THESIS

DEVELOPING INTEGRATED PEST MANAGEMENT TACTICS FOR ALFALFA MOSAIC VIRUS AND ITS APHID VECTOR IN CHILE PEPPERS

Submitted by

Taylor Janecek

Department of Agricultural Biology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2023

Master's Committee:

Advisor: Adrianna Szczepaniec

Punya Nachappa
Mark Uchanski

Copyright by Taylor Janecek 2023

All Rights Reserved

ABSTRACT

DEVELOPING INTEGRATED PEST MANAGEMENT TACTICS FOR ALFALFA MOSAIC VIRUS AND ITS APHID VECTOR IN CHILE PEPPERS

Alfalfa mosaic virus (AMV, Bromoviridae: Alfamovirus) is a virus transmitted to plants by aphids in a non-persistent manner. The virus was first identified in chile peppers *Capsicum annuum* L. (Solanales: Solanaceae) in Southern Colorado in 2019. The goal of this research was to explore management strategies to suppress the virus given its devastating impact on the yield and quality of chile peppers. The objectives were to: 1) determine whether chile peppers have innate resistance to AMV, 2) test the effectiveness of host plant resistance and planting date modifications to suppress the virus in the field, 3) determine whether AMV is seed transmissible, and 4) survey abundance and diversity of aphids (Hemiptera: Aphididae) that likely transmit AMV in the system. In the greenhouse experiment, I found significant differences among varieties of chile peppers in the severity of AMV symptoms and identified a variety suitable for a field experiment. In the field, I found that the susceptible variety, Joe Parker, which tended to have high AMV symptoms in the greenhouse, was also highly susceptible to AMV in the field. Conversely, Mira Sol, which appeared to have resistance to the virus in the greenhouse screening assay had low incidence of AMV symptoms and low AMV titers in the field as well. Planting date also played an important role in symptom severity, where late planted peppers (mid-June) had significantly lower severity of AMV symptoms than peppers planted at conventional and early planting dates (the middle and end of May). Despite this, the yield and quality of peppers planted early was significantly greater than that of peppers planted later in the season. In addition, there was evidence of seed transmission of AMV in chile

peppers, with 10% for Mira Sol and 2% for Joe Parker, from seeds collected from infected peppers had AMV. Lastly, I found high diversity of aphid species within my experimental plots (14-16 species) and lower diversity in nearby alfalfa fields (4-5 species). Moreover, severity and titers of AMV were positively correlated with earlier planting date, which was likely related to higher aphid densities early in the season. This research contributed to formulating integrated tactics that chile pepper producers can implement in their production to suppress the impact of AMV on the crop. Finally, this is the first report of AMV transmission through seed in peppers and is the first study describing this pathosystems in Colorado.

ACKNOWLEDGEMENTS

This research would not have been possible without the funding from the Colorado Department of Agriculture Specialty Crops Block Grants; the PIs of which were Dr. Nachappa and Dr. Szczepaniec (grant number 21SCBGPCO1008-00), and the National Institute of Food and Agriculture, Crop Protection and Pest Management-Extension Implementation Program (award number 2021-70006- 35439; PI: Szczepaniec). Thank you to Colorado State University and the department of Agricultural Biology for being such a welcoming and positive environment.

The field portion of this project was successful due to the contributions of the Arkansas Valley Research Center and the farm crew working there, of whom Kevin Tanabe was of significant help. Dr. Michael Bartolo provided all seeds used in this project, of which many were bred by him. His kindness and dedication to me and the chile pepper growers in Colorado inspired me throughout this research.

Thank you to my advisor, Dr. Adrianna Szczepaniec for teaching me how to do science and constantly improve upon my skills while also being so incredibly reliable. Thank you also to my committee members, Dr. Punya Nachappa who was of immense help with her expertise in insect vectors and plant pathology, and Dr. Mark Uchanski for his grounded approach to research and feedback. Thank you to my lab, as well as the entire Nachappa lab for accepting me with open arms, helping me, and making this experience so positive. Thank you to Dr. Amy Charkowski and Dr. Crystal Cooke for your guidance, mentorship, and advice.

Finally, I want to thank my family for believing in me for all these years and their unwavering support. To my grandpa in Texas, who always inspired me and brought a smile to my face. Thank you to my incredible spouse Liz, who makes me want to be a better person every day. She has changed my life and brought incredible meaning to so many things and thank

you for all your support. Time to rope you into the next scientific adventure with me. Thank you to my best friend, my cat Cornelius, who passed away before I finished this adventure. We went through so many things together, and I miss you.

DEDICATION

This is dedicated to those who kept their curiosity with them, and to those that have gone through so many hardships that it seems all is lost. I'm proof that you can do this, so keep fighting. To my wonderful cat Izzy and my recently passed best friend Cornelius who grounded me and made me take a breath every now and then, and my Khajito, may our home always have your favorite washers to play with. I also dedicate this to my incredible spouse Liz, who made me realize dreams don't have to stay dreams, and we can get there together.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

DEDICATION vi

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER 1: RESISTANCE TO ALFALFA MOSAIC VIRUS IN CHILE PEPPER VARIETIES .. 1

REFERENCES 10

CHAPTER 2: THE EFFECTS OF PLANTING DATE MODIFICATIONS AND RESISTANT VARIETIES ON THE INCIDENCE AND SEVERITY OF ALFALFA MOSAIC VIRUS IN CHILE PEPPERS 17

REFERENCES 28

CHAPTER 3: DIVERSITY AND ABUNDANCE OF APHIDS ASSOCIATED WITH CHILE PEPPERS 40

REFERENCES 48

CONCLUSIONS 65

APPENDIX A: CHAPTER 1 SUPPLEMENTARY MATERIAL 67

LIST OF TABLES

Chapter 1

Table 1. List of varieties used in greenhouse chile pepper resistance screening experiment……14

Chapter 3

Table 2. List of alfalfa fields and harvest dates…….51

Table 3. Results of statistics analyses comparing aphid abundance in pan traps on each sampling date……..52

Table 4. Results of statistics analyses comparing aphid abundance in alfalfa fields on each sampling date……..53

Appendix A: Chapter 1 supplementary material

Table 5. Mean separation using Tukey's HSD for proportion of leaf discoloration in greenhouse experiment…….....…...66

LIST OF FIGURES

Chapter 1

Figure 1. Effect of chile pepper variety on severity of AMV symptoms (A) and OD values (B). Bars are means ±1 SEM. Pepper varieties CSU-243-12, CSU-432-20, Joe Parker, and Sonora had the highest incidence of leaves with discoloration (over 50%), while Charger, Desperado, Pueblo Primrose, and Yellow Bardo had the lowest incidence of leaves with discoloration (less than 10%) (A). Pepper varieties Carne Duro, CSU-243-12, CSU-RLC, Joe Parker, and Lexus had the highest average OD values, while Desperado, Giant Marconi, Grenada, Mira Sol, Pueblo Popper, and Yellow Bardo pepper varieties displayed the lowest average OD values (B).……15

Figure 2. Discoloration incidence per variety. Bars are the number of plants with incidence of AMV symptoms divided by the total number of plants per variety. 23 of the 30 varieties had 100% incidence of AMV symptoms, while seven varieties had variable percentages of incidence.……16

Chapter 2

Figure 3. Incidence of leaf discoloration (A) and average O.D. values (B) in resistant and susceptible varieties across three planting dates in 2022. Bars are means ±1 SEM. Asterisks denote level of statistical significance (*P = 0.05, ***P = 0.001). Resistant variety had consistently lower incidence of discolored leaves than the susceptible variety across planting dates (A). Moreover, all late planted peppers had less than half the leaf discoloration than conventionally planted peppers. Similarly, the resistant variety had consistently lower O.D. values than the susceptible variety across planting dates (B)……33

Figure 4. Incidence of leaf discoloration (A) and average O.D. values (B) in resistant and susceptible varieties across three planting dates in 2023. Bars are means ±1 SEM. Asterisks denote level of statistical significance (***P = 0.001). Resistant variety had consistently lower incidence of discolored leaves than the susceptible variety across planting dates (A). Moreover, all late planted peppers had less than half the leaf discoloration than conventionally planted peppers. Similarly, the resistant variety had consistently lower O.D. values than the susceptible variety across planting dates (B)……35

Figure 5. Side by side comparison of resistant (Mira Sol, left) and susceptible (Joe Parker, right) varieties in the field. Joe Parker had severe discoloration caused by AMV, while Mira Sol had almost no visual symptom……37

Figure 6. Average number of peppers per variety across both years (A) and average number of peppers harvested per plant for resistant and susceptible varieties for each planting date (B). Bars are means ±1 SEM. Asterisks denote level of statistical significance (**P = 0.01, ***P = 0.001). The resistant variety yielded more than twice the number of peppers than the susceptible variety. On the

other hand, Joe Parker had the lowest peppers harvested per plant in the early plantings and had significantly lower yield than Mira Sol across all planting dates.
……..……...…..……..38

Figure 7. Proportion of seed germination from infected and uninfected peppers. Asterisks denote level of statistical significance (*P = 0.05, **P = 0.01). Infected seeds from Mira Sol plants had more than five times the germination rate of seeds from infected Joe Parker plants, while uninfected seeds had nearly 100% germination rates.....39

Figure 8. Number of seedlings out of 100 plants that tested positive for AMV. Mira Sol had 10% of seedlings that tested positive, while Joe Parker had 2%.....40

Chapter 3

Figure 9. Yellow pan traps interspersed throughout experimental pepper plot……..……..……55

Figure 10. Heatmap of total aphid diversity in 2022 in pan traps (A) and sweep nets (B). Darker colors indicate a higher percentage of total aphid community. Asterisks next to a species or genus mark known vectors of AMV. Pan traps (A) had almost three times the diversity as alfalfa field samples (B), Aphis spp. was the most abundant species collected in chile pepper plots throughout 2022, followed by *T. maculata* and *A. kondoi* as the next most abundant species. On the other hand, *T. maculata* was the most abundant species found in alfalfa fields……..……..…….....56

Figure 11. Heatmap of total aphid diversity in 2023 in pan traps (A) and sweep nets (B). Darker colors indicate a higher percentage of total aphid community. Asterisks next to a species or genus mark known vectors of AMV. Pan traps (A) had almost three times the diversity as alfalfa field samples (B), A. *kondoi* was the most abundant species collected in chile pepper plots throughout 2023, followed by *Aphis* spp. and *A. pisum* as the next most abundant species. On the other hand, *A. pisum* was the most abundant species found in alfalfa fields.……...…….…..….58

Figure 12**.** Densities of the most abundant aphid species in chile pepper plots (A) and alfalfa fields (B) in 2022. Markers represent means, bars are ±1 SEM, arrows point to alfalfa cut dates. Asterisks denote the level of statistical significance (*P = 0.05, ***P = 0.001). Aphids were collected weekly using 18 yellow pan traps filled with propylene glycol (A) and weekly to every 10 days in five alfalfa fields using sweep nets (B). *Aphis sp.* were the most abundant species of aphids in the pan traps on all sampling dates, whereas *T. maculata* was the most abundant species of aphids collected in alfalfa fields……...……..60

Figure 13. Densities of the most abundant aphid species in chile pepper plots (A) and alfalfa fields (B) in 2023. Markers represent means, bars are ±1 SEM, arrows point to alfalfa cut dates. Asterisks denote the level of statistical significance (**P = 0.01 ***P = 0.001). Aphids were collected weekly using 9 yellow pan traps filled with propylene glycol (A) and weekly to every 10 days in five alfalfa fields using sweep nets (B). *A. kondoi* was the most abundant species of aphids in the pan traps early

in the season, whereas *A. pisum* was the most abundant species of aphids collected in alfalfa fields and averaged over 200 aphids per sweep on 6 June 2023.
……..……63

Figure 14. Total aphids collected in chile pepper plots by species in 2022 (A) and 2023 (B). Late planted peppers had substantially lower total aphids collected from pan traps compared to conventional and early
planting……...…….…….……65

Conclusions

Figure 15. Conceptual model summarizing the outcomes of research on integrative tactics to suppress AMV in chile peppers. Severity and titers of AMV were positively correlated with earlier planting date, which was likely related to higher aphid densities early in the season (A). Yield of resistant peppers planted early was significantly greater than that of peppers planted later in the season and lacking resistance
(B)……..……64

CHAPTER 1: RESISTANCE TO ALFALFA MOSAIC VIRUS IN CHILE PEPPER VARIETIES

Introduction

Chile peppers, *Capsicum annuum* L. (Solanales: Solanaceae), are an important and profitable specialty crop in the U.S. In 2021 alone, nearly 11,000 acres of crop land were used to plant chile peppers, producing over $70 million in profit (USDA NASS 2021). The peppers are grown mainly in California, New Mexico, and the Southwest, including Colorado (USDA NASS 2021). Chile pepper yield in the U.S. was averaging nearly eight tons per acre in 2021, which was a slight decline from the previous years' yields of 9.7 tons in 2020 and a little over nine tons in 2019 and 2018 (USDA NASS 2021). The most commonly grown chile peppers in the U.S. are paprika, jalapeno peppers, Anaheim, and banana peppers (Hall and Skaggs 2003). In addition to being a highly profitable specialty crop, chile peppers have not required extensive inputs for pest management in Colorado (M. Bartolo, pers. comm., 2022) Some of the other regions of the U.S. such as the Southeast, on the other hand, have a more diverse assemblage of key pests of chile peppers and thus greater need for pest management. Pests known to affect chile pepper include several lepidopteran pests such as beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) (Sorenson et al. 2003, Smith et al. 2011), tobacco hornworm, *Manduca sexta* L. (Sphingidae) (Sorenson et al. 2003), corn earworm, *Helicoverpa zea* Boddie (Noctuidae) (Sorenson et al. 2003) and fall armyworm, *Spodoptera frugiperda* Smith (Noctuidae) (Sorenson et al. 2003), several species of flea beetles (Coleoptera: Chrysomelidae) (Sorenson et al. 2003), green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Smith et al. 2011), and potato leafhoppers, *Empoasca fabae* Harris (Hemiptera: Cicadellidae) (Sorenson et al. 2003).

However, a recent discovery of a new pathogen, *Alfalfa mosaic virus* (AMV, Bromoviridae: Alfamovirus) in chile peppers in southern Colorado has significantly reduced their yield and quality, requiring a more intense approach to pest management. AMV was first isolated and identified in alfalfa, *Medicago sativa* L. (Fabales: Fabaceae) and was identified in chile peppers in Arkansas Valley, Colorado in 2019 (M. Bartolo, pers. comm., 2021). It is capable of infecting over 400 species of plants (Yardimci et al. 2007), including many economically important vegetable and specialty crops throughout the world including solanaceous crops such as eggplant, *Solanum melongena* L. (Solanales: Solanaceae) (Sofy et al. 2021), tomatoes, *Solanum lycopersicum* L. (Solanales: Solanaceae) (Sofy et al. 2021) and potatoes, *Solanum tuberosum* L. (Solanales: Solanaceae) (Sofy et al. 2021), peas, *Pisum sativum* L., (Fabales: Fabaceae) (van Leur et al. 2013), and lentils, *Lens culinaris* Medik (Fabales: Fabaceae) (Jones and Coutts 1996).

