013; Sugio et al., 2011), or vice versa (i.e., suppression of SA and induction of JA; (Lu et al., 2014).

Other hormones, such as auxins, abscisic acid (ABA), and gibberellin (GA), play roles in plant development and growth regulation. ABA not only regulates the growth and developmental processes in plants but also acts as in adaptive responses to environmental stresses (Saito et al., 2004). GAs are plant hormones that regulate various developmental processes, including stem elongation, flowering, flower development, and leaf and fruit senescence. Recently, ABA and GA were reported as a key regulators of plant immunity (Denancé et al., 2013). In this study, we found two forms (sequences) of the gene that encodes a (+)-abscisic acid 8'-hydroxylase that is involved in ABA synthesis. One form is upregulated, while the other was down regulated (Table 4.2). Lower ABA levels were shown in phytoplasma-infected *Euphorbia coerulescens* and *Orbea gigantea* plants (Omar et al., 2014). In the Jujube plant, the genes related to ABA synthesis were also downregulated (Wang et al., 2018). It has been suggested that ABA is involved in the growth of axillary buds due to the loss of apical dominance (Dewir et al., 2015). This may be a factor in the excessive branching (witches' broom formation) induced by some phytoplasmas, including the causal agent of CFBD.

Table 4.2 Biosynthetic pathway, gene cluster, log_2Fold change, KO ID and the putative encoded proteins of selected genes of interest.

Biosynthetic pathway and gene cluster [a]	log_2 Fold Change [b]	KO ID [c]	Putative encoded protein [d]
ABA			
Cluster-15360.41209	Inf	K09843	(+)-abscisic acid 8'-hydroxylase
Cluster-15360.41206	2.6362	K09843	(+)-abscisic acid 8'-hydroxylase
Cluster-15360.4715	-2.9878	K09843	(+)-abscisic acid 8'-hydroxylase
Cluster-15360.4714	-Inf	K09843	(+)-abscisic acid 8'-hydroxylase
4CL and PAL			
Cluster-15360.33157	-1.6746	K01904	4-coumarate--CoA ligase
Cluster-15360.41311	-3.0314	K10775	phenylalanine ammonia-lyase
Amino acid			
Cluster-15360.40394	5.3782	K13427	nitric-oxide synthase, plant
Cluster-15360.37384	5.7445	K01755	argininosuccinate lyase
Cluster-15360.15514	-3.146	K00108	choline dehydrogenase
Cluster-15360.35040	-2.102	K00827	alanine-glyoxylate transaminase / (R)-3-amino-2-methylpropionate-pyruvate transaminase
Cluster-15360.55792	-Inf	K00600	glycine hydroxymethyltransferase
Cluster-15360.56873	-Inf	K13035	beta-cyano-L-alanine hydratase/nitrilase

[a] Biosynthetic pathway (bold) and gene cluster is the gene ID as determined using Corset.

[b] log_2 Fold Change is the log_2 ratio of genes' or transcript' expressed values between infected plants and healthy plants. ('positive value' indicates the values of expressed on infected plant is higher than healthy plant, 'negative value' indicates the values of expressed on infected plant is lower than healthy plant, 'Inf" indicates the expressed values of healthy plant is 0, '-Inf' indicates the expressed values of infected plant is 0)

[c] KO ID is the identifiers, or the number assigned to individual genes in the genome annotation in KEGG (Kyoto Encyclopedia of Genes and Genome) Orthology.

The ent-copalyl diphosphate synthase gene, which is involved in the GA biosynthetic pathway (Prisic, Xu, Wilderman, & Peters, 2004), was downregulated in infected plants. In coconut palm and tomato plant, the overexpression of GA 2 oxidase, an enzyme in the GA catabolic pathway, resulted in lower GA levels in infected plants (Ding et al., 2013; Nejat et al., 2015). The reduction of GA levels might be associated with the formation of abnormal flowers in cranberry with CFBD and other plants in which deformed flowers are a symptom of phytoplasma infection.

