GUT-CONTENT ANALYSIS OF SAP-FEEDING INSECTS

By

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri*, (ACP) is a sap-feeding insect that vectors bacterial plant pathogens in the genus *Candidatus* Liberibacter, notably associated with the incurable citrus greening disease. These pathogens disrupt the function of vital tissues responsible for transporting nutrients within the phloem plants. While feeding, ACP transmits the bacteria and ingests intracellular material, including DNA, from the plant. These insects possess valuable information within their gut contents that can be used to investigate the transmission of pathogens between host plant families and their associated insect vectors. However, validation of gut content analysis for distinguished host patterns has only been performed for a few psyllid species and has not been explored for other vectors that feed similarly. To address these knowledge gaps, we conducted DNA extractions with ACP to screen for the potential ingestion of plant DNA. PCR experiments with these extracts were designed using precise primer sets to amplify short and long conserved gene coding regions found across numerous host plant genomes. PCR amplicons sent for sanger sequencing were analyzed using bioinformatic tools and databases to confirm the presence and identity of plant DNA in the ACP extracts. To validate if this method of analysis is applicable to related vectors that feed similarly to psyllids, analogous experiments were performed using aphids cultured on various host plants. In this study, we demonstrate that both the aphids and psyllids are capable of ingesting and retaining plant DNA in their gut. The recovered genomic information can reveal potential hosts of *Candidatus* Liberibacter that ACP uses as nutrient reservoirs to migrate between viable habitats. This study and its techniques will guide future work in the exploration of emerging bacterial plant pathogens transmitted by sap-feeding insects and their migratory patterns across wild host plants within the ecosystem.

ACKNOWLEDGMENTS

I would like to extend my sincere thanks to the Mauck lab for guiding me through every step of this project. The patience and instruction of Dr. Kerry Mauck and PhD candidate Jaimie Kenney propelled my understanding of the mechanics behind each procedure. My knowledge and training as an undergraduate researcher are surely credited to their teachings. I would also like to thank Dr. Carolyn Rasmussen and U-SPARC for funding my research experience from beginning to end. This capstone project has been an invaluable experience and I am humbled to have had such an encouraging supporting cast. Thank you all.

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INTRODUCTION

Sap-Feeding Insect Vectors: Mechanisms of Feeding and Pathogen Transmission

The piercing-sucking order and suborder of insects, Hemiptera: Sternorrhyncha, includes economically important vectors of plant pathogens, such as aphids, psyllids, and whiteflies. The Sternorrhyncha possess specialized characteristics of their mouth parts whereby the labium and the labrum are modified into a rostrum and mandibles and maxillae form a needle-like stylet. The stylet is held within the rostrum in a rearward pointing position, also defined as opisthognathous, and appears to arise from between the legs. These striking modifications allow piercing-sucking insects to probe the epidermal and mesophyll layers of leaves in search for phloem sieve elements within vascular bundles which contain nutrients, namely sap (Brożek et al. 2015).

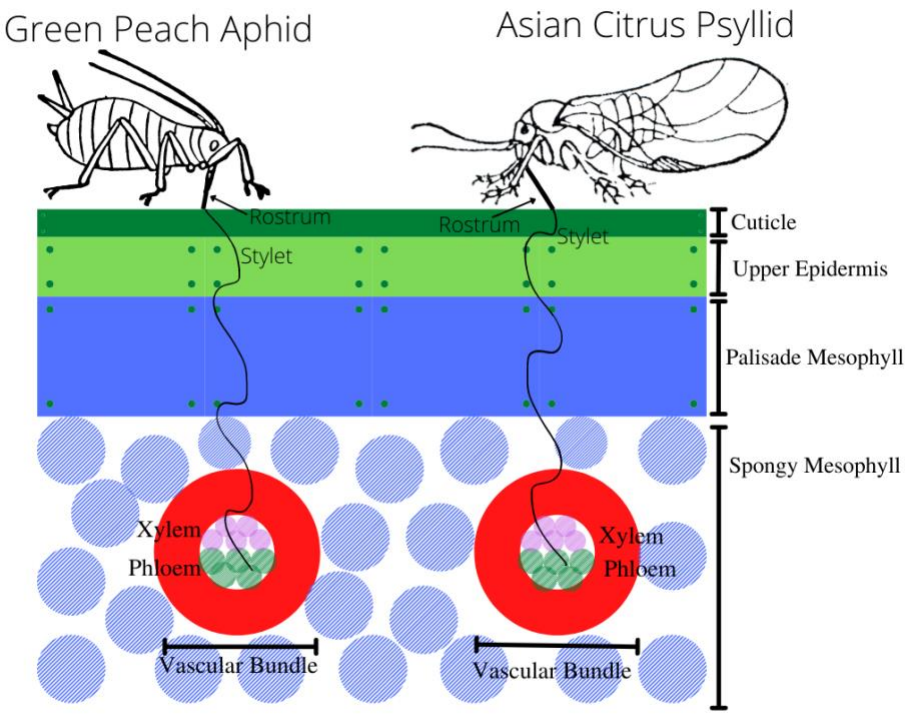


Figure 1: Diagram of stylet penetration to the phloem characteristic of piercing-sucking insects in Hemiptera: Sternorrhyncha.

In this process of gaining access to the phloem, Sternorrhyncha can acquire and inoculate plant pathogens, including viruses (aphids and whiteflies) and phloem-limited bacteria (psyllids). Upon feeding on the leaf or stem of an infected host plant, acquisition occurs as plant pathogens are stored in the saliva of Sternorrhyncha vectors. If moved to a new host, these insects transmit tainted salivary secretions into the phloem of plants, allowing the stored bacteria to inoculate and spread throughout the vascular system of the newly infected host (Milosavljević et al. 2017).

Tracking Hosts Used by Sap-Feeding Insects

In addition to pathogens, Sternorrhyncha often ingest DNA originating from punctured plant cells and their organelles, particularly chloroplasts, in the epidermal and mesophyll layers of leaves as seen in Figure 1. Since Sternorrhyncha ingest cell contents, it was hypothesized that they may be capable of acquiring DNA from their hosts. In 2016, Cooper et al. published a study on gut-content analysis of potato psyllids, *Bactericera cockerelli*, in which researchers used various PCR methods to provide the first evidence that host plant DNA can be successfully extracted and identified from a phloem-feeding insect (Cooper et al. 2016). Therefore, if a piercing-sucking insect obtains plant DNA, this newly developed method could reveal the identity of the plant being fed on.

In 2019, Cooper et al. expanded on this initial study with an analysis of non-crop host plants used by psyllids as nutrient reservoirs. The study proposed that psyllids were capable of ingesting and retaining plant DNA from many more plants than just their known crop hosts. Cooper et al. demonstrated that gut-content analysis of vectoring insects and agricultural pests like psyllids could identify alternative hosts used outside of crop environments for migratory

purposes, thus providing insight into predicting landscape-scale movements of psyllids and better management of vulnerable agricultural areas (Cooper et al. 2019).