AMV is most commonly transmitted by aphids such as green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Ahoonmanesh et al. 1990), bean aphid, *Aphis fabae* Scopoli, cowpea aphid, *Aphis craccivora* Koch (van Leur et al. 2019), pea aphid, *Acyrthosiphon pisum* Koch, blue alfalfa aphid, *Acyrthosiphon kondoi* Shinji, and spotted alfalfa aphid, *Therioaphis trifolii forma maculata* Buckton (Garran and Gibbs 1982, Jones 2013). Moreover, aphids transmit the virus in a non-persistent manner. This mode of transmission does not require prolonged feeding, as virus particles remain on the stylet and mere probing of plant tissue is sufficient to transmit the pathogen (Yardimci et al. 2007, Yang et al. 2019, Sofy et al. 2021). In addition, AMV can be transmitted mechanically through exposure to infected plant sap and has been shown to be transmitted through seed in lentils and chickpea, *Cicer arietinum* L. (Fabales: Fabaceae) (Yardimci et al. 2007, Sofy et al. 2021). However, seed transmission of AMV in chile peppers has not yet been confirmed.

AMV is a tripartite single stranded positive sense RNA virus (Sofy et al. 2021). Virus infection can lead to stunted shoots and seedlings in chickpea and lentils, while key symptoms of this virus in infected tomato, alfalfa, chickpea and lentils are leaf chlorosis, discoloration and mottling, leaf vein deformation, and leaf deformation (Sofy et al. 2021). Incidence of AMV infection tends to be high, with commonly over 50% of the crop infected (Rahman 1993, Colimba et al. 2016).

In general, there are three broad categories of plant responses to pathogens: resistance (i.e., the ability of the host to interfere with pathogen replication), tolerance (i.e., the ability of the host to mitigate the effect the pathogen has on its fitness), and susceptibility (i.e., the inability of the host to confer resistance or tolerance to the pathogen) (Pagán and García-Arenal 2018). There are two primary mechanisms of plant resistance: qualitative and quantitative. Qualitative resistance consists of a single gene or allele for a trait in resistance (*R* gene). For example, effector-triggered immunity (ETI) occurs when effectors trigger a downstream cascade, a hyper-sensitive response, which is considered monogenetic (Kushalappa et al. 2016). However, quantitative resistance can consist of many genes, and reduces the overall susceptibility of the plant, commonly after an ETI response. Quantitative resistance is considered more durable than qualitative resistance, as it is polygenic and pathogens have to overcome more than one gene (Kushalappa et al. 2016).

Historically, host plant resistance has been the most powerful tactic in the suppression of plant viruses and has been deployed in crop protection previously. For example, host plant resistance has been successfully used to suppress *bean yellow mosaic virus* (Potyviridae) in peas (van Leur et al. 2013). Resistant varieties of peas had less than 1% of incidence of the virus, while 90% of the susceptible varieties were infected. Likewise, resistance to *chile leaf curl virus* (Geminiviridae) in three specific chile pepper varieties decreased the impact of the disease that

would otherwise result in complete loss of the crop (Thakur et al. 2019).

Thus, the goal of this research was to screen chile pepper varieties for resistance to AMV. I hypothesized that AMV symptom severity and titer levels would differ among the varieties inoculated with the virus and predicted that some of these cultivars would exhibit resistance. This research will help inform management decisions for chile pepper producers given that host plant resistance is a powerful tactic that can be used in developing efficient management of AMV. This tactic is likely to reduce the economic impact of the virus and is an environmentally sound option to explore for management.

Methods

Plant cultivation

The greenhouse experiment was conducted in a greenhouse complex at Colorado State University (CSU), Fort Collins Colorado. All seeds were sown at the depth of ca. 0.5 cm in plug flats with high porosity potting mix (Lambert® LM-40, Riviére-Ouelle, Québec, Canada) and slow-release fertilizer (Osmocote® Plus 15:9:12 N-P-K, ICL, Summerville, SC, USA). All plants were maintained under a 16:8 h (L:D) supplemental lighting cycle (430W High-Pressure Sodium + 65W LED) and the day:night temperature was 31°C:27°C. When plants developed four true leaves, they were transplanted into 12.7 cm diameter pots with high porosity potting mix (Lambert® LM-40) and fertilized with liquid fertilizer (JR Peters Inc., 15-16-17 Peat Lite, Allentown, PA, USA). In order to exclude herbivores all plants were maintained in mesh cages (75 x 75 x 115 cm with an aperture of 680 µm mesh; MegaView Science Co. Ltd. Taichung, Taiwan). The plants were watered *ad libitum* when the soil was dry.

Virus sources

AMV sources were established from highly symptomatic chile peppers collected from the CSU Arkansas Valley Research Station, Rocky Ford, Colorado. The plants were transported to Fort Collins and used to establish the virus colony using a commercially available susceptible variety of chile peppers, Joe Parker (Botanical Interests®, Broomfield, CO, USA). All plants were maintained inside cages to exclude insects and mites. The virus colonies were mechanically inoculated with AMV at about five weeks after sowing, when plants had 2-3 pairs of true leaves. Peppers were inoculated based on previously established protocol (Malapi-Nelson et al. 2009). Briefly, mechanical inoculation consisted of processing infected plant tissue by grinding it into a paste in a mortar and pestle after adding 2-3 mL of buffer (0.1M K_2HPO_4, pH 7.2-7.4) to 2-3 g of the ground tissue (1 mL of buffer per 1 g of tissue). The resulting mixture was sprinkled onto leaves of the uninfected plants and the inoculum was rubbed into the leaf tissue using an abrasive powder (carborundum) for 15 s per leaf. Once virus symptoms were visible, PCR was used to confirm AMV presence using the primer sequence AMV CP for CACAAAAGAAAGCCGGTGGG and AMV CP REV AGAGCTCAGACTCAGAGGGG. The reaction was run using the following cycling conditions: 2 min incubation at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, and 30 s extension at 72°C, and a final 5 min incubation at 72°C, distilled water was used as a negative control. The virus source was maintained by inoculating additional plants every five to six wk.

Resistance screening assay in greenhouse

The experiment was a complete randomized design consisting of 30 chile pepper varieties (Table 1) that were sown on 21 December 2021 and exposed to AMV through mechanical inoculation. Each treatment within the experiment was replicated five times for each variety (N=150). Between two and five plants from each variety were set aside as controls and were not exposed to the virus. These plants were maintained in separate cages from plants exposed to AMV.

Plants were transplanted into 12.7 cm diameter pots on 14 and 15 January 2022. Pre-inoculation symptom data collection and mechanical inoculation occurred on 1 February 2022.

Peppers were assessed once every week for three weeks starting one wk after mechanical inoculation. The response variables were the proportion of discolored leaves per plant, incidence of symptoms across varieties, as well as virus presence, which was assessed at the end of the 21-d period through ELISA. The assays were conducted according to previously established protocol (Agdia®, User Guide: Compound-ELISA Reagent Set). Briefly, absorbance (OD) values were determined through triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA). Tissue was collected using sterile forceps on 23 February 2022. Plant tissue was placed within 2.0 mL centrifuge vials (GeneMate®, Milford, EN, UK) and immediately frozen in a -80°C ultracold freezer. Forceps were sterilized between sample collections to reduce the risk of cross contamination. Tissue was processed and tested according to the Agdia® AMV ELISA protocol, using Agdia® Compound-ELISA reagent and buffer sets. Plates were assessed using the ELx800 Universal Microplate Reader (Agilent technologies Inc, Santa Clara, CA, USA) plate reader at 405 nm. All assay wells were duplicated for each plate. Samples were considered positive if absorbance value was double that of the healthy average negative control. ELISA OD values were used as a semi-quantitative proxy for virus titers.

Statistical analyses

Proportions of leaf discoloration and OD values for each of the plants from each variety were used for analyses. One-way ANOVA (R Core Team 2023) was performed using linear models. Data were normally distributed (Shapiro-Wilk test; R Core Team 2023) and had homogeneous variances (Levene's test Fox and Weisberg 2019). Discoloration incidence per variety was calculated by dividing infect plants by total plants per variety. Means separation tests (Tukey's Honestly Significant Differences test) were performed where appropriate.

Results

There were significant differences in proportion of leaf discoloration among pepper varieties ($F_{29,121}$= 3.11, P <0.001; Figure 1A). Varieties with the highest proportion of discolored leaves were CSU-243-12, CSU-432-20, Joe Parker, and Sonora, while varieties Desperado and Yellow Bardo had the lowest proportion of leaf discoloration. Similarly, varieties of the peppers also differed in their OD values following mechanical inoculation with the virus ($F_{29,121}$= 1.90, P = 0.008; Figure 1B). Varieties Carne Duro, CSU-243-12, CSU-RLC, Joe Parker, and Lexus had the highest average OD values, which were over 1.2 (Figure 1B). On the other hand, pepper varieties Desperado, Giant Marconi, Grenada, Mira Sol, Pueblo Popper, and Yellow Bardo had the lowest average OD values, less than 0.6 (Figure 1B). None were statistically significant using Tukey's HSD (Appendix 1). The incidence of discoloration, defined as the number of plants in each variety exhibiting AMV symptoms divided by their totals was close to 100% (Figure 2). However, there were seven plants that did not have 100% incidence: Charger at 50%, Desperado at 60%, Giadone at 75%, Mira Sol at 88%, Pueblo Popper at 83%, Pueblo Primrose at 33%, and Yellow Bardo at 60%.

Discussion

I found evidence of host plant resistance to AMV in several chile pepper varieties. Varieties Desperado, Pueblo Primrose, Charger, and Yellow Bardo had less than 10% average leaf discoloration per plant while Joe Parker, CSU-432-20, Sonora, and CSU-243-12 had on average more than 50% discolored leaves. On the other hand, varieties Yellow Bardo, Desperado, Grenada, and Giant Marconi had the lowest OD values, less than 0.5, while almost half of the varieties tested had OD values higher than 1.0 (G76, CSU-442-20, Abay, Tam Vera Cruz, CSU-432-20, CSU-420-19, Joe Parker, Big Jim, CSU-RLC, Lexus, CSU-243-23, and Carne Duro).

Furthermore, there were two varieties that had consistently low OD values and proportion of leaf discoloration, Mira Sol and Pueblo Popper, which had lower than 0.6 average OD values and less than 25% leaf discoloration. On the other hand, varieties Joe Parker, Big Jim, CSU-RLC, Lexus, CSU-243-12 and Carne Duro had consistently high OD values (over 1.18) and leaf discoloration (over 40%). This is the first study to illustrate host plant resistance to AMV in chile peppers.

The effectiveness of innate host resistance to viruses has been demonstrated before and can be a powerful tactic in suppression of plant viruses. For example, *barley yellow dwarf virus* (BYDV) (Tombusviridae), transmitted by bird cherry-oat aphid *Rhopalosiphum padi* L. (Hemiptera: Aphididae) in grain crops has caused yield losses reported up to 80% (Perry et al. 2000). With the use of resistant varieties, however, yield loss was reduced by up to 63% in wheat (Chrpová et al. 2020). In another system, resistant maize (*Zea mays* L.) infected with *maize streak virus* (MSV) transmitted by leafhoppers in the genus *Cicadulina* spp. (Hemiptera: Cicadellidae) can cause significant yield loss. A resistant variety of maize was shown to have a 40-60% greater yield when compared to the susceptible variety (Bosque-Pérez et al. 1998).

Although host plant resistance is a powerful tool in managing many pathosystems, the mechanisms that enable resistance can be complex. The most common form of host plant resistance is driven by an underlying genetic arms race for survival between the pathogen and the plant (Kang et al. 2005). There are multiple ways in which resistance is exhibited and can have impressive amounts of variations and permutations, which can come from a singly expressed gene or multiple alleles (Kang et al. 2005). For example, there are four identified resistance genes to BYDV in barley, the most used of these is the *Ryd2* gene (Jarošová et al. 2016). This gene reduces the rate of virus replication in the plants phloem, reducing the virus's ability to impact the health of the plant (Jarošová et al. 2016). However, a resistance gene (*N*) in tobacco *Nicotiana tobacum* L. (Solanales: Solanaceae) provides a different mechanism of resistance to *tobacco mosaic virus* (TMV) (Virgiviridae) (Garcia-Ruiz 2019). A protein encoded by the *N* gene recognizes a TMV

replication protein and triggers a hypersensitive response, localizing the infectious areas in noticeable lesions, but quarantining them from the rest of the plant (Garcia-Ruiz 2019). Identifying these underlying mechanisms is an important facet of host plant resistance research.

This study consisted of one successful greenhouse screening experiment, and further replication of this screening will provide more robust evidence for resistant chile pepper cultivars. Further, virus testing consisted of ELISA assays when PCR would likely provide more accurate titer and presence/absence evidence as PCR is a more sensitive method than ELISA with more quantitative information for virus titers. Genetic mechanisms responsible for resistance were also not investigated and could provide more insight into chile pepper resistance to AMV. However, this research provides an important cultural control tool to manage AMV using host plant resistance. Throughout history and in recent times, this management strategy has proven to be a potent and extensively employed approach for mitigating and minimizing the impact of viruses on agricultural crops, and the use of AMV resistant chile pepper varieties may be imperative to continuing their sustainable production.

REFERENCES

Ahoonmanesh, A., M. R. Hajimorad, B. J. Ingham, and R. I. B. Francki. 1990. Indirect double antibody sandwich ELISA for detecting alfalfa mosaic virus in aphids after short probes on infected plant. Journal of Virological Methods. 30: 271–281.

Bak, A., and J. B. Emerson. 2020. Cauliflower mosaic virus (CaMV) Biology, management, and relevance to GM plant detection for sustainable organic agriculture. Frontiers in Sustainable Food Systems. 4: 21.

Bosque-Pérez, N. A., S. O. Olojede, and I. W. Buddenhagen. 1998. Effect of maize streak virus disease on the growth and yield of maize as influenced by varietal resistance levels and plant stage at time of challenge. Euphytica. 101: 307–317.

Chrpová, J., O. Veškrna, J. Palicová, and J. K. Kundu. 2020. The evaluation of wheat cultivar resistance and yield loss thresholds in response to barley yellow dwarf virus-pav infection. Agriculture. 10: 20.

Colimba, J., E. Falcón, E. R. Castro, D. Davila-Aldas, V. Pallas, J. A. Sanchez-Navarro, and G. Gomez. 2016. First report of alfalfa mosaic virus in red pepper plants in Ecuador. Plant Disease. 100: 1026–1026.