Plant secondary compounds, such as flavonoids, lignin, alkaloids, and terpenoids, play important roles in the defense mechanisms of plants. During the infection, plants commonly use phenylpropanoid compounds to synthesize flavonoids, lignin, and phytoalexins. The 4-coumarate-CoA ligase and PAL are key enzymes in this metabolic pathway (Dixon et al., 2002). In our study, 4-coumarate-CoA ligase and PAL are downregulated (Fig. 4.3 and Table 4.2) in infected plants relative to uninfected plants. The downregulation of these genes can result in lower levels of phenolic compounds in infected cranberry plants. In fact, Pradit et al. (2019) reported lower levels of proanthocyanidins, a class of polyphenols, in CFBD-infected than uninfected plants. In contrast, phytoplasma infection in apple and corn plants causes an increase in phenolic compounds and overproduction of hydrogen peroxide (Junqueira et al., 2004; Musetti et al., 2004).

2. Photosynthesis- and carbohydrate-related genes

Only two genes involved in photosynthesis, cytochrome b599, an important component of photosystem II, and photosystem II reaction center I protein, were highly influenced by phytoplasma infection in cranberry. Phytoplasma infection causes an inhibition of the entire electron transport chain in photosystem II in grape leaves (Bertamini & Nedunchezhian, 2001), significantly depresses photosystem I activity in

Paulownia leaves (Mou et al., 2013), and downregulates the genes associated with the electron transport chain, such as ferredoxin, photosystem I, and photosystem II, in jujube (Wang et al., 2018). The grana and stroma lamellae, structures of chloroplasts, were destroyed, and original lamellae were formed in the infected jujube leaves (Xue et al., 2018). Our results show the suppression of photosystem II reaction center I protein gene expression, while the gene for cytochrome b599 showed induction, so the effects of CFBD on photosynthesis are unclear. The stage of infection is important in the expression levels of photosynthetic- and chlorophyll-related genes (Wang et al., 2018). Chlorophyll degradation in the later infection stages contributes to the yellowing and/or reddening symptoms of phytoplasma infection.

Photosynthetic products are first used for sucrose synthesis, and a complex enzyme system is used in the carbohydrate metabolism in plants (Hassid & Putman, 1950; Stein & Granot, 2018; Xue et al., 2018). Phytoplasma infection results in an accumulation of sugar in *Catharanthus roseus* (Lepka et al., 1999) and in coconut palm (Maust et al., 2003), whereas sugar content was reduced in maize (Junqueira et al., 2004). In jujube, sugar metabolism was downregulated at the first infection stage but then upregulated in the actively infecting stage (Wang et al., 2018; Xue et al., 2018). In our study, the genes associated with carbohydrate metabolism were both up- and downregulated (Fig. 4.4). Interestingly, the genes that show upregulation include fructokinase and malate dehydrogenase (MDH). Fructokinase phosphorylates fructose to form fructose 6-phosphate, which is then used for glycolysis (Stein & Granot, 2018). Fructose is the preferential energy source for *Spiroplasma citri*, a plant-pathogenic bacterium in the same taxonomic class as phytoplasmas (André et al., 2005). MDH is involved in the oxidation of malate to form oxaloacetate. This enzyme acts in the citric acid cycle and is involved in energy release from carbohydrates and other substrates.

Thus, phytoplasma infection seems to interfere with photosynthesis while increasing the metabolism of carbohydrates.