Study Objectives

These studies indicate that non-crop and non-reproductive hosts may be critical for survival and persistence of Sternorrhyncha vectors, especially psyllids. Additionally, gut content analysis may provide a way of revealing what hosts are playing this role. However, knowledge gaps remain in both areas of study. Gut-content analysis of sap-feeding insects has only been tested on a few psyllid species and has not been rigorously tested with other Sternorrhyncha vectors that feed similarly, such as aphids. Additionally, more studies are needed that test the survival of psyllid vectors on alternative hosts. The purpose of this project was to address these knowledge gaps by developing PCR-based methods of gut-content analysis and designing host survival experiments. Various PCR trials were conducted to examine the potential acquisition, detection, and identification of host plant DNA from the gut of the Asian citrus psyllid, *Diaphorina citri,* and another Sternorrhyncha species that feeds similarly, *Myzus persicae,* also known as the green peach aphid. Concurrently, a psyllid survival experiment using *Diaphorina citri* aimed to investigate the use of alternative host plants as viable nutrient reservoirs over time.

Studied Organisms: Importance and Reasons for Inclusion.

The Asian Citrus Psyllid (ACP), *Diaphorina citri*, is a small yet nimble sap-feeding insect that is a known vector of *Candidatus* Liberibacter asiaticus (CLas), the bacterial plant pathogen associated with Huanglongbing (HLB) also known as citrus greening disease. This

insect-pathogen relationship is the most economically important and destructive association that the citrus industry currently faces (Pelz-Stelinski et al. 2010).

ACP was first found in the U.S. in Florida in 1998, and CLas was detected later in 2005 (USDA APHIS 2020a). In less than a decade, both had spread across the southeastern United States, finally reaching California in 2008. Today, ACP and HLB can be found in every citrus-producing region in the country (USDA APHIS 2020a). Net losses attributed to the spread of HLB have been estimated to be in the billions of dollars along with the loss of thousands of jobs and a massive increase in production costs (Milosavljević et al. 2017).

The strain of *Candidatus* Liberibacter that causes HLB restricts the flow of nutrients supplied throughout the plant by disrupting the function of vascular tissues (Hansen et al. 2008) This eventually results in reduced quantities of fruit, blotchy yellow discoloration of leaves, unripe fruit, loss of fruit symmetry, and ultimately tree death (CDFA 2021). In response to the spread of CLas, ACP and HLB now both lead the USDA's list of the five most destructive exotic citrus pests due to their potential to damage every acre of citrus in the United States.

Candidatus Liberibacter bacterial pathogens currently have no cure and cannot be sustained in culture (Pelz-Stelinski et al. 2010). Unfortunately, the most effective treatment is also the most devastating and expensive. Rapid tree removal and extensive use of insecticides are the only methods used to suppress this invasive insect (Milosavljević et al. 2017). Some states, including California and Florida, have adopted more proactive prevention methods to monitor and regulate the transportation of fruit across both state and county lines. Quarantine zones have been implemented to prohibit the movement of all citrus plant nursery stock outside designated areas unless inspected by the U.S. Department of Agriculture. Provisions in California's quarantine spanning 22 counties require that citrus must be harvested, cleaned, packaged, and

shipped in accordance with new practices and procedures installed by the USDA's Citrus Health Response Program (Milosavljević et al. 2017). Nonetheless, these preventive measures will only effectively address the vulnerability that the citrus industry faces for so long. More than ever, there is a need for a biological investigation and response to insect-pathogen relationships.

Another Sternorrhyncha vector, *Myzus persicae,* or the green peach aphid, was selected to test the acquisition of plant DNA alongside ACP because of its similar mouth parts and feeding behaviors. This aphid species was used to explore the stylet-borne and ingestion hypotheses originally investigated by Fereres et. al 1997. The less popular 'stylet-borne' hypothesis proposed that an analysis of plant DNA localized to the stylet of aphids from probing for phloem vessel elements would thus detect and identify the DNA of only the recently fed on plant. The more accepted 'ingestion' hypothesis was tested to determine if plant DNA was being consumed and stored long term in the saliva and foregut. Therefore, the DNA of the plant used for long term feeding, not just probing, should be detected (Fereres et al. 1997).

Research Importance and Potential for Impact

In 2021, California and Florida together accounted for over 90% of the United States' production of citrus (USDA NASS 2021). While these two states strive to maintain their command over the industry, the Asian citrus psyllid thrives in the background with the capability to cripple the supply of oranges, lemons, tangerines, and grapefruits if left unaddressed. Therefore, this study is paramount to guiding future work using gut-content analysis and progressing its application to invasive sap-feeding insects including, but not limited to, the Asian citrus psyllid.

MATERIALS AND METHODS

Host Plant Selection

To begin this study, several different species and families of host plants were selected to serve as nutrient reservoirs for potential colony propagation specific to ACP or the green peach aphid. Three host plants were selected for the psyllid model *Diaphorina citri*: curry (*Murraya koenigii*), potato (*Solanum tuberosum*), and black nightshade (*Solanum nigrum*). ACP typically fix their attraction to the pungent odors of the citrus plant family Rutaceae of which curry leaf is a member, while potato and black nightshade both belong to the Solanaceae nightshade family (Liu et al. 2021). Four host plants were selected for the aphid model *Myzus persicae*: pepper (*Capsicum annuum*) from the Solanaceae family, beet (*Beta vulgaris*) from the Amaranthaceae family, canola (*Brassica napus*) from the Brassicaceae family, and melon (*Cucumis melo*) from the Cucurbitaceae family. Although *Myzus persicae* is a generalist species capable of feeding on a wide taxonomic range of plants, it has been documented that these aphids prefer the Brassicaceae and Solanaceae families (Jhou et al. 2021). All seeds and insects for this study were supplied by the UC Riverside Department of Entomology and were grown and preserved in laboratory conditions optimal for subsequent gut-content and insect survival experiments.

A primary colony of aphids and ACP was reared to supply experiments, procedures, and other host plants with plenty of their corresponding insects. For the duration of this study, a colony of ACP was maintained on curry leaf while a colony of *Myzus persicae* was maintained on pepper. These two plants were chosen based on the insect's familiarity with the respective host family and their capacity to act as a suitable long term nutrient reservoir and reproductive host.

Plant and Insect Containment

It was vital to thoroughly isolate host plants and insect colonies to prevent attempts at escape and damage from other pests like thrips and parasitic wasps. Plastic cages equipped with mesh netting and plastic trays allowed for effective routine watering and simple monitoring of insect and plant health. Aspirators and fine point paint brushes allowed for simple placement and sampling of psyllids and aphids to and from host plants. All plants were germinated in growth chambers and later moved to greenhouses for further isolated growth. Insect colonies were storied in insectaries and quarantine facilities on campus throughout all stages of development.