Fox, J., and S. Weisberg. 2019. An R Companion to applied regression, Third. ed. Sage.

Garcia-Ruiz, H. 2019. Host factors against plant viruses. Molecular Plant Pathology. 20: 1588–1601.

Garran, J., and A. Gibbs. 1982. Studies on alfalfa mosaic virus and alfalfa aphids. Australian Journal Agricultural Research. 33: 657.

Hall, T. Y., and R. K. Skaggs. 2003. New Mexico's Chile Pepper Industry: Chile types and product sourcing. College of Agricultural, Consumer and Environmental Sciences, New Mexico State University (accessed on 10/11/23, https://pubs.nmsu.edu/research/horticulture/CTF8/index.html).

Jarošová, J., E. Beoni, and J. K. Kundu. 2016. Barley yellow dwarf virus resistance in cereals: Approaches, strategies and prospects. Field Crops Research. 198: 200–214.

Jones, R. A. C. 2013. Virus diseases of perennial pasture legumes in Australia: incidences, losses, epidemiology, and management. Crop Pasture Sci. 64: 199.

Jones, R. A. C. 2021. Global Plant Virus Disease Pandemics and Epidemics. Plants. 10: 233.

Jones, R. A. C., and B. A. Coutts. 1996. Alfalfa mosaic and cucumber mosaic virus infection in chickpea and lentil: incidence and seed transmission. Annals of Applied Biology. 129: 491–506.

Kang, B.-C., I. Yeam, and M. M. Jahn. 2005. Genetics of Plant Virus Resistance. Annual Review of Phytopathology. 43: 581–621.

Karasev, A. V., and S. M. Gray. 2013. Continuous and Emerging Challenges of Potato virus Y in Potato. Annual Review of Phytopathology. 51: 571–586.

Kushalappa, A. C., K. N. Yogendra, and S. Karre. 2016. Plant Innate Immune Response: Qualitative and Quantitative Resistance. Critical Reviews in Plant Sciences. 35: 38–55.

Latham, L. J., R. A. C. Jones, and B. A. Coutts. 2004. Yield losses caused by virus infection in four combinations of non-persistently aphid-transmitted virus and cool-season crop legume. Australian Journal Experimental Agriculture. 44: 57.

van Leur, J., Z. Duric, J. George, and S. Boschma. 2019. Alfalfa mosaic virus infects the tropical legume Desmanthus virgatus in Australia and the potential role of the cowpea aphid (Aphis craccivora) as the virus vector. Australasian Plant Disease. Notes. 14: 3.

van Leur, J., S. Kumari, M. Aftab, A. Leonforte, and S. Moore. 2013. Virus resistance of Australian pea (Pisum sativum) varieties. New Zealand Journal of Crop and Horticultural Science. 41: 86–101.

Malapi-Nelson, M., R.-H. Wen, B. H. Ownley, and M. R. Hajimorad. 2009. Co-infection of soybean with Soybean mosaic virus and Alfalfa mosaic virus results in disease synergism and alteration in accumulation level of both viruses. Plant Disease. 93: 1259–1264.

Moradi, Z., and M. Mehrvar. 2021. whole-genome characterization of alfalfa mosaic virus obtained from metagenomic analysis of Vinca minor and Wisteria sinensis in Iran: With implications for the genetic structure of the virus. Plant Pathology Journal. 37: 619–631.

Najar, A., and H. Ben Ghanem. 2017. Introgression of barley yellow dwarf virus resistance into Tunisian barley varieties. Canadian Journal of Plant Science. 97: 210–213.

Nalam, V., J. Louis, and J. Shah. 2019. Plant defense against aphids, the pest extraordinaire. Plant Science. 279: 96–107.

Pagán, I., and F. García-Arenal. 2018. tolerance to plant pathogens: theory and experimental evidence. International Journal of Molecular Sciences. 19: 810.

Perry, K. L., F. L. Kolb, B. Sammons, C. Lawson, G. Cisar, and H. Ohm. 2000. Yield effects of barley yellow dwarf virus in soft red winter wheat. Phytopathology®. 90: 1043–1048.

R Core Team, C. T. 2023. R: A language and environment for statistical computing: reference index. R Foundation for Statistical Computing, Vienna.

Rahman, F. 1993. Incidence of viruses on alfalfa in western North America. Plant Disease. 77: 160.

Saucke, H., M. Juergens, T. F. Döring, S. Fittje, D. E. Lesemann, and H. J. Vetten. 2009. Effect of sowing date and straw mulch on virus incidence and aphid infestation in organically grown faba beans (Vicia faba). Annals of Applied Biology. 154: 239–250.

Smith, R., J. L. Aguiar, A. Baameur, M. Cahn, M. Cantwell, M. De La Fuente, T. Hartz, S. Koike, R. Molinar, E. Natwick, T. Suslow, and E. Takele. 2011. Chile Pepper Production in California. University of California, Agriculture and Natural Resources.

Sofy, A. R., M. R. Sofy, A. A. Hmed, R. A. Dawoud, E. E. Refaey, H. I. Mohamed, and N. K. El-Dougdoug. 2021. molecular characterization of the alfalfa mosaic virus infecting solanum melongena in Egypt and the control of its deleterious effects with melatonin and salicylic acid. Plants. 10: 459.

Sorenson, K., J. Baker, and D. Stephan. 2003. Pests of pepper. North Carolina State University Extension. (accessed on 9/2/2022 https://vegetables.ces.ncsu.edu/peppers-insects/)

Thakur, H., S. K. Jindal, A. Sharma, and M. S. Dhaliwal. 2019. A monogenic dominant resistance for leaf curl virus disease in chili pepper (Capsicum annuum L.). Crop Protection. 116: 115–120.

Whitfield, A. E., B. W. Falk, and D. Rotenberg. 2015. Insect vector-mediated transmission of plant viruses. Virology. 479–480: 278–289.

Yang, Q., S. Arthurs, Z. Lu, Z. Liang, and R. Mao. 2019. Use of horticultural mineral oils to control potato virus Y (PVY) and other non-persistent aphid-vectored viruses. Crop Protection. 118: 97–103.

Yardimci, N., H. Eryigit, and I. Erda. 2007. Effect of Alfalfa mosaic virus (AMV) on the content of some macro-and micronutrients in alfalfa. Journal of culture collections. 5: 90–93.

Zaffaroni, M., L. Rimbaud, L. Mailleret, N. J. Cunniffe, and D. Bevacqua. 2021. Modelling interference between vectors of non-persistently transmitted plant viruses to identify effective control strategies. PLoS Computational Biology. 17: e1009727.

Zhu, J., Z. Zhu, Y. Zhou, Q. Lu, X. Sun, X. Tao, Y. Chen, H. Wang, and J. Cheng. 2009. effect of rice sowing date on occurrence of small brown planthopper and epidemics of planthopper-transmitted rice stripe viral disease. Agricultural Sciences in China. 8: 332–341

FIGURES AND TABLES

Table 1. List of varieties used in the greenhouse screening for AMV resistance.

Variety
Abay
Anaheim118
Aristotle
Big Jim
California Wonder
Carne Duro
Charger
CSU-243-12
CSU-420-19
CSU-432-20
CSU-442-20
CSU-RLC
Desperado
G76
Giadone
Giant Marconi
Grenada
Joe Parker
Karisma
Lexus
Masivo
Milena
Mira Sol
Mosco
Pueblo Popper
Pueblo Primrose
Sonora
Sweet Delilah
Tam Vera Cruz
Variety
Yellow Bardo

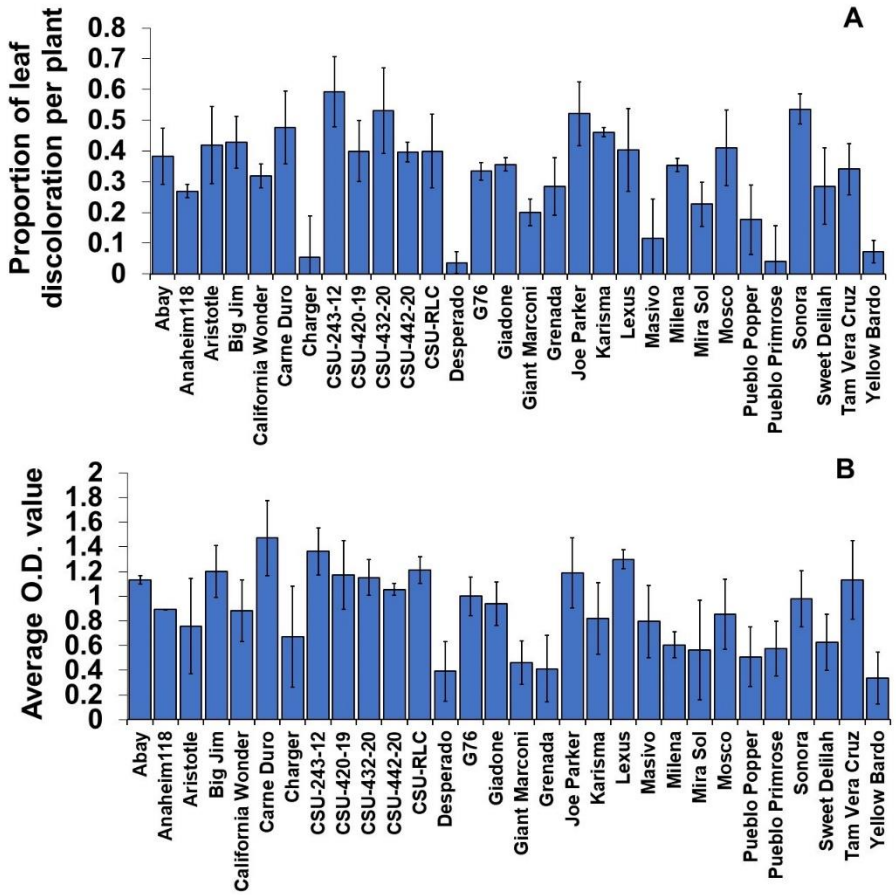


Figure 1. Effect of chile pepper variety on severity of AMV symptoms (A) and OD values (B). Bars are means ±1 SEM. Pepper varieties CSU-243-12, CSU-432-20, Joe Parker, and Sonora had the highest incidence of leaves with discoloration (over 50%), while Charger, Desperado, Pueblo Primrose, and Yellow Bardo had the lowest incidence of leaves with discoloration (less than 10%) (A). Pepper varieties Carne Duro, CSU-243-12, CSU-RLC, Joe Parker, and Lexus had the highest average OD values, while Desperado, Giant Marconi, Grenada, Mira Sol, Pueblo Popper, and Yellow Bardo pepper varieties displayed the lowest average OD values (B).

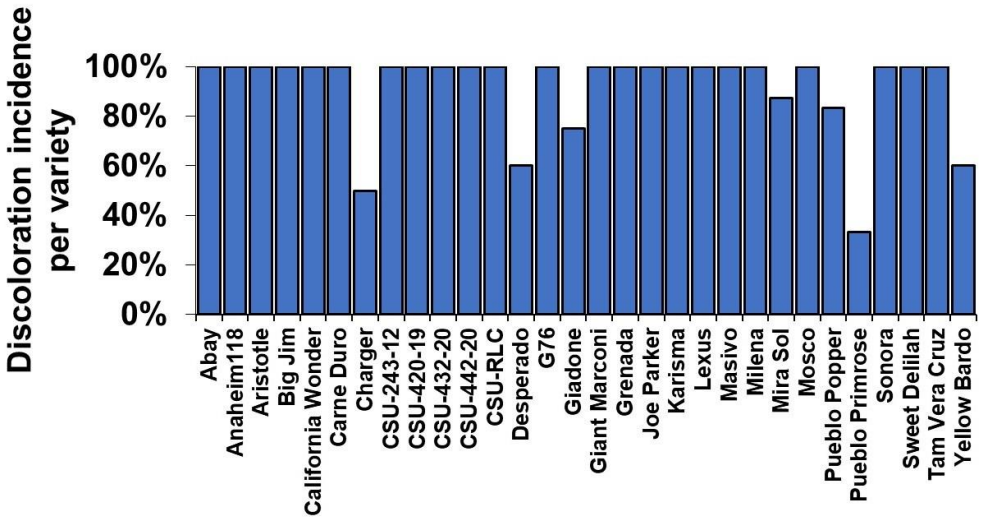


Figure 2. Discoloration incidence per variety. Bars are the percentage of plants with incidence of AMV symptoms. Twenty three of the 30 varieties had 100% incidence of AMV symptoms. Charger and Pueblo Primrose had the lowest incidence of symptoms with only 45 and 35% of the plants displaying discoloration due to AMV infection.

CHAPTER 2: THE EFFECTS OF PLANTING DATE MODIFICATIONS AND RESISTANT VARIETIES ON THE INCIDENCE AND SEVERITY OF ALFALFA MOSAIC VIRUS IN CHILE PEPPERS

Introduction

Integrated Pest Management (IPM) is a set of tactics focused on prevention of pest issues and their suppression using a wide variety of strategies (Barzman et al. 2015). Host plant resistance against insect pests and pathogens is a powerful and important IPM tactic that has effectively reduced economic losses and pesticide use (Barzman et al. 2015). Additionally, cultural practice of altering planting date is another preventative measure used in IPM, which can aid in desynchronizing pest abundance and the most vulnerable crop stages (Szczepaniec 2018). This has been demonstrated to effectively reduce losses in yield and increase overall plant health over the growing season (Stoddard et al. 2010, Barzman et al. 2015).

Discovery of *alfalfa mosaic virus* (AMV) in Colorado chile peppers in 2019 has prompted a need for management guidelines to suppress its impact on the crop. The use of host plant resistance has historically been especially effective against insect-transmitted diseases in crop plants. For example, resistant maize infected with *maize streak virus* transmitted by leafhoppers in the genus *Cicadulina* spp. can cause significant yield loss. A resistant variety of maize was shown to have a 40-60% greater yield when compared to the susceptible variety (Bosque-Pérez et al. 1998). In another study, barley yield was assessed on candidate varieties for resistance and susceptibility to the virus *barley yellow dwarf virus.* Yield of the resistant variety increased by up to 55% when compared to that of the susceptible barley (Najar and Ben Ghanem 2017).

Additionally, in potato production systems, *potato virus Y* (PVY) can cause yield losses of up to 70%, and host-plant resistance has been one of the primary tactics in managing this pathogen (Karasev and Gray 2013). These and many other examples illustrate the importance and benefits of using host plant resistance in decreasing the impact of pathogens infecting crop plants.