3. Flowering- and development-related genes

Cranberry plants with CBFD display abnormalities in plant growth and flower formation (De Lange & Rodriguez-Saona, 2015; Dobroscky, 1931). In this study, we expected some changes in the expression of flowering and development genes. We found that CFBD induces the expression of zinc finger SWIM domain-containing protein 3 and pentatricopeptide repeat-containing protein (PPR), while suppressing protein FAR1-related sequence (FRS). The zinc finger proteins are a super family of proteins involved in protein-protein interactions and are associated with many activities of plant growth and development, photosynthesis, and resistance mechanisms for biotic and abiotic stress (Feurtado et al., 2011; Lu et al., 2011). The pentatricopeptide repeat-containing protein can alter RNA sequences, turnover, processing. or translation. It is targeted to mitochondria or chloroplasts. This protein affects organelle biogenesis and function and, consequently, has an effect on photosynthesis, respiration, plant development, and environmental responses (Barkan & Small, 2014). FRS is the family of proteins that influence the transcription process. FRS family members play multiple roles in cellular processes, including light signal transduction, circadian rhythm and flowering, shoot meristem and floral development, plant immunity, ABA response, and chlorophyll biosynthesis (Ma & Li, 2018). Together, these proteins (zinc finger SWIM domain-containing protein 3, pentatricopeptide repeat-containing protein, and FRS) have a potentially wide range of functions in plant cells, including growth and development and response to biotic stress. Alterations in their expression patterns due to CFBD may be partly responsible for the visible symptoms (witches' broom and development of malformed flowers) of the disease.

Higher expression was noted in NAC domain-containing protein, PHS1, PAS2, and AP2. NAC is a transcription factor that acts as a floral repressor, controlling flowering time. NAC is normally expressed in response to cold temperatures (Nuruzzaman et al., 2013). PHS1 and PAS2 are synthesized by an endoplasmic reticulum-localized elongase multiprotein complex. PHS1 and PAS2 are involved in multiple biological processes, especially proliferation control of meristematic and, nonmeristematic cells and cell dedifferentiation and proliferation. PHS1 and PAS2 expression can be enhanced by cytokinins, leading to callus-like structure development of the apical part of seedlings (Bach et al., 2008; Bellec et al., 2002). AP2 or APETALA2 is a gene and a member of a large family of transcription factors that play various roles throughout the plant lifecycle. The function of AP2 is to control floral organ identity determination and to respond to biotic or abiotic stresses (Riechmann & Meyerowitz, 1998). Increasing NAC gene expression can repress flower formation, whereas PHS1, PAS2, and AP2 manipulate the organ development at the apical part of plant. Thus, this set of genes may also play a role in the bushy appearance and/or flower abnormality in CFBD-affected plants.

Phytoplasma infection caused downregulation on FARs, AGL42, EID1-like F-box protein 3, phytochrome A, and transcription factor TGA. FARs play an important role in long-chain fatty acid alcohol metabolism, which can be found in the root, seed coat, and wound-induced leaf tissue (Doan et al., 2009; Domergue et al., 2010). AGL42 interacts genetically with CONSTANS 1 and FLOWERING LOCUS T. The two interacting components (CONSTANS 1 and Flowering T) have been shown to regulate flowering in *Arabidopsis* (Yoo et al., 2005). EID1-like F-box protein 3 is an F-box protein that functions as a negative regulator in phytochrome A-specific light signaling ubiquitin ligase complexes. Phytochrome A is important in light sensing for plant

flowering (Marrocco et al., 2006). Transcription factor TGAs are implicated as regulators of pathogenesis-related genes. Transcription factor TGA proteins plays a role not only in defense against pathogens but also in processes involved in plant development (Zander et al., 2012). Thus, CFBD suppresses genes related to flowering, development, and defenses.

4. Vector interactions

Phytoplasmas require insects as vectors. For phytophagous insects, nitrogen content is an important factor in food selection (Minkenberg & Ottenheim, 1990). The DEGs show both up- and downregulation of amino acid metabolism (Table 4.2). Arginine and proline metabolism show upregulation, whereas alanine, glycine, serine, and threonine metabolism were suppressed by phytoplasma infection. Pradit et al. (2019) reported higher nitrogen content in CFBD-infected compared to uninfected plants. Thus, there is some manipulation of nitrogen metabolism in infected plants, but it is unclear how these changes in gene expression affect leaf nitrogen content and availability.