Colony Propagation

After a selected host plant had matured, colonies were propagated by introducing 20 to 50 adult psyllids or aphids. After two days, all adult insects were removed from the plant to ensure that there was no carry-over from the host that the parent was raised on and that the gut content across all future adults on a host was uniform. Keeping the host plant healthy was critical to colony maintenance as the stress from hundreds of insects takes its toll over time. A colonized plant can suffer from overcrowding leading to sooty mold build-up and leaf shriveling which can gradually cause a plant to yield less nutrients for the colony. To maintain a colony, host plants used for rearing were replaced if signs of deterioration became apparent.

Psyllid Survival Experiment

A survival experiment with ACP was devised to test the ability of the psyllids to persist on different non-citrus hosts for extended periods of time. For this experiment, we used three different host plant species, along with a non-plant negative control (total of four treatment

groups). Host plant species included curry leaf, as a positive control, as well as two species in the nightshade family (Solanaceae), potato (*Solanum tuberosum*) and black nightshade (*Solanum nigrum*). These two species were selected for testing due to promising amplification results with potato psyllid, *Bactericera cockerelli* (Cooper et al. 2016).

For the first trial, 5 fully grown curry, potato, and nightshades (15 plants total) were selected and placed into individually assembled BugDorm® cages with a water saucer under each plant's plastic potting. As a negative control, 5 membrane feeders filled with water were custom-made with cut falcon tubes and parafilm wax sheets. One end of the tube was capped while the other had 2 thin layers of parafilm with 200 μL of water in-between to allow the stylet of the psyllids to siphon water. 20 collection tubes each containing 5 recently matured ACP were aspirated from the primary curry colony (100 ACP total). Adult ACP were stunned in their collection tubes by cooling before being transferred to the water traps with a paintbrush, while tubes for the plants were simply uncapped in their cages. All plants and negative controls were inspected and tallied once every 24 hours for the first 4 days then every other day up until day 10. On every inspection, three conditions were tallied: number of ACP alive, number feeding, and number confirmed dead. Both trials were conducted, in their entirety, at an insect greenhouse inside the UC Riverside Insectary & Quarantine Facility.

The main struggle learned from the first experiment was ensuring the ACP in each cage did not get stuck in the water provided for the plant, essentially drowning themselves in the first few days. Thus, to prevent this from masking potential host plant effects, a second trial was performed with some minor adjustments. Another 15 BugDorm® cages were assembled but this time the saucer under all plants was covered with a mesh bag or aluminum foil to deter the psyllids from landing in the plants water supply. 5 curry, potato, nightshade, and water traps (20

samples total) were each assigned 1 collection tube containing 10 adult ACP aspirated from the primary curry colony (200 ACP total).

Aphid Probing Experiment

Adult *Myzus persicae* reared on pepper were relocated to a canola leaf in a petri dish and allowed to probe for three different time intervals: 5 minutes, 10 minutes, and 15 minutes. Each time interval was tested three times using 10 aphids (90 aphids total). Aphids were monitored under a dissecting scope, and folded-back antennae were used as a visual cue to confirm that aphids had initiated feeding. The timer began as soon as the aphids were introduced to canola. At the end of the time allotment, each group of aphids was frozen in separately labeled 1.7mL tubes of 100% ethanol for future DNA extraction and subsequent PCR assessment.

DNA Extraction Methods

Prior to every extraction, psyllid and aphid samples stored in a -80 C freezer were surface sterilized to ensure that the presence of a plant DNA signal was from ingested material and not from any contaminates on the surface of the insects. Using a tube with a mesh net, the insects were submerged in separate 50mL beakers of 70% ethanol for 1 minute, then 2% bleach for 1 minute, and finally rinsed in ddh_2O (Cooper et al. 2016).

Two distinct methods were used to extract DNA from sterilized insect samples. The first was the cetryltrimethylammonium bromide (CTAB) method. Although it is originally designed to purify DNA from bacterial pathogens, it is equally effective in producing insect extracts and correlating plant positive controls (Crosslin et al. 2011). Small pieces of plant tissue (2-3mm^2) or 1 to 10 psyllids or aphids were placed in Eppendorf tubes equipped 2 steel grinding balls. All

samples were then submerged in liquid nitrogen until completely frozen to make the tissue brittle and easy to homogenize with a GenoGrinder at 1100 rpm for 1 minute. A series of buffers and EconoSpin column purification steps isolates 40µL of high molecular weight nucleic acids from the frozen, pulverized tissue (Zhang et al. 1998).

A secondary DNA extraction method using a Qiagen DNeasy® Blood and Tissue Kit was performed exclusively with insect samples to test if an alternative streamlined method would result in a higher DNA concentration yield. Like the CTAB method, this extraction kit was initially designed to isolate microbial DNA; however, it uses high-end buffers and reagents optimized to purify DNA while simultaneously removing contaminants and potential PCR inhibitors. Psyllids and aphids were still surface sterilized before conducting this procedure and were homogenized manually with plastic pestles. Both methods had similar stepwise instructions for purification resulting in a 40µL insect extracts.

All resulting insect and plant DNA extracts were evaluated using a Thermo Scientific NanoDrop™ 2000 Spectrophotometer to measure the total DNA concentration recorded in nanograms/microliter. This was done to ensure the extraction method was successful and that the yield was high enough for use in future PCR experiments.

Primer Selection

PCR-based methods of gut content analysis utilized four "universal" primer sets capable of amplifying highly conserved regions of nuclear or chloroplast DNA over a wide taxonomic range (Taberlet et al. 1991). ITS 2F/3R and ITS 174/294 were chosen to amplify a 500bp and 120bp region of ITS respectively (Cooper et al. 2016). Likewise, primer sets *trnL* c/d (Taberlet et

al. 1991) and *trnL* 575F/755R (Cooper et al. 2016) were used to amplify a 500bp and 180bp region of *trnL*.

Primer Set	Average Melting Temperature
trnL c/d (500 bp)	54.9℃
trnL 575F/755R (200bp)	56.5℃
ITS 2F/3R (500bp)	48℃
ITS 294/174 (200bp)	58.1℃

Table 1: Recommended primer set annealing temperatures.

PCR Reagents and Conditions

PCR conditions and reagent measurements were determined by the optimal temperatures recommended by the manufacturer of each primer set as well as the polymerase used in the master mix. Each master mix consisted of either Taq or Phusion polymerase, a correlating buffer, 2mm DNTPS, forward and reverse primers, and ddh_2O. 1 µL of DNA was used for each sample.

To ensure the primer sets successfully amplify DNA from the selected host plants, every PCR contained a plant positive control and a negative control. Multiple positive controls were often used to ensure that the desired PCR primers can successfully amplify DNA from the selected plant hosts. They also served as visual references to determine whether the resulting amplicons were of the expected size for each host plant species.

Reagent	1 reaction (in µL)
2mm DNTPS	2 µL
HF Buffer	4 µL
Forward Primer	1 µL

Reagent	1 reaction (in µL)
Reverse Primer	1 µL
Phusion Polymerase	0.2 µL
ddh_2O	10.8 µL

Table 2: Phusion polymerase PCR master mix recipe. 19 µL of master mix per reaction.