It is important to note that in these and other examples of employing innate defenses to the pathogen, i.e., the host's ability to limit the impact of the pathogen on its fitness, is the most frequently used mechanism of resistance (Pagán and García-Arenal 2018). When plants are resistant to pests or diseases the densities of insects or titers of pathogens are not reduced, but the crop yield and quality are improved despite lack of change in pest fitness (i.e., antibiosis) or preference for the crop (i.e., antixenosis). Tolerance to pathogens or their vectors can be expressed through various strategies. For example, plants can compensate for injury caused by a pathogen (Paudel and Sanfaçon 2018). The plant *Nicotiana benthamiana* Domin (Solanales: Solanaceae) showed tolerance to *tomato ringspot virus* (Nepoviridae), where the new growth of the plant did not exhibit symptoms even though virus titers in the rest of the plant tissue did not change significantly (Jovel et al. 2007). Priming is another example of a common mechanism of resistance, where plants with previous exposure to the virus suffer lower fitness costs (Paudel and Sanfaçon 2018). For example, in *Arabidposis thaliana* L. (Brassicales: Brassicaceae), exposure to specific pathogens resulted in production of plant metabolites specialized in pathogen disruption and suppression. These metabolites were still present during reinoculation events, priming the plant's defenses against subsequent pathogen exposure (Jung et al. 2009).

In addition to plant resistance, a cultural practice of modifying planting dates to introduce asynchrony between the vectors of plant pathogens, or allow plants to outgrow their most vulnerable stages has been used effectively to protect crops from diseases. For example, early sowing of fava bean resulted in almost 30% lower incidence of a virus transmitted by pea

aphids, *pea enation mosaic virus* (Solemoviridae), across all three years of the experiment (Saucke et al. 2009). In another example, late planted rice (*Oryza sativa* L., Poales: Poaceae) was less susceptible to *rice stripe tenuivirus* (Phenuiviridae) transmitted by a planthopper, *Laodelphax striatellus* Fallen (Hemiptera: Delphacidae) (Zhu et al. 2009). The researchers found that late planted rice had up to 25% lower virus incidence than the early planted rice (Zhu et al. 2009). While the mechanism underlying benefits of planting date modification varies, allowing plants to avoid exposure to the vectors during their most vulnerable growth stages is the most common driver of the benefits of this approach.

In addition to vector transmission, seed transmission is another common mode of pathogen infection. It is not known whether AMV can be seed transmitted in chile peppers. However, previous research has demonstrated that the virus can be transmitted via seeds in other crops. For example, in a study examining seed transmission of AMV in field and forage crops, researchers found that AMV is seed transmissible in *Trifolium clypeatum* L. (Fabales: Fabaceae) and *Trigonella balansae* L. (Fabales: Fabacaeae) (Latham and Jones 2001). In another study, seed transmission of AMV was identified in chickpea and lentil (Jones and Coutts 1996). Seed transmission is an important part of understanding viral pathosystems and the risks of infection.

The goal of this work was to explore how host plant resistance and planting date modifications affected incidence and severity of the virus, and yield of peppers in the field. I also examined whether the virus was seed-transmissible in chile peppers, which has not yet been demonstrated. I hypothesized that innate resistance to the virus in chile pepper varieties in combination with planting date modifications would reduce virus symptoms and its impact on the yield. This is the first report of employing IPM tactics to suppress AMV in chile peppers. This work has the potential to provide chile pepper growers in Colorado and elsewhere with management guidelines to greatly reduce the impact of AMV on their pepper production.

Methods

Field experiment

The field experiment was conducted at the CSU Arkansas Valley Research station in Rocky Ford, Colorado over two growing seasons in 2022 and 2023. The experiment was embedded within a 1.38 ha field planted next to a 0.4 ha field of grain sorghum to the east, a 4-ha alfalfa field to the west, a 1.6 ha field of alfalfa to the north, and a 12.15 ha pasture to the south. The plot consisted of 12 rows, each were 0.91 m apart and 18.3 m in length totaling 658.4 m^2 of experimental area. The experiment was a split plot design with a planting date factor as a whole-block factor (early, conventional, and late) and plant variety as a split-block factor (susceptible variety Joe Parker, and a resistant variety Mira Sol). Each of these treatments was replicated six times (N=36), and each replicate plot consisted of eight chile peppers, four resistant and four susceptible. The peppers were planted 15.24 cm apart and two-row buffers separated each planting block.

Seeds for the field experiment were sown and germinated as described in *Plant cultivation* (Chapter 1) in a greenhouse complex at CSU, Fort Collins. Seeds (48 per variety per planting date) were sown into flats and sowing was staggered by two weeks to ensure plants of the same approximate stage could be transplanted into the field. Once the peppers reached four true leaf stage, they were transported to the research farm in Rocky Ford, CO and planted into rows assigned to the early (13 May), conventional (26 May), and late (8 June) planting date treatment in 2022; and early (10 May), conventional (23 May), and late (13 June) planting date treatment in 2023.

Plants were assessed for symptom incidence of AMV on June 22, July 12, and July 22 in 2022 and June 14, July 5, August 7 in 2023. The response variables were incidence of leaf discoloration among plants in each replicate as present or absent, and ELISA optical density values,

which were used as a semi-quantitative proxy for AMV titers. Symptomatic leaf tissue was collected from each plant on June 22 and July 22 in 2022, and June 14 and August 7 in 2023 and placed in 2 mL centrifuged tubes and kept in a chilled cooler until placed in a -80°C ultracold freezer on the same day. ELISA assays were conducted as described in *Resistance screening assay* (Chapter 1).

Chile peppers were collected at the end of the season when fruits reached red-mature stage. defined as 50% or more of the fruit becoming red. Peppers were sorted into labeled paper and plastic bags denoting planting date, replicate, and plant number. The response variables collected were the number of peppers per plant and weight. Chile pepper harvest continued weekly from 2 August to 19 September in 2022 and from 17 August to 21 September in 2023 until the plants stopped producing fruits. Peppers from AMV positive plants with high absorbance values were used for the seed transmission experiments.

Seed transmission assay

Seeds were collected from six AMV-positive pepper plants from each variety used in the field (Joe Parker and Mira Sol) in 2022 to assess whether AMV is seed-transmitted. The seeds were sown from 15 September 2022 through 5 January 2023 and cultivated as described in *Plant cultivation* (Chapter 1). Due to low germination rates, seeds were sown continually until 100 plants from each variety germinated. Tissue was collected as described in *Resistance screening assay* (Chapter 1), except that seeds were germinated to their first two true leaves. Leaves were assayed for AMV using ELISA as described in *Resistance screening assay* (Chapter 1).

Statistical analyses

The incidence of leaf discoloration among plants, OD values, and yield were averaged across replicates and were compared among varieties and planting dates for both years. Incidence of leaf discoloration were analyzed with logistic regression using generalized linear mixed-effects models

with the lme4 package (Bates et al. 2015). Yield and OD values were analyzed using ANOVA (R Core Team 2023) with linear models which met assumptions of parametric test (Shapiro-Wilk test) (R Core Team 2023) test for normality and Levene's test (Fox and Weisberg 2019) for homogeneity of variance. Means separation tests (Tukey's Honestly Significant Differences test) were performed where appropriate. Kruskal-Wallis was used for data that did not meet assumptions for ANOVA, with Dunn's test for means separation (R Core Team 2023).

Results

Field experiment

There was a significant interactive effect of year, variety, and planting date on incidence of discoloration ($F_{2,547} = 7.89$, $P < 0.001$), and marginally significant interaction among year, variety, and planting date on OD values ($F_{2,60} = 2.44$, $P = 0.096$). Thus, both years were analyzed separately to assess the effects of treatments on these variables. However, there was no significant interactive effect among year, variety, and planting date on yield of peppers ($F_{2,54} = 1.42$, $P = 0.251$), and these data were combined for analyses across the two years of the experiment.

In 2022 there was an interactive effect of variety and planting date on incidence of discoloration ($F_{2,265} = 6.91$, $P = 0.001$). Mira Sol had significantly lower incidence of leaf discoloration than Joe Parker across the planting dates ($F_{1,265}=31.05$, $P<0.001$; Figure 3A). The estimated proportion of plants with symptoms of AMV for Mira Sol was 0.05, while the estimated proportion of plants with symptoms of AMV for Joe Parker was 0.29 ($p < 0.001$). Planting date also had a significant effect on incidence of AMV ($F_{1,265}=35.68$, $P<0.001$; Figure 3A). The effects of planting date modifications were more variable, however. Regardless of variety, peppers planted early (13 May 2022) and at conventional planting date (26 May 2022) had significantly higher incidence of leaf discoloration than the late plantings (8 June 2022), with an estimated proportion of plants with symptoms of AMV of 0.65 for early, 0.53 for conventional, and 0.001 for late ($p <$

0.001). Early and conventional plantings had, on average, more than twice the incidence of leaf discoloration than late planted peppers in 2022 (Figure 3A). There was also a significant interactive effect of variety and planting date on OD values ($F_{2,413}$= 12.8, P < 0.001) in 2022. Mira Sol had significantly lower OD values than Joe Parker (X^2 = 6.94, df = 1, *P* = 0.001), and this effect was evident across all planting dates (Figure 1B). There was also evidence of significant differences in OD values when comparing late to early and conventional planting dates, with early planted peppers having the highest OD values (X^2 = 36.12, df = 2, *P* = 0.001, Figure 3B).

Similarly, in 2023 there was an interactive effect of variety and planting date on incidence of discoloration ($F_{2,282}$=36.76, P<0.001). Mira Sol had significantly lower incidence of leaf discoloration than Joe Parker across the planting dates ($F_{1,282}$=118.1, P<0.001 Figure 4A). The estimated proportion of plants with symptoms of AMV for Mira Sol was 0.01, while the estimated proportion of plants with symptoms of AMV for Joe Parker was 0.11 ($p < 0.001$). Planting date also had a significant effect on incidence of AMV ($F_{2,282}$=56.04, P<0.001 Figure 4A). Similar to 2022, peppers planted early (10 May 2023) and at conventional planting date (23 May 2023) had significantly higher incidence of leaf discoloration than the late plantings (13 June 2023) regardless of the variety, with an estimated proportion of plants with symptoms of AMV of 0.06 for early, 0.12 for conventional, and 0.008 for late ($p < 0.001$). As in 2022, the early and conventional plantings had, on average, more than twice the incidence of leaf discoloration than late planted peppers. In both years of the experiment the incidence of discoloration was evident in peppers and was characterized by severely deformed leaves and mosaic-like symptoms. These were especially pronounced in the susceptible variety (Figure 5). Moreover, there was a significant interactive effect of variety and planting date on OD values in 2023 as well ($F_{2,221}$= 2.93, P < 0.048). Mira Sol had significantly lower OD values than Joe Parker ($F_{1,221}$= 23.03, P < 0.001, Figure 4B), which was evident across all planting dates (Figure 3B). Peppers planted early had the highest OD values, similar to 2022 (X^2 = 43.19, df = 2, *P* = 0.001, Figure 4B). OD values, on average, were much higher in magnitude in 2023

when compared to 2022.

Chile pepper variety ($F_{1,30}$= 21.49, P < 0.001) and planting date ($F_{2,30}$= 10.55, P < 0.001) had a significant effect on pepper yield, while there was no significant interactive effect of the two treatments on yield ($F_{2,30}$= 2.02, P = 0.15). Resistant peppers produced more than twice as many peppers as the susceptible plants (Figure 6A), and Mira Sol had consistently higher average yield than Joe Parker across all planting dates and both years. However, conventional and early plantings had significantly higher yields than the late planted peppers, regardless of the variety ($F_{2,33}$= 10.55, *p* = 0.004, Figure 6B).

Seed transmission

There was a significant difference in germination rates between seeds collected from the resistant and susceptible chile pepper varieties infected with AMV and uninfected seeds of the same varieties (X^2 = 6.94, df = 1, *P* = 0.008) as well as a significant difference between germination rates among varieties of infected peppers ($F_{1,6}$= 8.9, P = 0.025). Seeds collected from infected plants from Joe Parker and Mira Sol germinated at the 5% and 29% level, respectively, compared to seeds from uninfected plants, which had a 100% germination rate (Figure 7). Moreover, I detected AMV in a total of 12 seedlings, which represented a 6% transmission rate (Figure 8). Two percent of Joe Parker seedlings tested positive for AMV using ELISA, while 10% of Mira Sol seedlings were positive.

Discussion

I found evidence that the variety Mira Sol is resistant to AMV and that planting date modifications can lower the incidence and severity of AMV in the field in both years. Joe Parker had four times the incidence of leaf discoloration compared to Mira Sol, as well as significantly higher OD values. Moreover, these symptoms were dramatically apparent in the field. Mira Sol had little to no symptoms throughout the entire season, and if the symptoms were apparent, they

were limited to single leaves. On the other hand, many of the individual Joe Parker plants had 100% leaf discoloration on every leaf, a staggering difference to Mira Sol especially in visual observation and side-by-side comparison (Figure 2). The titers of AMV in Mira Sol were also consistently lower than that of Joe Parker, though the scale of these values was vastly different in 2023 where AMV levels were much higher on average than in 2022. In June, much of Colorado had already received the average annual precipitation, making 2023 one of the wettest years on record, bringing the state out of a drought for the second time in 23 years (Butzer 2023). This could be a contributing factor to the increased scale in OD values in the second year, as aphid abundance may have been higher. However, incidence and severity of AMV was lower which may be attributed to increased plant health due to more water throughout the season. Similar effects of high precipitation and greater abundance of viruliferous aphids has been noted before. For example, the average fall temperatures and precipitation were linked with the probability of the presence and abundance of BYDV viruliferous aphids in Kansas (Enders et al. 2018). It is also worth noting that plant insect interactions can be complex, and what is true for one vector pathosystem may not be for others.

Furthermore, I found evidence that early planted peppers were at the highest risk of AMV infection, while late planted peppers were at the lowest risk of infection. This was more evident in the susceptible variety, Joe Parker, which exhibited symptoms more conspicuously. Higher abundances of aphids within neighboring fields, specifically alfalfa earlier in the season could contribute to this outcome. For example, earlier flight activity and abundant aphid vectors are likely to contribute to higher incidence and prevalence of PVY in potato fields, although key aphid vector species may differ depending on location (Hlaoui et al. 2020). In another study, researchers determined that the primary indicator of the likelihood of melon *Cucumis melo* L. (Cucurbitales: Cucubitaceae) being infected with the plant virus *cucurbit aphid-borne yellows virus* (Luteoviridae) was the early-season abundance of *Aphis gossypi* Glover (Hemiptera: Aphididae)

(Schoeny et al. 2020).