In this study, we tested the expression of selected target genes by RT-qPCR. The genes we selected include those related to defense (CML and PAL), carbohydrate metabolism (FK), and flowering (AP2). The relative gene expression data show that phytoplasma infection significantly induced the expression of CML, FK, and AP2 in the host plant, but there was no significant effect on PAL expression. This confirms that in cranberry with CFBD, defense signaling, carbohydrate metabolism, and flowering were impacted. PAL expression was lower in infected plants in the transcriptome data (Fig. 4.3), suggesting a dampening of the phenylpropanoid pathway defense response. However, the RT-qPCR data (Fig. 4.6) show PAL expression was not significantly impacted. Changes in PAL expression could be transient and ‘missed’ in the samples

used for RT-qPCR. Alternatively, changes in this pathway and its products (phenylpropanoids) may occur primarily downstream of PAL.

CONCLUSIONS

Cranberry false blossom disease was found to influence the expression of 358 unigenes (132 upregulated and 225 downregulated) in the leaves of young cranberry plants. The DEGs show that phytoplasma infection influences plant defense, photosynthesis and carbohydrate metabolism, and flowering and development of cranberry plants. Interestingly, the JA and SA phytohormone pathways are not influenced by this phytoplasma, Thus, the defense responses induced by the JA and SA signaling pathways appear to be uninduced in infected plants. Furthermore, the genes associated with secondary plant metabolism were suppressed in the phytoplasma-infected plant, while expression of carbohydrate metabolism genes was enhanced. Phytoplasma infection also caused the induction of malformed flowers and, thus, no fruit load, which may also contribute to nutrient availability and attraction of the leafhopper vector of this CFBD. Pradit et al. (2019) showed higher nutrient content, reduced defenses, and enhanced performance of phytophagous insects on plants with CFBD as compared with uninfected plants. Together, our data support the “host manipulation hypothesis” (Eigenbrode et al., 2018), wherein substantial manipulation of host plant gene expression facilitates the survival of the bacteria in the host plant and fosters dispersal of this vector-borne pathogen by depressing plant defenses while enhancing nutritional benefits to insect herbivores. It has long been known that some cranberry cultivars are less attractive to the insect vector (Wilcox and Beckwith, 1933). The next step in this research is to see how universal the changes described herein are across other cranberry cultivars, and specifically, what differences might affect vector attraction.

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CHAPTER FIVE

CONCLUSIONS

Vector manipulation by pathogens is critical for the transmission of vector-borne diseases in plants. Here, I used a phytoplasma that causes false blossom disease in cranberries, *Vaccinium macrocarpon* Ait., as a model system to study the plant-mediated effects of diseases on insect vectors and non-vectors. I showed the capacity of the phytoplasma to change the chemistry of the host plant to manipulate the insect vector, the blunt-nosed leafhopper (*Limotettix vaccinii* Van Duzee). This host-plant manipulation by phytoplasmas also had an effect (positive) on the insect community, as it improved the performance of three herbivores: spotted fireworm (*Choristoneura parallela* Robinson), Sparganothis fruitworm (*Sparganothis sulfureana* Clemens), and gypsy moth (*Lymantria dispar* L.). Further studies need to investigate the relative importance of visual versus chemical cues used by *L. vaccinii* during foraging for host plants. Additional studies are also needed to determine the long-term effects of phytoplasma infection on vector and non-vector herbivores. The role of elicitors that activate defenses against diseases and herbivores in plants should be tested to suppress phytoplasma infection in cranberries. This study is a first step into a better understanding of phytoplasma manipulation of an insect vector in cranberries and the broader implications of phytoplasma infection on the insect community. This knowledge will help in the development of sustainable management practices, such as host-plant resistance, to reduce transmission of diseases in cranberries.