Reagent	1 reaction (in µL)
2mm DNTPS	2.5 µL
10x Taq Buffer	2.5 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Taq Polymerase	0.125 µL
ddh_2O	17.875 µL

Table 3: Taq polymerase PCR master mix recipe. 24 µL of master mix per reaction.

Thermocycler Conditions

Both the Phusion and Taq polymerase's have distinct thermocycler conditions. Note that the annealing temperature was often accommodated to the selected primer set using Table 1.

Step	Temperature	Time
Initial Denaturation	98°C	5 min
Denaturation	98°C	10 s
Annealing	Variable	30 s
Extension	72°C	1 min
Final Extension	72°C	10 Min

Table 4: Phusion polymerase thermocycler conditions. 20 µL per reaction. 40 cycles.

Step	Temperature	Time
Initial Denaturation	95°C	30 s
Denaturation	95°C	30 s
Annealing	Variable	30 s
Extension	68°C	30 s
Final Extension	68°C	5 min

Table 5: Taq Polymerase thermocycler conditions. 25 µL per reaction. 30-35 cycles.

Gel Electrophoresis & Sequencing

PCR products were mixed with 2µL of loading dye and pipetted into a 1% agarose gel for visualization. Every gel was photographed and annotated after each trial. Visible amplicons were excised and purified using a Zymoclean™ Gel DNA Recovery kit before being sent for Sanger sequencing to verify their identity. Resulting DNA sequences were quality-checked and trimmed using BioEdit sequence alignment editing software. Resulting sequences were input into BLASTn on the NCBI database to identify each amplicon.

RESULTS

DNA Extracts

DNA extractions and recovered DNA concentration results were typically uniform and had minimal complications. As expected, more homogenized material resulted in higher concentration yields. 10 aphids or psyllids was determined to be the most comparable measurement to ~3 mm^2 of plant tissue, both producing ~250-350 ng/µL of DNA. For insect samples, there was no significant difference between the Qiagen and CTAB method in DNA concentration yield or in their performance in PCR experiments.

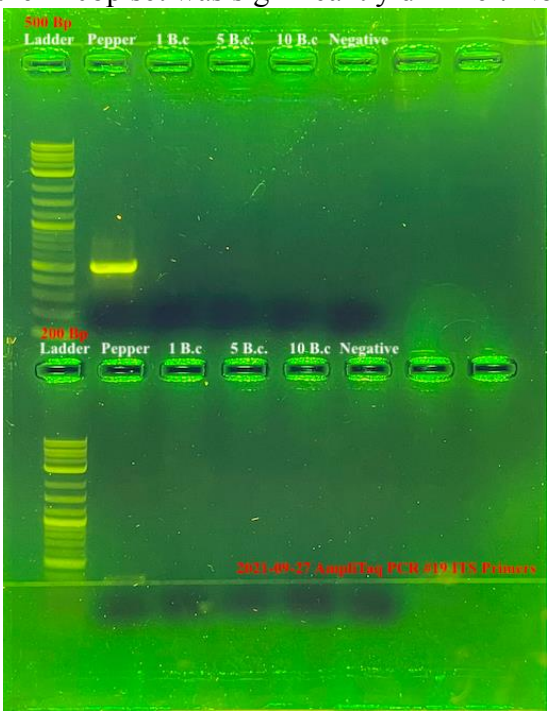
However, amplifying DNA from a positive control curry leaf extract required more labor than other plant samples to produce similar results. It was hypothesized that this may have been due to the presence of PCR inhibitors specific to this plant, so in response, a DNA dilution trial was attempted. Adding ddh_2O to a curry leaf extract was done to dilute any inhibitors enough for better amplification while also keeping a relatively high DNA concentration. A 1:100 curry DNA to ddh_2O dilution successfully produced amplicons for all further PCR experiments using *Murraya koenigii* as a positive control.

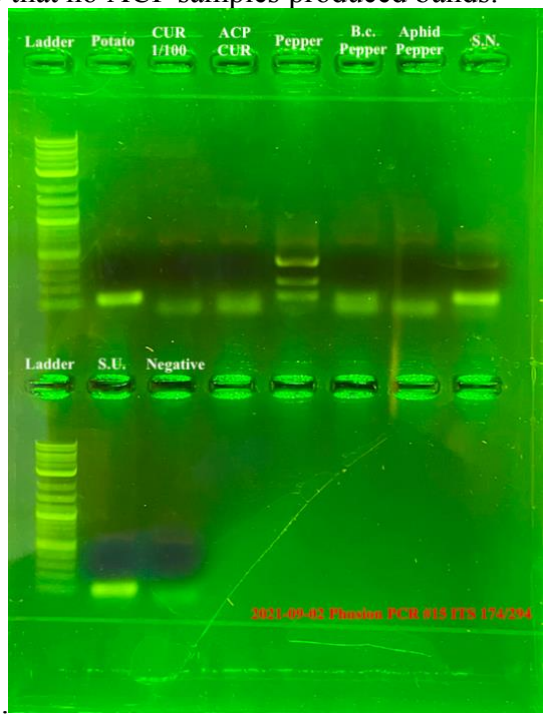
PCR Reagent Selection

The selected ITS and *trnL* primer sets using either Taq or Phusion polymerase produced visible amplicons at varying efficiencies. DNA from all plant species (pepper, curry, beet, potato, nightshade, canola, and melon) was successfully amplified using any of the primer sets and with either of the polymerases. Insect DNA extracts were much more sensitive to the designated reagents and thermocycler conditions.

ITS 2F/3R (500bp region) and ITS 174/294 (120bp region) were both unable to produce any significant bands using insect extracts with either Phusion or Taq polymerase. Both primer sets were tested 3 times each, only clearly amplifying the plant positive controls for every trial. Of these trials, ITS 2F/3R using Taq polymerase generated the brightest bands for positive controls, while ITS 174/294 with Taq led to notably dimmer yet visible bands. Both sets produced faint amplicons when using Phusion polymerase.

Figure 2:
A: Taq PCR with ITS 2F/3R (500bp) and ITS 174/294 (120bp) successfully amplified plant samples with different intensities. The ITS 500bp primer set produced a bright band for pepper while the 120bp set was significantly dimmer. Note that no ACP samples produced bands.