Delaying planting date in my experiment decreased AMV incidence, but the late-planted peppers had significantly lower yields when compared to early planted chile peppers in particular. Modifying planting date is an effective IPM tactic for suppressing plant viruses, but its impact on pests and pathogens is context dependent. In several published studies delayed planting benefited crop productivity. For example, *zucchini yellow mosaic virus* (Potyviridae) can cause substantial losses in cucurbit crops with high incidence (Coutts et al. 2011). However, delaying seed sowing can reduce incidence by up to 49% (Coutts et al. 2011). In other examples, planting earlier or later than common planting window offers comparable benefits, as was the case for *mungbean yellow mosaic virus* (Geminiviridae) in mungbean *Vigna radiata* L. (Fabales: Fabaceae). Planting mungbean earlier or later than conventional dates resulted in up to 28% reduction in virus incidence (Swamy et al. 2023). In my experiment, early planted peppers likely had enough time to compensate for AMV injury and also had more time to grow, and this was reflected in higher yield and quality of the peppers. Strong resistance to the virus in Mira Sol was clearly reflected in its yield. Mira Sol produced significantly higher yields than Joe Parker in both years and across all planting dates.

Germination rates of seeds collected from infected resistant and susceptible peppers were extremely low. On average, the germination rate of seeds from infected Joe Parker peppers was only 5%. This rate was also considerably lower than the germination rate of seeds from infected Mira Sol peppers (nearly 30%). Despite these differences in germination rates, the incidence of AMV in seeds of both varieties was consistent. Only 6% of all plants grown from infected peppers were positive for AMV. Two of the previous studies noted in the *Introduction* corroborate this outcome. For example, in a study examining seed transmission of AMV in field and forage crops, researchers found that seed transmission varied from 0.05% in *T. clypeatum* to seven percent in *T. balansae* (Latham and Jones 2001). In another study, seed transmission of AMV was identified in

chickpea (up to 1%) and lentil (up to 5%) (Jones and Coutts 1996). This is the first experiment to demonstrate that AMV can be transmitted through seed in chile peppers, and its transmissibility rates were similar to that reported in other crops.

The experiments had a few limitations that are worth noting. I standardized the collection of peppers at their red mature stages, with Mira Sol displaying generally faster maturity rates compared to Joe Parker. Joe Parker and Mira Sol produced peppers of varying sizes and shapes, and restricting the collection to only red mature peppers may have introduced data bias across the varieties. Further, weed control in our plots was inconsistent over the two years, especially in 2023 due to substantial precipitation. Furthermore, our seed transmission assays from infected peppers were exclusively conducted on germinated seedlings. We did not bleach seed coats or score the seeds for these assays, which may have affected our findings. Nonetheless, this study marks the first exploration of IPM strategies for suppressing AMV in chile peppers and the first documented case of AMV seed transmissibility in chile peppers. Our research introduces two crucial cultural control strategies for managing AMV in chile peppers: host plant resistance and modifications to planting dates. These strategies have historically and contemporarily proven effective in mitigating virus impact on agricultural crops. Incorporating AMV-resistant chile pepper varieties and adjusting planting dates could be key in the sustainable production these peppers. The potential seed transmission of the virus in chile peppers highlights the importance of understanding the risks associated with seed production. Future research focused on preventing the planting of infected seeds holds the potential to mitigate these risks.

REFERENCES

Barzman, M., P. Bàrberi, A. N. E. Birch, P. Boonekamp, S. Dachbrodt-Saaydeh, B. Graf, B. Hommel, J. E. Jensen, J. Kiss, P. Kudsk, J. R. Lamichhane, A. Messéan, A.-C. Moonen, A. Ratnadass, P. Ricci, J.-L. Sarah, and M. Sattin. 2015. Eight principles of integrated pest management. Agronomics and Sustainable Development 35: 1199–1215.

Bates, D., M. Machler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models using {lme4}. Journal of Statistical Software.

Bosque-Pérez, N. A., S. O. Olojede, and I. W. Buddenhagen. 1998. Effect of maize streak virus disease on the growth and yield of maize as influenced by varietal resistance levels and plant stage at time of challenge. Euphytica. 101: 307–317.

Butzer, S. 2023. Colorado is drought-free for only the second time in 23 years. Denver 7 Colorado News. (Accessed 10/11/2023 https://www.denver7.com/news/local-news/colorado-is-drought-free-for-only-the-second-time-in-23-years#:~:text=releases%20the%20data.-,According%20to%20its%20map%20published%20Thursday%20morning%2C%20which%20depicts%20conditions,is%20100%25%2Ddrought%20free.&text=This%20is%20only%20the%20second,a%20hydrologist%20with%20the%20NWS.)

Coutts, B. A., M. A. Kehoe, and R. A. C. Jones. 2011. Minimising losses caused by Zucchini yellow mosaic virus in vegetable cucurbit crops in tropical, sub-tropical and Mediterranean environments through cultural methods and host resistance. Virus Research. 159: 141–160.

Enders, L. S., T. J. Hefley, J. J. Girvin, R. J. Whitworth, and C. M. Smith. 2018. Spatiotemporal distribution and environmental drivers of barley yellow dwarf virus and vector abundance in Kansas. Phytopathology®. 108: 1196–1205.

Fox, J., and S. Weisberg. 2019. An {R} Companion to Applied Regression, Third. ed. Sage.

Hlaoui, A., E. Mazzoni, R. Souissi, and S. Boukhris Bouhachem. 2020. Diversity and abundance of landing aphids in two areas of seed potato production in Tunisia; relative to the incidence of potato viruses. Redia. 103: 75–85.

Jones, R. A. C., and B. A. Coutts. 1996. Alfalfa mosaic and cucumber mosaic virus infection in chickpea and lentil: incidence and seed transmission. Annals of Applied Biology. 129: 491–506.

Jovel, J., M. Walker, and H. Sanfaçon. 2007. Recovery of Nicotiana benthamiana plants from a necrotic response induced by a nepovirus is associated with RNA silencing but not with reduced virus titer. Journal of Virology. 81: 12285–12297.

Jung, H. W., T. J. Tschaplinski, L. Wang, J. Glazebrook, and J. T. Greenberg. 2009. Priming in systemic plant immunity. Science. 324: 89–91.

Karasev, A. V., and S. M. Gray. 2013. Continuous and emerging challenges of potato virus y in potato. Annual Review of Phytopathology. 51: 571–586.

Latham, L. J., and R. A. C. Jones. 2001. Alfalfa mosaic and pea seed-borne mosaic viruses in cool season crop, annual pasture, and forage legumes: susceptibility, sensitivity, and seed transmission. Australian Journal of Agriculture. Res. 52: 771.

Latham, L. J., R. A. C. Jones, and B. A. Coutts. 2004. Yield losses caused by virus infection in four combinations of non-persistently aphid-transmitted virus and cool-season crop legume. Australian Journal of Experimental Agriculture. 44: 57.

Najar, A., and H. Ben Ghanem. 2017. Introgression of barley yellow dwarf virus resistance into Tunisian barley varieties. Canada Journal of Plant Science. 97: 210–213.

Pagán, I., and F. García-Arenal. 2018. Tolerance to plant pathogens: Theory and experimental evidence. International Journal of Molecular Sciences. 19: 810.

Paudel, D. B., and H. Sanfaçon. 2018. Exploring the diversity of mechanisms associated with plant tolerance to virus infection. Frontiers in Plant Science. 9: 1575.

R Core Team, C. T. 2023. R: A language and environment for statistical computing: reference index. R Foundation for Statistical Computing, Vienna.

Rahman, F. 1993. Incidence of viruses on alfalfa in western North America. Plant Disease. 77: 160.

Saucke, H., M. Juergens, T. F. Döring, S. Fittje, D. E. Lesemann, and H. J. Vetten. 2009. Effect of sowing date and straw mulch on virus incidence and aphid infestation in organically grown faba beans (Vicia faba). Annals of Applied Biology. 154: 239–250.

Schoeny, A., L. Rimbaud, P. Gognalons, G. Girardot, P. Millot, K. Nozeran, C. Wipf-Scheibel, and H. Lecoq. 2020. Can winged aphid abundance be a predictor of cucurbit aphid-borne yellows virus epidemics in melon crop? Viruses. 12: 911.

Stoddard, F. L., A. H. Nicholas, D. Rubiales, J. Thomas, and A. M. Villegas-Fernández. 2010. Integrated pest management in faba bean. Field Crops Research. 115: 308–318.

Swamy, S. M., N. Sandra, S. K. Lal, A. Kumar, H. K. Dikshit, B. Mandal, and A. D. Munshi. 2023. Evaluation of sowing dates for managing yellow mosaic disease caused by mungbean yellow mosaic India virus in mungbean. 3 Biotech. 13: 207.

Szczepaniec, A. 2018. Interactive effects of crop variety, insecticide seed treatment, and planting data on population dynamics of sugarcane aphid (Melanaphis sacchari) and their predators in late-colonized sorghum. Crop Protection. 109: 72–79.

Zhu, J., Z. Zhu, Y. Zhou, Q. Lu, X. Sun, X. Tao, Y. Chen, H. Wang, and J. Cheng. 2009. Effect of rice sowing date on occurrence of small brown planthopper and epidemics of planthopper-transmitted rice stripe viral disease. Agricultural Sciences in China. 8: 332–341.

FIGURES

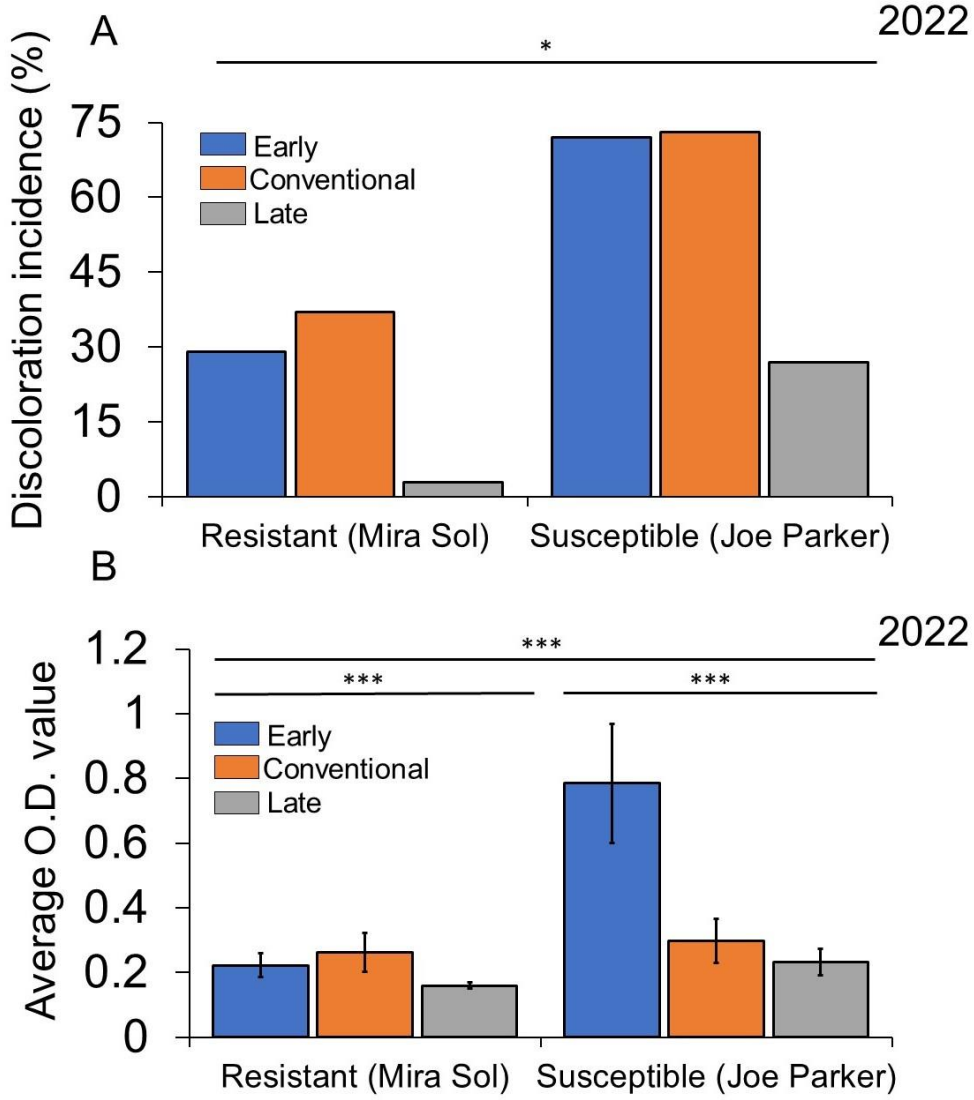


Figure 3. Incidence of leaf discoloration (A) and average O.D. values (B) in resistant and susceptible varieties across three planting dates in

2022. Bars are means ±1 SEM. Asterisks denote level of statistical significance (*P = 0.05, ***P = 0.001). Resistant variety had consistently lower incidence of discolored leaves than the susceptible variety across planting dates (A). Moreover, all late planted peppers had less than half the leaf discoloration than conventionally planted peppers. Similarly, the resistant variety had consistently lower O.D. values than the susceptible variety across planting dates (B).

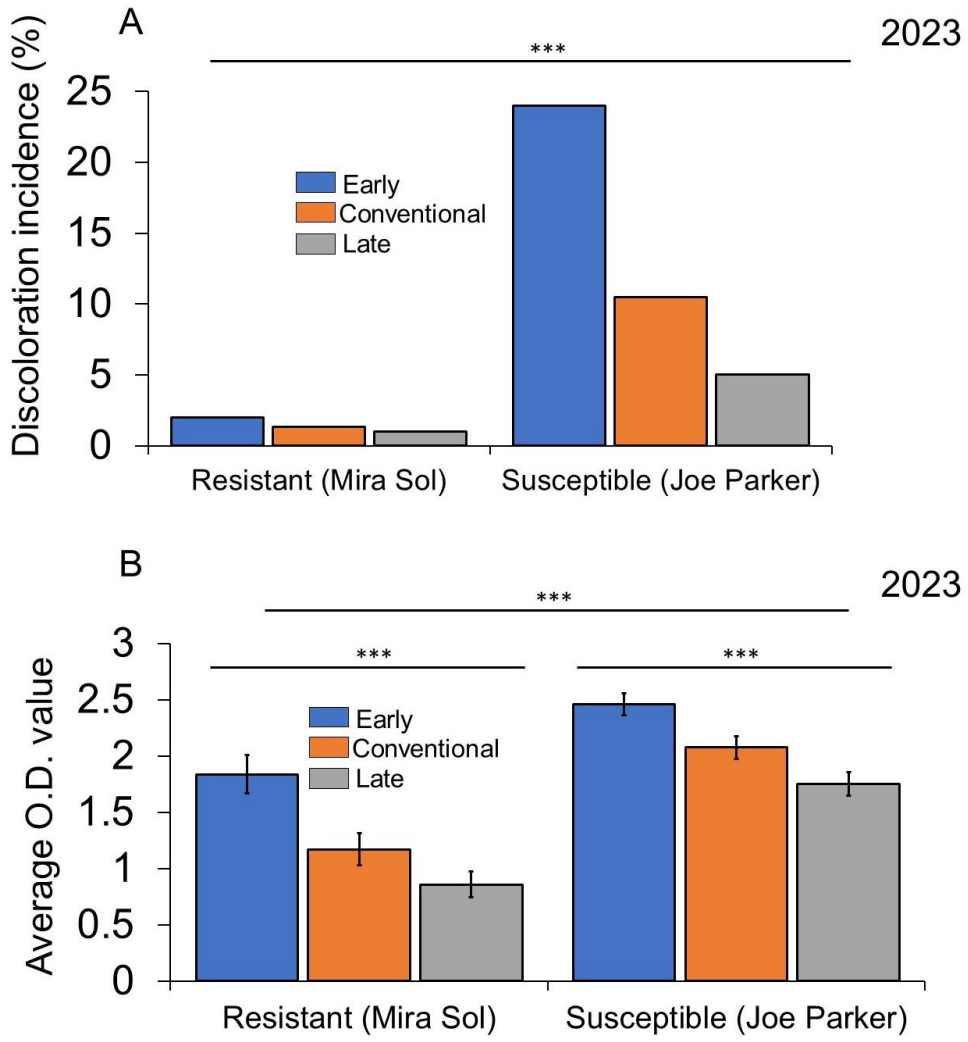

A
2023
***
Discoloration incidence (%)
25
20
15
10
5
0
Early
Conventional
Late
Resistant (Mira Sol)
Susceptible (Joe Parker)
B
2023
***
***
***
Average O.D. value
3
2.5
2
1.5
1
0.5
0
Early
Conventional
Late
Resistant (Mira Sol)
Susceptible (Joe Parker)

Figure 4. Incidence of leaf discoloration (A) and average O.D. values (B) in resistant and susceptible varieties across three planting dates in 2023. Bars are means ±1 SEM. Asterisks denote level of statistical significance (***$P = 0.001$). Resistant variety had consistently lower incidence of discolored leaves than the susceptible variety across planting dates (A). Moreover, all late planted peppers had less than half the leaf discoloration than conventionally planted peppers. Similarly, the resistant variety had consistently lower O.D. values than the susceptible variety across planting dates (B).



Figure 5. Side by side comparison of resistant (Mira Sol, left) and susceptible (Joe Parker, right) varieties in the field. Joe Parker had severe discoloration caused by AMV, while Mira Sol had almost no visual symptoms.

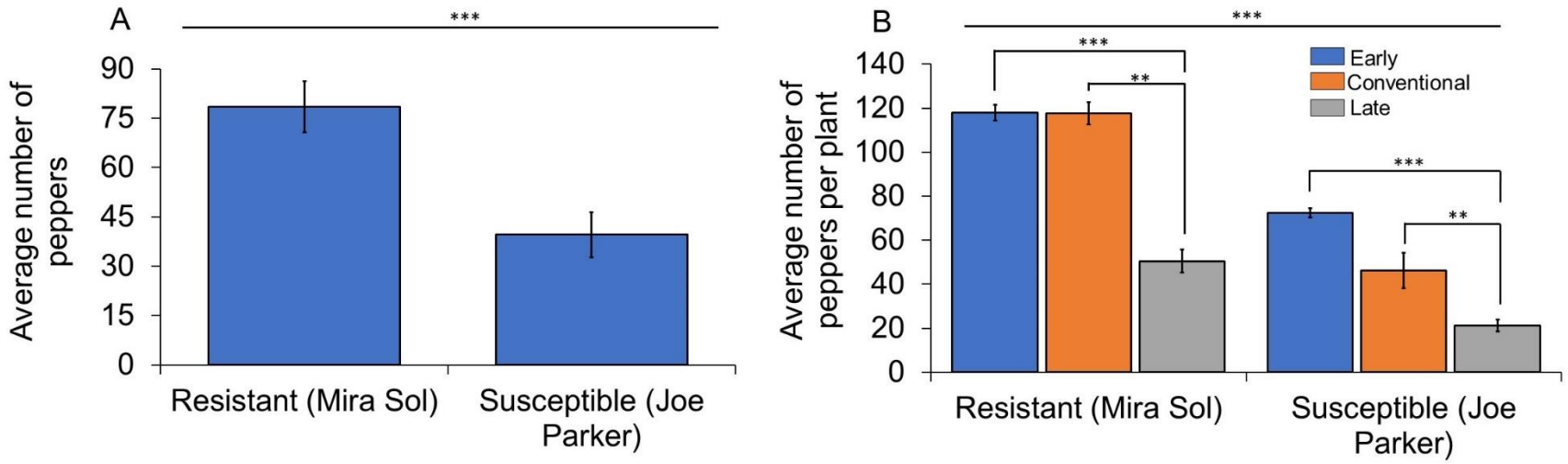


Figure 6. Average number of peppers per variety across both years (A) and average number of peppers harvested per plant for resistant and susceptible varieties for each planting date (B). Bars are means ±1 SEM. Asterisks denote level of statistical significance (**P = 0.01, ***P = 0.001). The resistant variety yielded more than twice the number of peppers than the susceptible variety. On the other hand, Joe Parker had the lowest peppers harvested per plant in the early plantings and had significantly lower yield than Mira Sol across all planting dates.

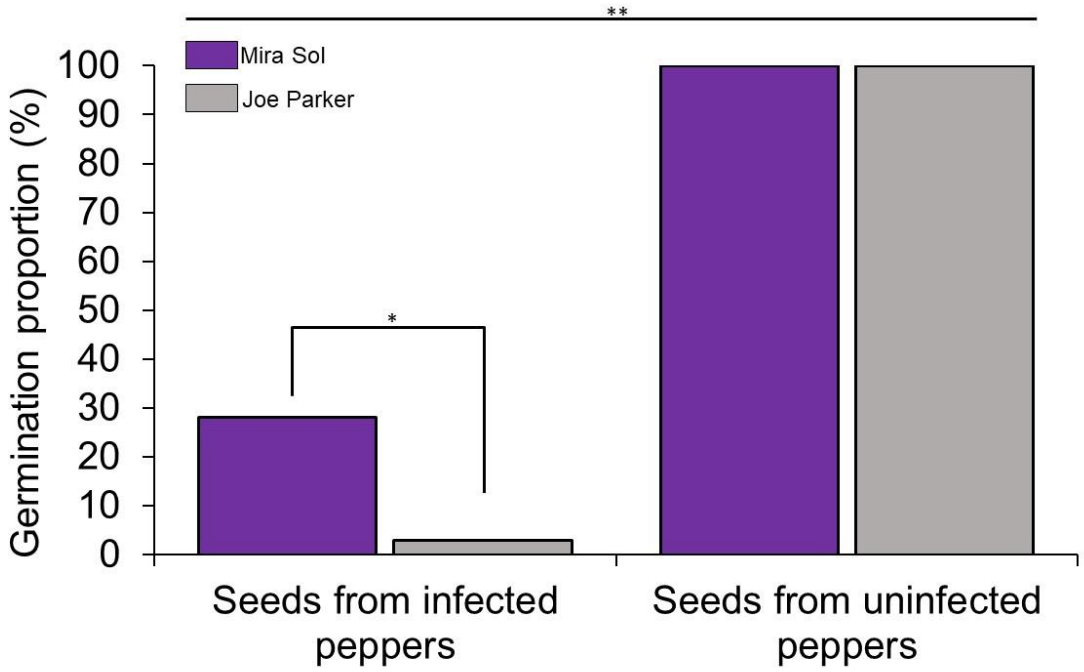


Figure 7. Proportion of seeds that successfully germinated for infected and uninfected peppers. Asterisks denote level of statistical significance (*$P = 0.05$, **$P = 0.01$). Infected seeds from Mira Sol plants had more than five times the germination rate, uninfected seeds had similar germination rates between varieties at approximately 100%.

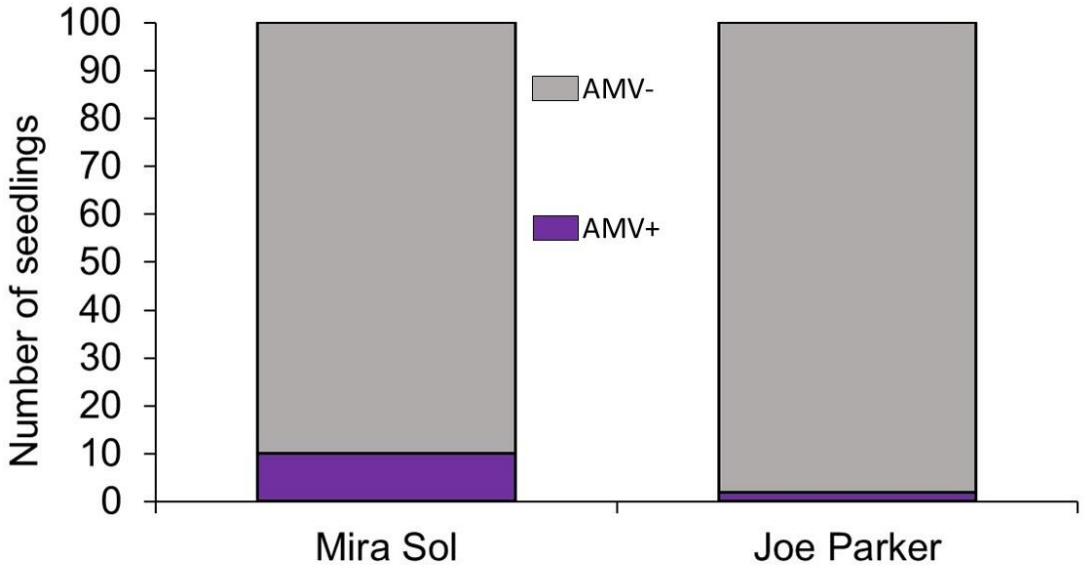


Figure 8. Number of seedlings that tested AMV positive for each variety out of 100 using ELISA. Mira Sol had 10% of seedlings that tested positive, while Joe Parker had 2%.

CHAPTER 3: DIVERSITY AND ABUNDANCE OF APHIDS ASSOCIATED WITH CHILE PEPPERS

Introduction

Aphids (Hemiptera: Aphididae) are some of the most important insect pests in the world and transmit over 200 plant pathogens, including nearly 30% of all known plant viruses (Nalam et al. 2019, Zaffaroni et al. 2021). Furthermore, aphids are also responsible for transmission of most of the known non-persistently transmitted viruses (Zaffaroni et al. 2021). This mode of transmission does not require prolonged feeding, as virus particles remain on the insect stylet and mere probing of plant tissue is sufficient to transmit the virus (Yardimci et al. 2007, Yang et al. 2019, Sofy et al. 2021). Given the brief period of transmission, suppression of aphids that transmit viruses non-persistently as means of reducing virus incidence is frequently ineffective.

Many of these aphid transmitted viruses are economically important worldwide, such as PVY, *Faba bean necrotic yellows virus* (FBNYD) (Nanoviridae: Nanovirus), and *Cauliflower mosaic virus* (CaMV) (Caulimoviridae: Caulimovirus) (Whitfield et al. 2015). In potatoes, PVY can cause the potato tuber necrotic ringspot disease (PTNRD), where symptom appearance and severity depend on the site of infection: the tuber or the foliage. The diseases caused by PVY can cause economic losses by reducing yields by as much as 70%, or by lowering the quality of tubers making them unmarketable (Karasev and Gray 2013). In fava bean, FBNYD causes fava bean necrotic yellows disease which can be economically devastating, reducing fava bean yields by up to 90% (Jones 2021). Furthermore, CaMV impacts on crops in the family Brassicaceae (e.g., cauliflower, broccoli) can also be quite severe, with yield losses of up to 50% in some cases (Bak and Emerson 2020). In many cases across the United States, regionality and localization of pathosystems have been changing temporally, and spatially.

The recent detection of a virus in chile peppers in the southern region of Colorado has had a significant impact on their yield and quality. Given that the virus, AMV, is transmitted non-persistently, the approach to virus suppression through pesticide use to decrease the aphids that transmit it is not likely to be effective. However, identifying the major species of aphids and peaks of their activity in peppers may facilitate effective IPM tactics such as planting date modifications to escape the greatest risk for aphid activity during the most vulnerable stages of plants. AMV was identified in chile peppers in the Arkansas Valley of Colorado in 2019 and has the ability to infect more than 400 plant species (Yardimci et al. 2007), including various economically important crops worldwide, such as tomatoes (Sofy et al. 2021), potatoes (Sofy et al. 2021), peas (van Leur et al. 2013) and lentils (Jones and Coutts 1996). AMV is transmitted by various aphid species, such as the green peach aphid (Ahoonmanesh et al. 1990), bean aphid (van Leur et al. 2019), cowpea aphid (van Leur et al. 2019), pea aphid, blue alfalfa aphid, and spotted alfalfa aphid (Garran and Gibbs 1982).

The goal of this work was to survey diversity and abundance of aphids in chile pepper plots and nearby alfalfa fields. I predicted that alfalfa would be the major source of aphids and harvests of this crop would trigger increase in abundance of aphids in chile plots as well. Understanding the diversity, abundance, and seasonal peaks of aphid densities will help assess the risk of virus transmission to nearby vegetable crops, thereby contributing to improved pest management and protecting chile peppers from the impacts of AMV infection.

Methods

Aphid survey in alfalfa fields

Aphid surveys were conducted within five alfalfa fields adjacent to the chile pepper plots used in field experiments (Chapter 2, Methods)**.** Each of the sampled alfalfa fields was located within a 600 m radius from experimental plots (Table 1). Each field was sampled bi-weekly following the early planting of peppers on 13 May in 2022 and 10 May 2023. Aphids were sampled in a V-shaped

pattern starting approximately 10 m in from the edge of each alfalfa field. A sweep net was used to sample the aphids at five sampling points in 2022 separated by 5 m along each arm of the V-shaped pattern, and two sampling points in 2023. Sampling consisted of three sweeps of the net at each sampling point. Aphids were carefully transferred to 50 mL conical tubes (Falcon™, Thermo Fisher Scientific®, Waltham, MA, USA) filled with 90% ethanol. All alfalfa fields were harvested multiple times, though in 2023, field four was not used for alfalfa (Table 1). Samples were brought back to Colorado State University (CSU) campus, where aphids were identified to genus or species using a dissecting microscope and counted. Sweeping of alfalfa fields ceased on 8 August 2022 and 20 July 2023. Pan traps were not used in alfalfa fields as cutting dates were variable with risk of sample loss.