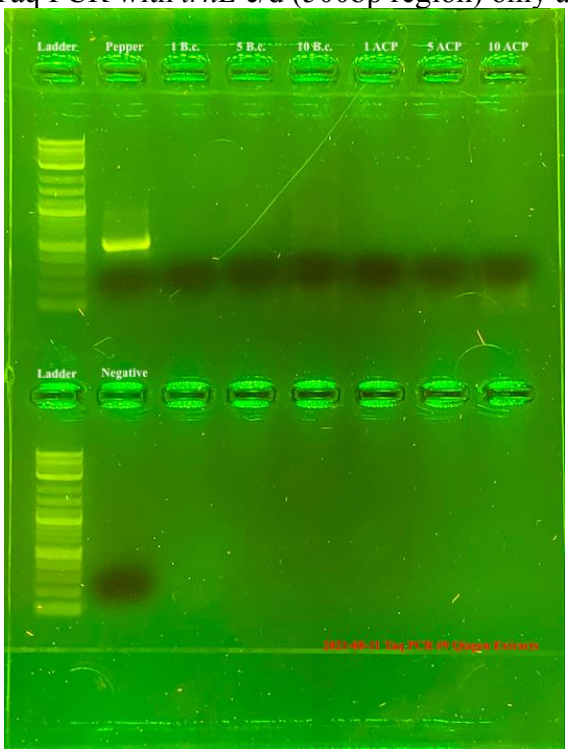
A. 

B. 

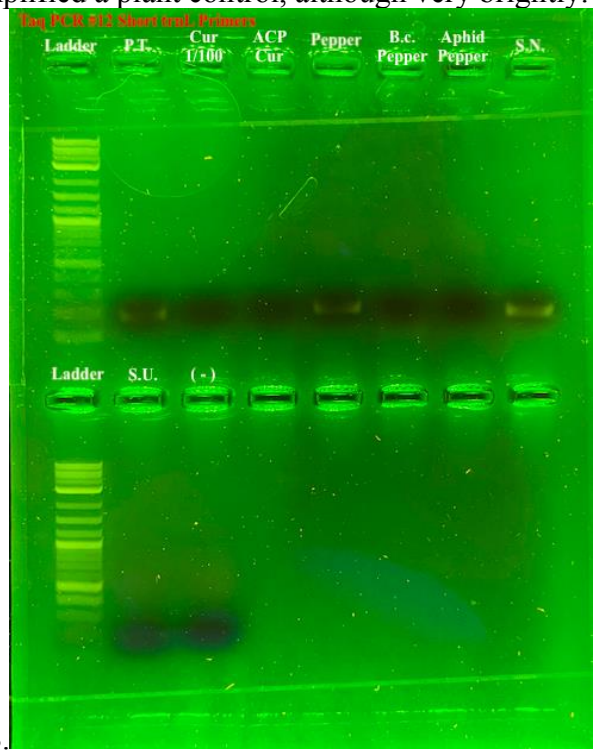
B: Phusion PCR with ITS 174/294 (120bp) produced very faint bands for positive controls and insignificant amplification of insect gut samples.

PCR with *trnL* c/d (500bp region) using Taq polymerase was featured heavily in the first attempts at amplification of plant DNA in ACP and *Myzus persicae* extracts because of its applications over a wide taxonomic range. However, only the positive controls produced amplicons for each trial. No amplicons were visible for any insect extracts using *trnL* c/d with Taq polymerase. Likewise, *trnL* 575F/755R (180bp region) with Taq had no bands for any psyllid or aphid DNA extracts and produced only faint bands for corresponding plant positive controls.

Figure 3:
A: Taq PCR with *trnL* c/d (500bp region) only amplified a plant control, although very brightly.



A.



B.

B. Taq PCR with *trnL* 575F/755R (180bp region) produced faint amplicons for plant positives but none for insect samples.

The most promising results came from using the *trnL* primer sets with Phusion polymerase. In the first trial using *trnL* 575F/755R with Phusion, visible bands were obtained for plant positive controls and *Myzus persicae* extracts with no indication of contamination. However, ACP samples continued to not show any amplification with these conditions. Figure 4 showed that a test with a DNA extract from a potato psyllid, *Bactericera cockerelli,* managed to generate a visible band while ACP did not. A second trial with the same reagent combination was performed, confirming these results. With this first minor success in obtaining amplicons from insect samples, future PCR experiments shifted towards the primary use of *trnL* 575F/755R and Phusion polymerase.

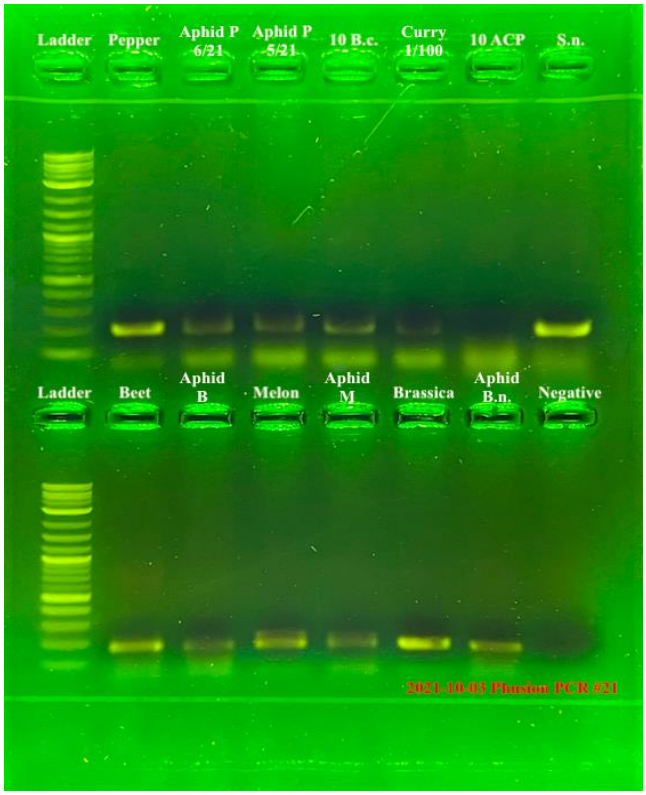


Figure 4:
Phusion PCR with *trnL* 575F/755R (180bp region) produced numerous bands. All aphid samples produced amplicons. No contamination indicated in negative control. Yet again, no bands were reported for ACP gut samples; however, *Bactericera cockerelli* showed amplification.

Gut-Content Analysis by Sanger Sequencing

Amplicons from each PCR and subsequent gel electrophoresis were excised and purified using a Zymoclean™ Gel DNA Recovery kit before being sent for Sanger sequencing to confirm the identity of an amplified PCR product. BioEdit and BLASTn were the two primary tools used to analyze sequencing results.

The BioEdit software generates a chromatogram showing peaks of individual nucleotides from a recovered sequence. These peaks were an initial determinant of successful amplification as they guide the manual analysis of a sequence, clearly identifying ambiguous nucleotides and poorly amplified regions. While plant positive controls typically provided clear chromatograms, it was common for insect samples to require more labor in generating a definitive sequence for BLASTn.