Aphid surveys in experimental research plots

In addition to sweeping alfalfa fields, the aphids were also sampled within pepper plots using yellow pan traps. These traps were interspersed within field research plots described in Chapter 2 (Figure 1). The dimensions of the yellow pan traps were 34.29 cm x 20.32 cm x 12.7 cm (JO-ANN STORES LLC, Top Notch, Hudson, OH, USA). Pans were filled with 1.25 L of propylene glycol (Sanco Industries, Inc, Fort Wayne, IN, USA) and placed between rows of chile peppers as soon as the peppers were planted. Six traps were placed within each planting date block in 2022, and three in 2023, and were spaced 3 m apart and weighed down with three small rocks 0.54 kg each within the pan trap. Surveys of aphids within the pepper plots ceased on 8 August 2022 and 14 July 2023.

Collection of samples from pan traps occurred weekly by filtering the contents of yellow trap pans over an organza fabric filter (JO-ANN STORES LLC, Hudson, OH, USA) into 50 mL conical tubes filled with 90% ethanol. Pan traps were refilled with up to 1.25 L of recycled or fresh propylene glycol depending on the condition of the solution. In 2023, pan traps were

collected every three to four days throughout the sampling period. Samples were brought back to the laboratory and processed as described above. Sweep nets were not used in chile pepper plots as they were not applicable and would potentially damage pepper plants.

Statistical analyses

Heatmaps were created using total species counts divided by the entire aphid community identified that year (Wickham 2016). Data for aphid abundance in alfalfa and the chile pepper plots were analyzed by comparing mean species densities within each sampling date using ANOVA. Data that did not meet assumptions of ANOVA were transformed (square root or log) or analyzed using non-parametric tests Kruskal-Wallis and Dunn's test. All analyses were performed in R statistical analysis software (R Core Team 2023).

Results

Samples collected from pan traps in experimental peppers plots had almost three times the number of species compared to aphids surveyed in alfalfa fields in 2022 (Figure 9). Up to 14 different species were identified in pan trap samples, and *Aphis* spp. was the dominating taxa comprising almost 60% of the total aphid community (Figure 9A). On the other hand, only five species of aphids were identified in alfalfa fields, with spotted alfalfa aphid as the most abundant species at approximately 86% of the aphid community (Figure 9B). Similar trends were seen in 2023, with chile pepper plots having higher diversity of aphid species than the alfalfa fields (Figure 10). Pan traps samples had a total of 15 species, with the blue alfalfa aphid being the most prevalent species, constituting around 35% of captured aphids (Figure 10A). *Aphis* spp. accounted for 29% of the aphid community, and pea aphids comprised nearly 20% of the samples. On the other hand, the most abundant species collected in alfalfa fields was the pea aphid, which comprised more than 90%

of the total aphid community (Figure 10B).

There were significant differences in densities of aphid species in experimental pepper plots across most of the growing season in 2022 (Table 3). *Aphis* spp. was more abundant than all other species across all sampling dates in 2022 and dominated most of the samples (Figure 12A). There were some notable peaks in *Aphis* spp. densities in early June, July, and August, and two of these dates (8 June, 8 August) were preceded by the harvesting of nearby alfalfa fields (Table 2), which may account for the sharp increases in density found in pan traps. Likewise, the densities of aphid species in alfalfa fields had significant variations during the majority of the 2022 growing season (Table 12). Between 8 June, and 28 June, spotted alfalfa aphid had substantially higher densities compared to all other species, surpassing the next most abundant species, pea aphid, by more than eightfold. Conversely, *Aphis* spp. had notably lower densities compared to all other species throughout the entire 2022 season (Figure 12B).

There were also significant differences in the densities of aphid species in pan traps in 2023 (Table 4). Surprisingly, blue alfalfa aphid was the most abundant species in pan trap samples, while *Aphis* spp. became more abundant later in the season (Figure 13A). From 2 June to 9 June there was a steep increase in density of *Aphis* spp., pea aphid, spotted alfalfa aphid, and blue alfalfa aphid, which was followed by a sharp decline in mid to late June. *Aphis* spp. made a small resurgence in early- to mid-July. On the other hand, in alfalfa fields, pea aphid was the most abundant species, with a distinct peak in their abundance noted in early June (Figure 13B). Samples on this date were some of the most highly abundant of all samples between both years, with pea aphid densities averaging over 200 individuals per sweep. The least abundant species in these samples was *Aphis* spp., with only one individual across the season. Of the aforementioned species, there are several that are confirmed vectors of AMV, such as the green peach aphid (Ahoonmanesh et al. 1990), bean aphid (van Leur et al. 2019), cowpea aphid (van Leur et al. 2019), pea aphid, blue alfalfa aphid, and spotted alfalfa aphid (Garran and Gibbs 1982).

Discussion

Aphid abundance and diversity were highly variable in each year. This is not surprising, as in June of 2023, much of Colorado had already received the average annual precipitation, making 2023 one of the wettest years on record, bringing the state out of a drought for the second time in 23 years (Butzer 2023). On the other hand, the year prior was characterized by more typical precipitation and generally drier conditions. Precipitation and climatic differences between years can have varying influences on aphid densities, with some species responding positively to excess moisture, while other species being unaffected by precipitation. For example, in a study examining aphid population dynamics and climate variation in wheat fields, researchers found that abundance of rose grass aphid *Metopolophium dirhodum* Walker (Hemiptera: Aphididae) was strongly correlated with precipitation while climatic factors had little to no impact on densities of bird cherry-oat aphid (Davis et al. 2014). Further, in another study analyzing 40 years of data, researchers in Korea found that spring flights for aphids was starting earlier as overall temperatures increased, and that the annual total of aphids had increased by over 3 times across those 40 years (Kim and Kwon 2019). Therefore, it is likely that the precipitation trends that were significantly different in 2022 and 2023 in my study had significant impact on the outcome of the surveys.

Although temperature and precipitation can influence aphid abundance, host plants and landscape diversity are likely to play a significant role in regulating aphid populations. For example, in a study using aphid population data over 24 years, aphid populations oscillated regularly over four years periods (Brabec et al. 2014). Aphid densities in this survey were highest in the early parts of the season, followed by an abrupt decline in density, which can be due to numerous factors including plant age, diversity, and impacts of natural enemies (Honek and Martinkova 2004, Davis et al. 2014). The high diversity of crops grown in the vicinity of my chile

pepper plots likely contributed to the significantly greater number of species of aphids I captured in my plots compared to alfalfa fields. Pepper plots were surrounded by a diverse array of crops including *Sorghum bicolor* L. (Poales: Poaceae), alfalfa, melons, cowpea *Vigna unguiculata* L. (Fabales: Fabaceae), corn, and numerous weeds such as kochia *Bassia scoparia* L (Caryophyllales: Amaranthaceae), and lamb's quarter *Chenopodium album* L. (Caryophyllales: Amaranthaceae).

There are few species of aphids that are known pests of chile peppers, although green peach aphid has been noted in this crop as its most common and damaging aphid pest (Sun et al. 2018, Chen et al. 2020). Green peach aphid was scarcely present in pan trap samples, and none were identified in alfalfa field samples. Further, its densities were much lower than many of the other aphids, such as pea aphid. It is important to note that I did not note any active feeding or infestation on any of the chile pepper plants in either of the years of the field experiment. Moreover, the abundance and diversity of aphids found in chile pepper plots was not driven by their densities and diversity in alfalfa fields.

In 2022, *Aphis* spp. were dominant in pan traps while spotted alfalfa aphids were dominant in alfalfa field sweeps. *Aphis* spp. is a highly diverse genus with over 600 species and has a broad host range (Kim et al. 2011). On the other hand, spotted alfalfa aphids prefer alfalfa and most clover (*Medicago* spp.), which was apparent in the 2022 alfalfa field surveys. Other studies corroborate my findings of spotted alfalfa aphid as the most abundant species in alfalfa. For example, in a study on alfalfa field ecology, spotted alfalfa aphid was the most abundant species found in over 40 alfalfa fields, and surprisingly, blue alfalfa aphid was not collected at any of the fields (Jovičić et al. 2016).

The trends observed in 2022 were not evident in 2023, however. Blue alfalfa aphid was the dominant species in pan traps that year, and pea aphid was the dominant species in alfalfa field surveys. Alfalfa and clover are common hosts for blue alfalfa aphid, as well as closely related genera such as yellow sweet clover *Melilotus officinalis* L. (Fabales: Fabaceae) (Kodet and Nielson

1980). Pea aphids tend to be legume specialists, rarely found colonizing other plants (Ramsey et al. 2010). Furthermore, there was higher species diversity and abundance in 2023, and pea aphid densities averaged more than 200 per sweep on 6 June. This significant spike in aphid abundance may have been reflected in the average OD values for 2023 (Chapter 2, Results), which were almost twice as high as the previous year's. While this preliminary finding should be explored further, it suggests that the abundance of pea aphids may be correlated with higher risk of AMV in peppers.

There were, however, several limitations to this survey. First, aphids were not tested for AMV, which should be explored in future research. This may be challenging due to the short length of time that the virus remains viable on the stylet of the aphids. Further, *Aphis* spp. were not identified to species, and although the level of identification was sufficient for this study, more information about species-specific host preference for these highly abundant insects could be relevant to their impact on AMV transmission. Lastly, deploying suction traps in the field at various locations to aid in determining aphid spatiotemporal flight patterns may aid in the scope and breadth of sampling as well.

This work helps us better understand the composition, and abundance of possible vectors of AMV in chile peppers and is the first survey of aphids in this system in Colorado. While more research is required to validate these findings, harvesting of neighboring alfalfa fields may not be as correlated to AMV incidence in peppers as first thought, as the populations found within each were composed of different species and variable densities throughout both years. Investigating aphids and their effectiveness of transmitting AMV may allow for management of their preferred hosts, which in turn could reduce the risk of AMV incidence in producers chile pepper fields.

REFERENCES

Ahoonmanesh, A., M. R. Hajimorad, B. J. Ingham, and R. I. B. Francki. 1990. Indirect double antibody sandwich ELISA for detecting alfalfa mosaic virus in aphids after short probes on infected plant. Journal of Virological Methods. 30: 271–281.

Brabec, M., A. Honěk, S. Pekár, and Z. Martinková. 2014. Population dynamics of aphids on cereals: Digging in the time-series data to reveal population regulation caused by temperature. PLoS ONE. 9: e106228.

Butzer, S. 2023. Colorado is drought-free for only the second time in 23 years. Denver 7 Colorado News. (Accessed 10/11/2023 https://www.denver7.com/news/local-news/colorado-is-drought-free-for-only-the-second-time-in-23-years#:~:text=releases%20the%20data.-,According%20to%20its%20map%20published%20Thursday%20morning%2C%20which%20depicts%20conditions,is%20100%25%2Ddrought%20free.&text=This%20is%20only%20the%20second,a%20hydrologist%20with%20the%20NWS.)

Chen, Q., X. Liang, and C. Wu. 2020. Trait inheritance in pepper (Capsicum spp.) cultivars identified as resistant to green peach aphid (Myzus persicae). Plant Breeding. 139: 996–1002.

Davis, T. S., J. T. Abatzoglou, N. A. Bosque-Pérez, S. E. Halbert, K. Pike, and S. D. Eigenbrode. 2014. Differing contributions of density dependence and climate to the population dynamics of three eruptive herbivores. Ecological Entomology. 39: 566–577.

Garran, J., and A. Gibbs. 1982. Studies on alfalfa mosaic virus and alfalfa aphids. Australian Journal of Agricultural Research 33: 657.

Honek, A., and Z. Martinkova. 2004. Host plant age and population development of a cereal aphid, Metopolophium dirhodum (Hemiptera: Aphididae). Bulletin of Entomological Research 94: 19–26.

Jones, R. A. C., and B. A. Coutts. 1996. Alfalfa mosaic and cucumber mosaic virus infection in chickpea and lentil: incidence and seed transmission. Annals Applied Biology. 129: 491–506.

Jovičić, I., A. Radonjić, and O. Petrović-Obradović. 2016. Aphids (Hemiptera: Aphididae) on alfalfa and their coccinellid predators in Serbia: seasonal abundance. Acta zoologica bulgarica. 68: 581–587.

Kim, H., S. Lee, and Y. Jang. 2011. Macroevolutionary patterns in the Aphidini Aphids (Hemiptera: Aphididae): Diversification, host association, and biogeographic origins. PLoS ONE. 6: e24749.

Kim, J., and M. Kwon. 2019. Population dynamics of aphid species in Korean seed potato cultivation area over four decades. Entomological Research. 49: 179–184.

Kodet, R. T., and M. W. Nielson. 1980. Effect of temperature and photoperiod on polymorphisms of the blue alfalfa aphid, Acyrthosiphon kondoi. Environmental Entomology. 9: 94–96.

van Leur, J., Z. Duric, J. George, and S. Boschma. 2019. Alfalfa mosaic virus infects the tropical legume Desmanthus virgatus in Australia and the potential role of the cowpea aphid (Aphis craccivora) as the virus vector. Australasian Plant Disease. Notes. 14: 3.

van Leur, J., S. Kumari, M. Aftab, A. Leonforte, and S. Moore. 2013. Virus resistance of Australian pea (Pisum sativum) varieties. New Zealand Journal of Crop and Horticultural Science. 41: 86–101.

R Core Team, C. T. 2023. R: A language and environment for statistical computing: reference index. R Foundation for Statistical Computing, Vienna.

Ramsey, J. S., D. S. Rider, T. K. Walsh, M. De Vos, K. H. J. Gordon, L. Ponnala, S. L. Macmil, B. A. Roe, and G. Jander. 2010. Comparative analysis of detoxification enzymes in Acyrthosiphon pisum and Myzus persicae. Insect Molecular Biology. 19: 155–164.

Sofy, A. R., M. R. Sofy, A. A. Hmed, R. A. Dawoud, E. E. Refaey, H. I. Mohamed, and N. K. El-Dougdoug. 2021. Molecular Characterization of the Alfalfa mosaic virus Infecting Solanum melongena in Egypt and the Control of Its Deleterious Effects with Melatonin and Salicylic Acid. Plants. 10: 459.

Sun, M., R. E. Voorrips, G. Steenhuis-Broers, W. Van'T Westende, and B. Vosman. 2018. Reduced phloem uptake of Myzus persicae on an aphid resistant pepper accession. BioMed Central (BMC) Plant Biology. 18: 138.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysts. Springer-Verlag New York.

Yardimci, N., H. Eryigit, and I. Erda. 2007. Effect of Alfalfa mosaic virus (AMV) on the content of some macro-and micronutrients in alfalfa. Journal of culture collections. 5: 90–93.

Table 2. List of alfalfa fields and harvest dates

Field	Coordinates	Harvest dates	
		2022	2023
1	38°02'16.3"N, 103°41'39.6"W	31 May	24 May, 18 July, 22 August
2	38°02'20.2"N, 103°41'44.7"W	31 May, 13 July, 2 August	24 May,18 July, 22 August
3	38°02'27.3"N, 103°41'38.6"W	31 May, 13 July, 2 August	24 May, 18 July, 22 August
4	38°02'21.8"N, 103°41'28.4"W	31 May	N/A
5	38°02'22.2"N, 103°41'22.8"W	31 May, 2 August	24 May, 18 July, 22 August

Table 3. Results of statistical analyses comparing aphid abundance in alfalfa fields on each sampling date.