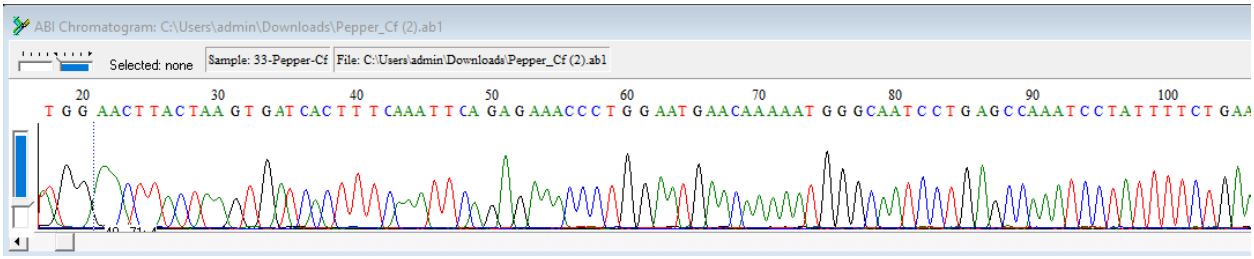


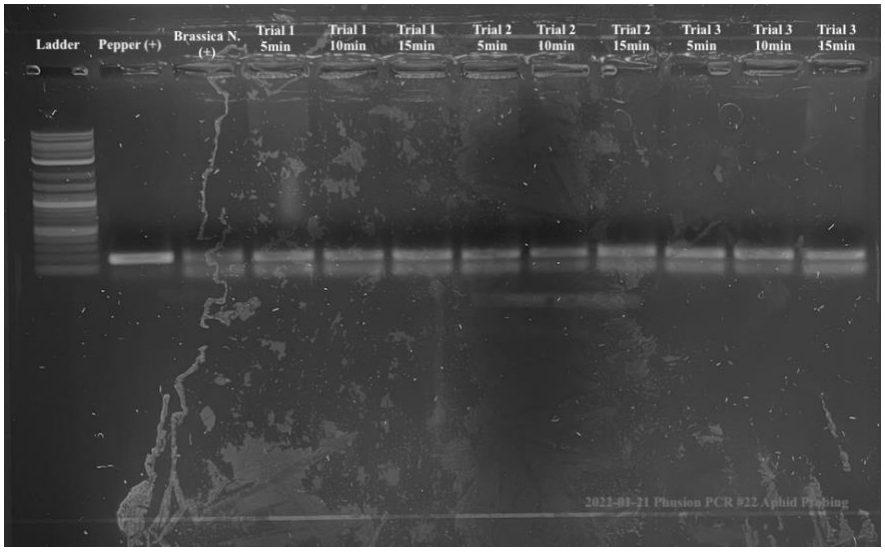
Figure 5: A BioEdit chromatogram reveals the clear recovered sequence of an excised pepper amplicon. Note that there is a single definitive peak for each nucleotide.

Across 25 different PCR trials with insect DNA extracts, the most success was found with *Myzus persicae,* as these as plant was consistently amplified under the described conditions and subsequently identified using PCR and bioinformatic tools. Very little success was found with psyllid samples as plant DNA from ACP was never successfully amplified with the designed PCR-based methods of this study.

Aphid Probing PCR

DNA was extracted from the 5-, 10-, and 15-minute aphid probing trials and used for PCR after the success of *Myzus persicae* samples generating visible bands with *trnL* 575F/755R and Phusion polymerase. These PCR conditions yet again produced visible bands for all *Myzus persicae* samples and plant positive controls. However, two bands were discernible for each aphid sample, with one brighter than the other as seen in Figure 6. Nonetheless, a second PCR was performed this time using *trnL* c/d (500bp region) with Phusion polymerase to confirm the results of the previous PCR trial. Multiple bands were again visible for aphid extracts some containing 1 to 3 amplicons per sample, but like the previous gel, one band was clearly brighter than the others in the same column. All amplicons from both aphid probing PCR trials were excised, purified, and sent for Sanger sequencing to analyze the results of amplification.

A.



B.

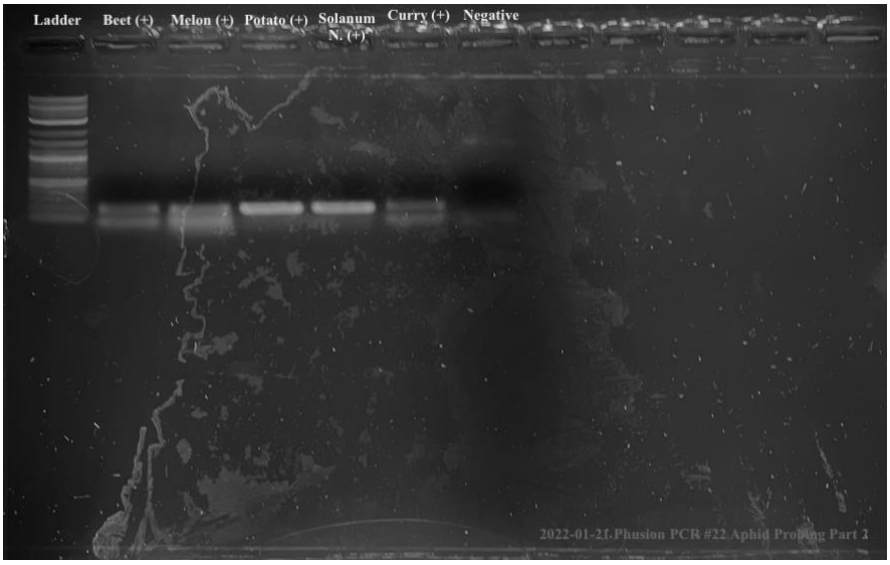


Figure 6: Black and white imaging of the aphid probing PCR using *trnL* 575F/755R and Phusion polymerase revealed two distinguishable bands for many samples. Both images are from the same PCR trial.

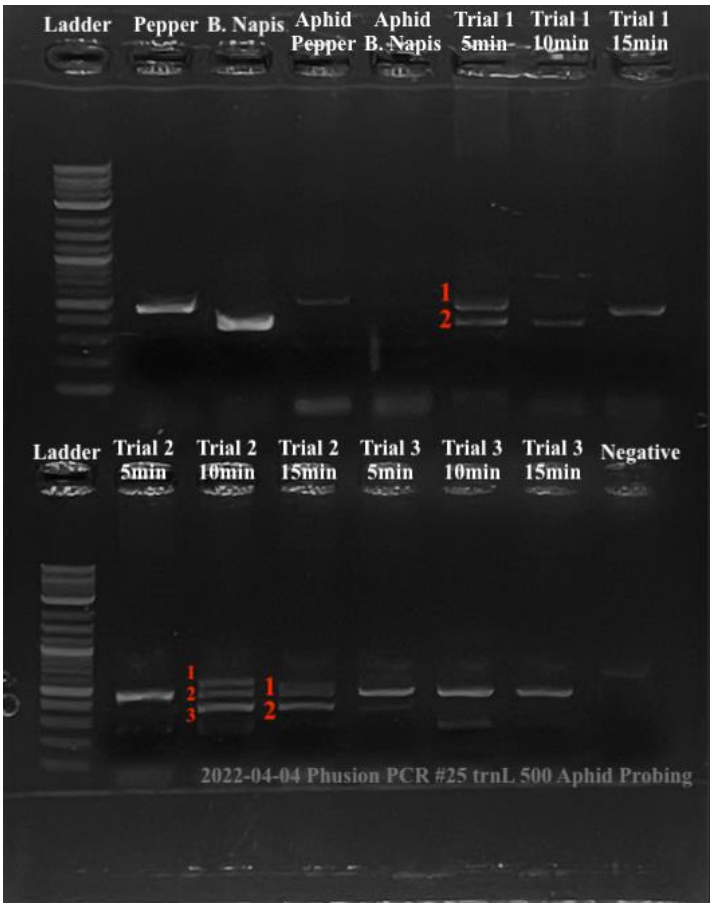


Figure 7: A PCR with *trnL* c/d (500bp region) and Phusion produced several bands for 5-, 10-, and 15-minute *Myzus persicae* probing trials). Trial 1 5 min generated 2 amplicons. #1 was identified as pepper while #2 was canola. Trial 2 10 min had 3 bands but only the brightest one, number 3, produced a definitive sequence which was identified as canola. Band #2 of Trial 2 15 min was also canola and #1 was inconclusive.

Sequencing results from the second PCR with *trnL* c/d (500bp region) and Phusion revealed the presence of both pepper and canola in the gut-content of probing aphids. Samples with multiple bands often only recovered one plant species, usually from the brightest amplicon in the column. Although, one probing sample generated two bright bands with one identified as *Capsicum annuum* and the other as *Brassica napus* as seen in Figure 7, Trial 1 5min. Clear sequences were recovered from bright amplicons while dimmer ones from the same sample produced extremely 'noisy' chromatograms that had several double peaks on BioEdit and hence generated poor quality sequences that had no definitive matches in the BLASTn NCBI database.

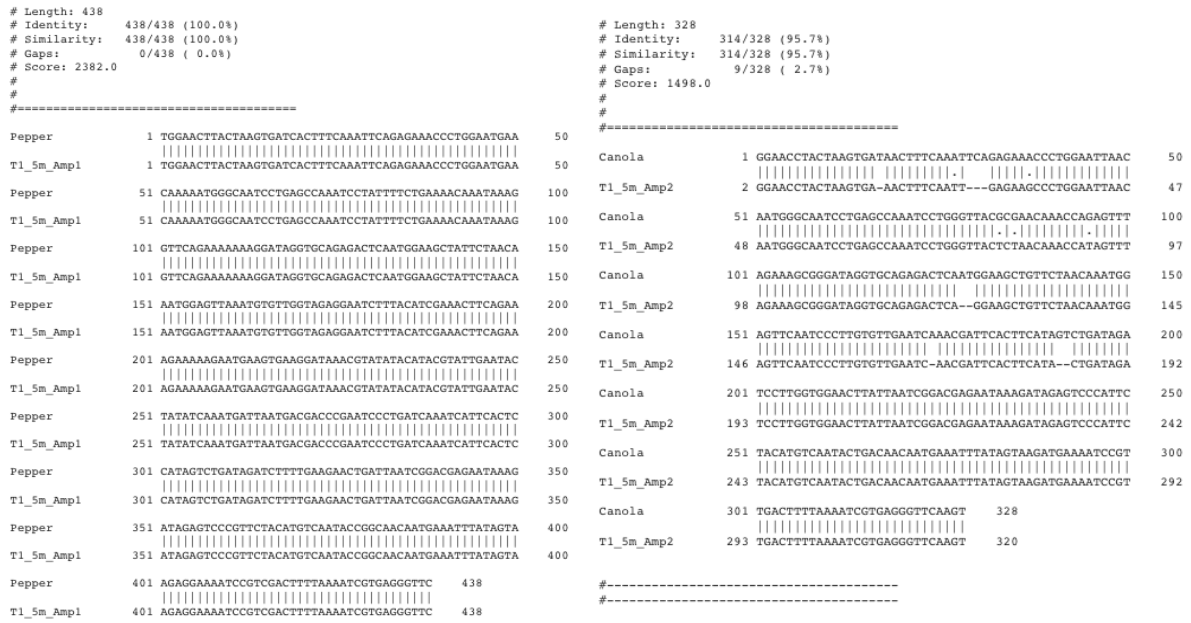


Figure 8: A local alignment of the sequences generated from amplicons 1 and 2 from Figure 7 Trial 1 5 min reveal a 100% identity to *Capsicum annuum* (pepper) and 96% identity to *Brassica napus* (canola) respectively.

Psyllid Survival Experiment

Data collected from ACP on curry leaf, potato, and nightshade showed that after ~10 days the number of alive ACP on non-citrus plants had reached 0 in both trials. Meanwhile, a water negative control showed that if ACP had no plant to feed on, death typically occurred in the first 3 days. Due to the small sample size of only 5 or 10 psyllids to an individual plant, a significant standard deviation was observed particularly for potato and black nightshade. While the conditions that forced ACP to use Solanaceae plants as nutrient reservoirs ultimately resulted in death, access to these plants sustained ACP for far longer than the compared negative control.

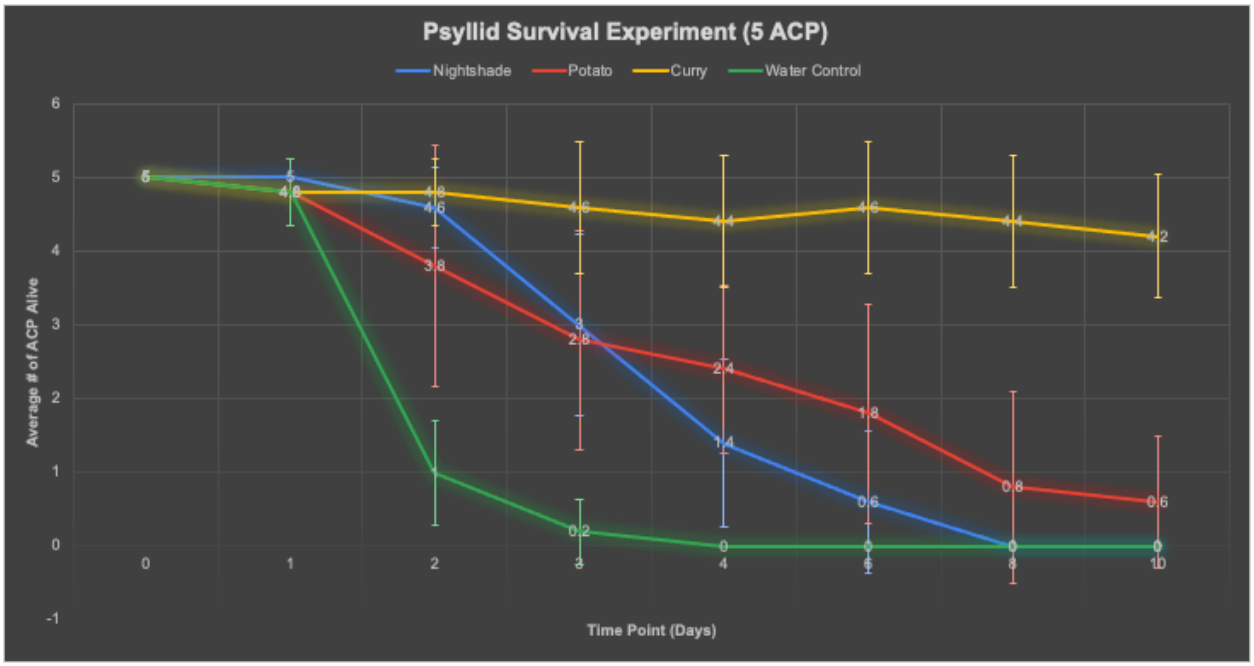


Figure 9: This data collection displaying a line graph of 5 ACP on nightshade, potato, curry, and a water negative control initially infers that while 5 psyllids is a very small sample size, there is a significant difference in the life expectancy of ACP when given access to a plant even if it is not citrus.

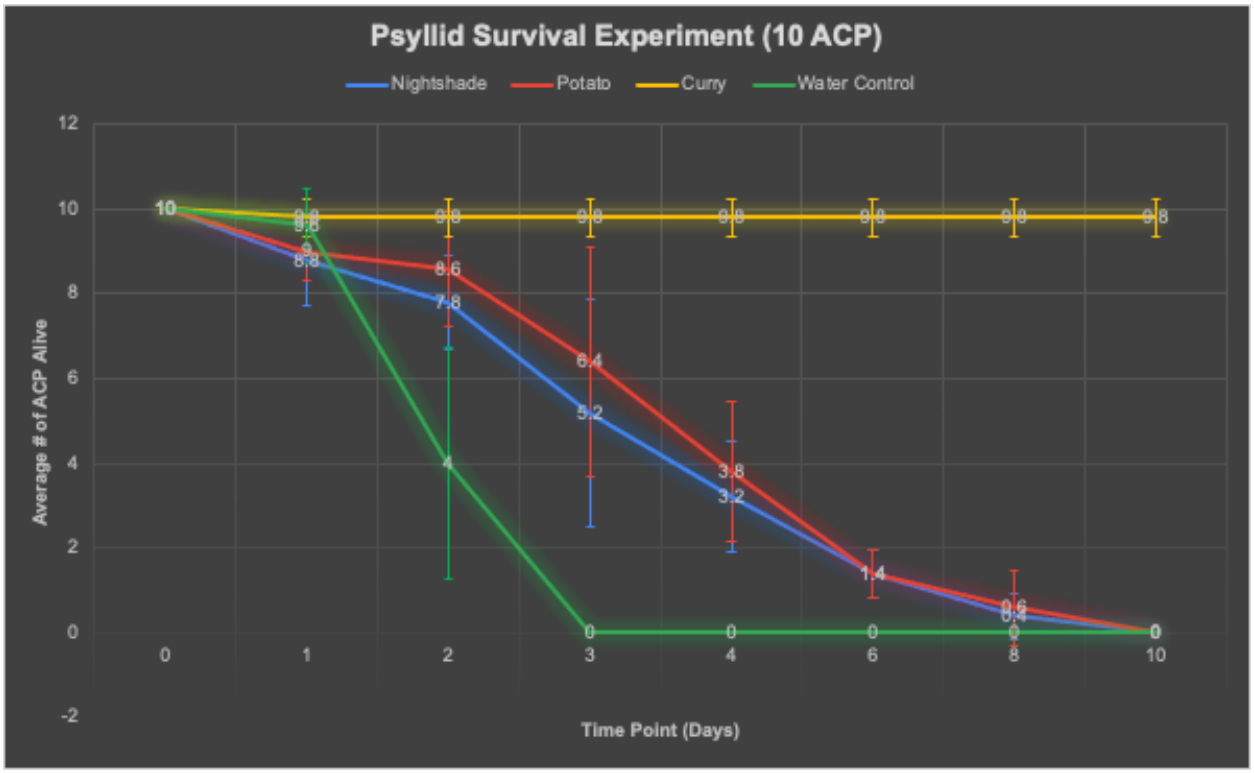


Figure 10: A line graph of 10 ACP on nightshade, potato, curry, and a water negative control shows a rapid decline in number of ACP alive in water controls contrasted with a steady decline for ACP on Solanaceae plants.

DISCUSSION

This study demonstrates the use of PCR-based methods of analysis and host survival experiments to address knowledge gaps related to plant DNA acquisition and alternative host use by *Diaphorina citri.* DNA extractions and various PCR trials show that plant DNA can be isolated, amplified, and identified from the gut-content of Sternorrhyncha vectors, particularly *Myzus Persicae;* however, ACP extracts failed to show any indication of plant DNA acquisition from selected hosts plants. These PCR results with *Diaphorina citri* correlate with Cooper et al. 2019, as ACP did not produce amplicons or sequences for citrus DNA using any of the attempted PCR conditions. Methods used to isolate DNA from ACP may have been limiting the ability to cleanse potential inhibitors within the gut-content of *Diaphorina citri* and potentially involved with DNA from the citrus family Rutaceae, as seen with the need to dilute DNA from curry leaf.

Using *Myzus persicae* as a concurrent model with ACP proved that the methods described are indeed capable of amplifying plant DNA from the gut-content of sap-feeding insects. PCR trials using *trnL* 575F/755R (180bp) and *trnL* c/d (500bp) primer sets with Phusion polymerase provided the most consistent amplification and sequencing identification results from *Myzus persicae* extracts. PCR from probing experiments showed that *Myzus persicae* was ingesting DNA from *Brassica napus* during the allotted probing times, while the DNA of the plant that they were reared on, *Capsicum annuum*, was still stored and preserved in their gut. This challenges both the stylet-borne and ingestion hypotheses proposed by Fereres et al. 1997 as DNA from both a recently fed on plant and from long-term feeding was detected.

The survival experiment data of ACP on non-crop hosts reveals that *Diaphorina citri* can use alternative host plants as nutrient reservoirs for a significant amount of time as compared to not having access to a plant at all. This proves that while ACP has a clear preference for the

citrus family Rutaceae, alternative host plants from the nightshade family Solanaceae may allow ACP to persist in regions outside of normal crop environments. However, these non-citrus plants did not allow for propagation nor were they a long-term feeding solution as both trials using 5 and 10 ACP show their ability to sustain themselves on these plants for only ~10 days. Using these findings, future studies can work towards developing a high efficiency method of identifying plants surrounding agricultural areas that may be used by ACP.

Without a method of predicting landscape movements or identifying viable alternative hosts, ACP will continue to burden the citrus industry by spreading HLB. The results of this study show that gut-content analysis has promising applications in identifying and amplifying plant DNA from Sternorrhyncha vectors. Although, more research is needed to provide conclusive evidence that *Diaphorina citri* ingests and retains plant DNA from citrus and non-citrus hosts. This study will guide future research on using gut-content analysis as a method to help advance the current understanding of the relationship between sap-feeding insects and vectored pathogens and the degree of vulnerability that several agricultural industries and the ecosystem faces today.

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