Date	Test	df	*P*
2022			
2 June	F = 4.41	3, 12	$P = 0.026$
8 June	$X^2 = 21.74$	3	$P < 0.001$
22 June 2022	$X^2 = 53.57$	5	$P < 0.001$
28 June 2022	$X^2 = 66.42$	3	$P < 0.001$
2 August 2022	F = 1.67	3, 16	$P = 0.214$
8 August 2022	$X^2 = 22.32$	3	$P < 0.001$
2023			
24 May 2023	$X^2 = 25.02$	4	$P < 0.001$
6 June 2023	$X^2 = 16.25$	4	$P = 0.003$
14 June 2023	$X^2 = 32.57$	3	$P < 0.001$
21 June 2023	$X^2 = 9.08$	4	$P = 0.06$
30 June 2023	$X^2 = 3.17$	2	$P = 0.53$
20 July 2023	$X^2 = 5.82$	4	$P = 0.211$

Table 4. Results of statistical analyses comparing aphid abundance in pan traps on each sampling date.

Date	Test	df	*P*
2022			
26 May 2022	$X^2 = 19.4$	13	$P = 0.11$
2 June 2022	$X^2 = 69.47$	11	$P < 0.001$
8 June 2022	$X^2 = 135.26$	11	$P < 0.001$
16 June 2022	$X^2 = 111.13$	12	$P < 0.001$
22 June 2022	$X^2 = 199.15$	12	$P < 0.001$
28 June 2022	$X^2 = 25.02$	11	$P < 0.001$
8 July 2022	$X^2 = 16.25$	12	$P = 0.003$
12 July 2022	$X^2 = 32.57$	13	$P < 0.001$
16 July 2022	$X^2 = 9.08$	11	$P = 0.06$
27 July 2022	$X^2 = 3.17$	11	$P = 0.53$
2 August 2022	$X^2 = 5.82$	11	$P = 0.211$
8 August 2022	$X^2 = 72.99$	11	$P < 0.001$
2023			
2 June 2023	$X^2 = 48.69$	11	$P < 0.001$
9 June 2023	$X^2 = 53.04$	11	$P < 0.001$
16 June 2023	$X^2 = 11.21$	11	$P = 0.443$
23 June 2023	$X^2 = 7.76$	12	$P = 0.774$
30 June 2023	$X^2 = 22.63$	11	$P = 0.02$
7 July 2023	$X^2 = 57.96$	11	$P < 0.001$
14 July 2023	$X^2 = 52.52$	11	$P < 0.001$



Figure 9. Yellow pan traps interspersed throughout experimental pepper plot.

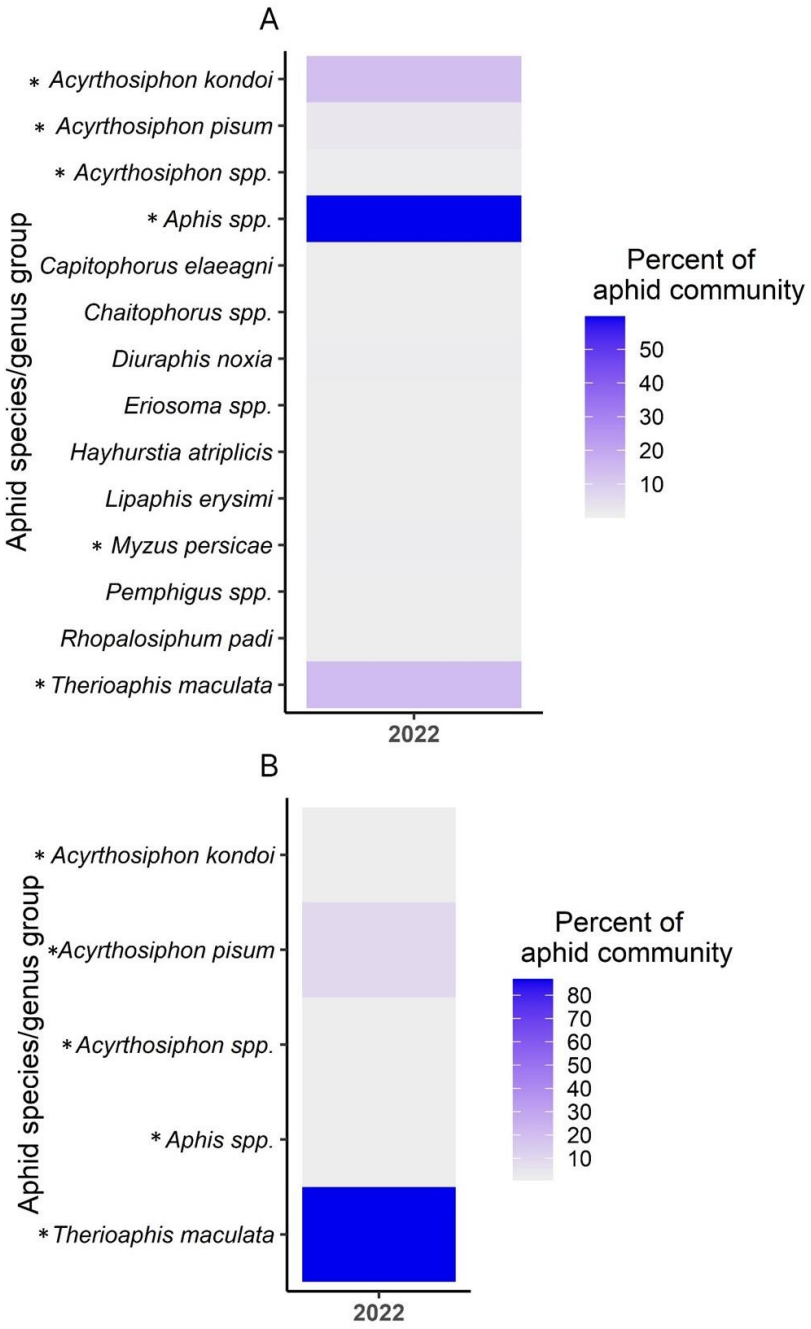


Figure 10. Heatmap of total aphid diversity in 2022 in pan traps (A) and sweep nets (B). Darker colors indicate a higher percentage of total aphid community. Asterisks next to a species or genus mark known vectors of AMV. Pan traps (A) had almost three times the diversity as alfalfa field samples (B), Aphis spp. was the most abundant species collected in chile pepper plots throughout 2022, followed by *T. maculata* and *A. kondoi* as the next most abundant species. On the other hand, *T. maculata* was the most abundant species found in alfalfa fields.

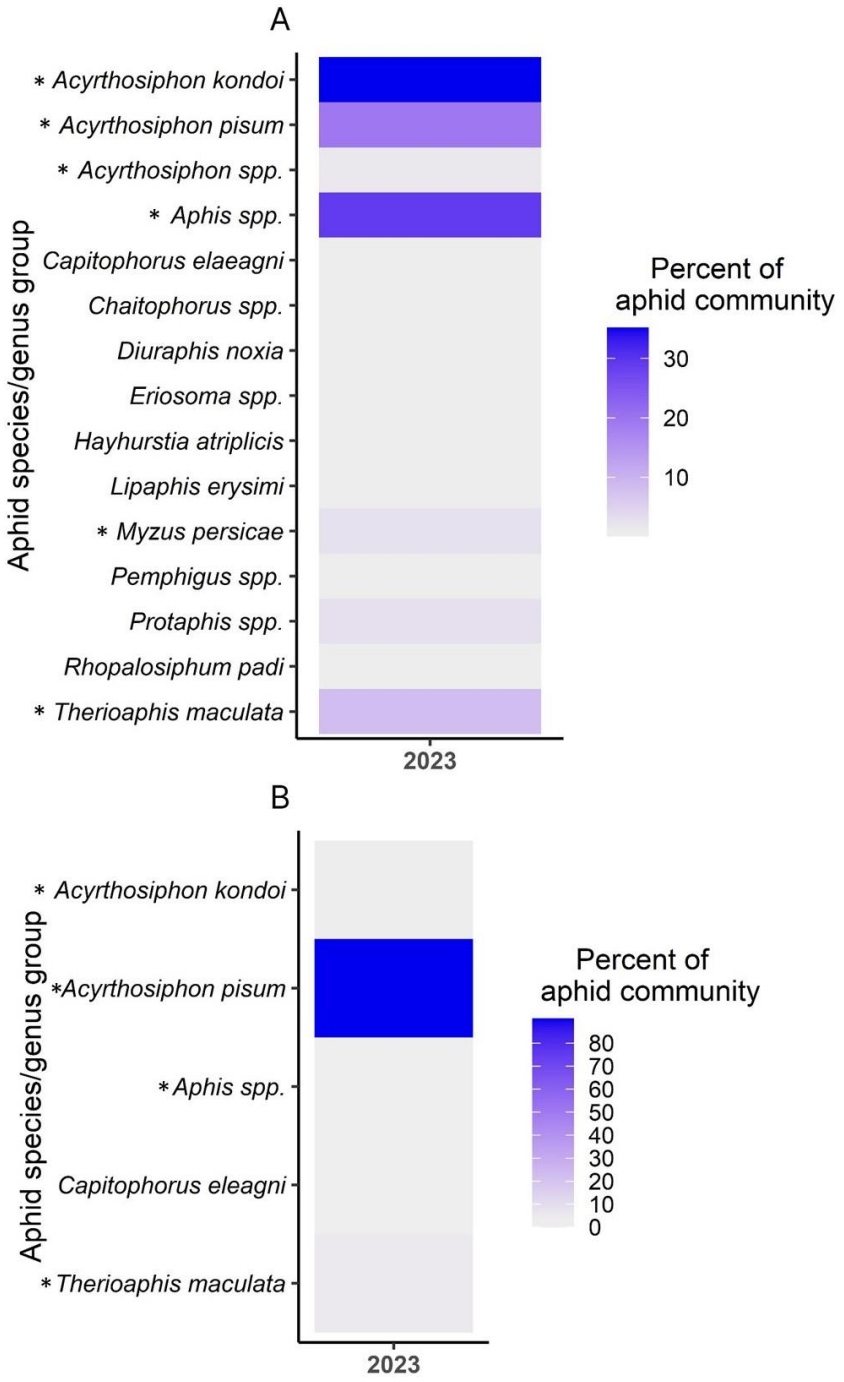


Figure 11**.** Heatmap of total aphid diversity in 2023 in pan traps (A) and sweep nets (B). Darker colors indicate a higher percentage of total aphid community. Asterisks next to a species or genus mark known vectors of AMV. Pan traps (A) had almost three times the diversity as alfalfa field samples (B), A. *kondoi* was the most abundant species collected in chile pepper plots throughout 2023, followed by *Aphis* spp. and *A. pisum* as the next most abundant species. On the other hand, *A. pisum* was the most abundant species found in alfalfa field.

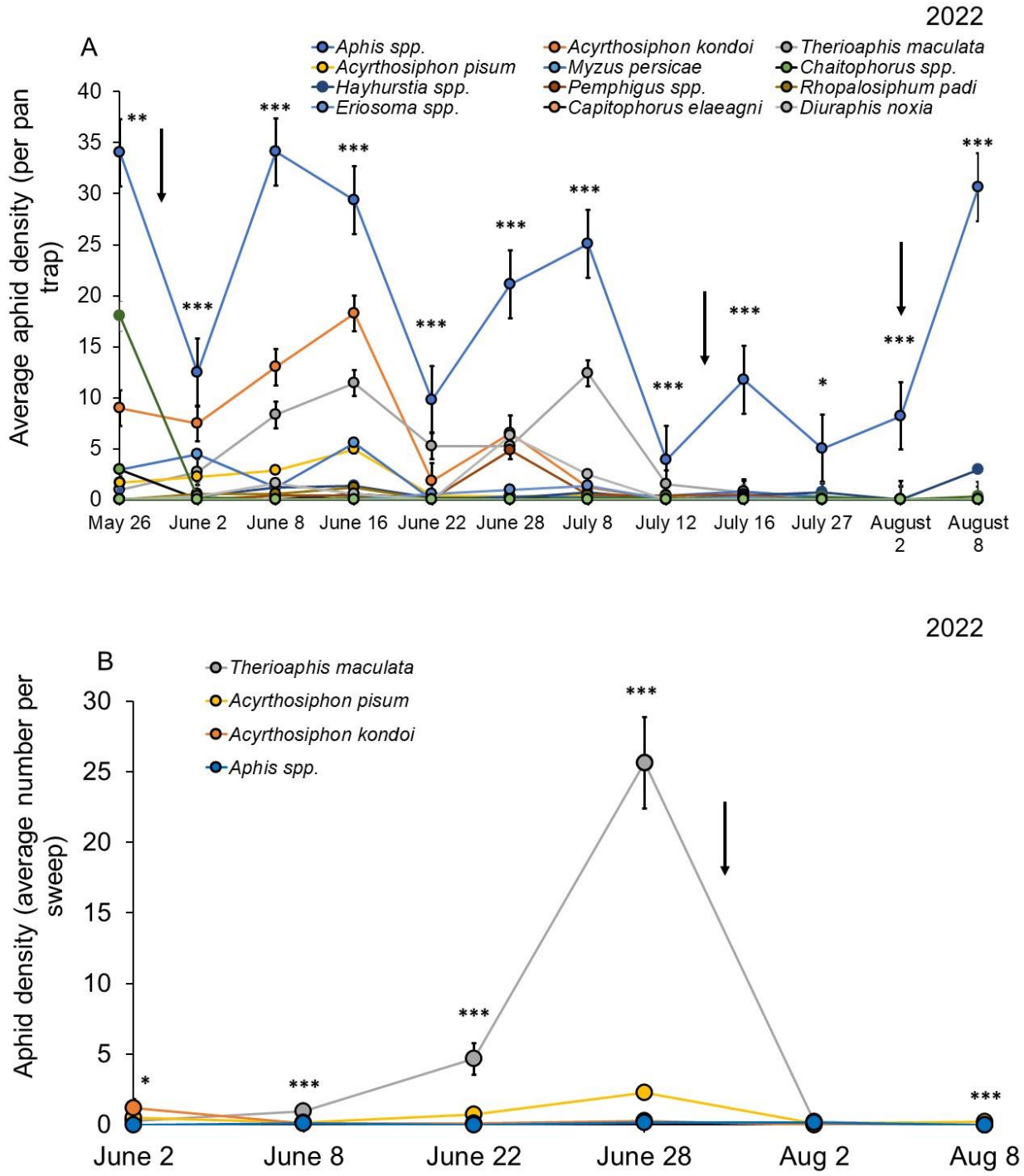


Figure 12**.** Densities of the most abundant aphid species in chile pepper plots (A) and alfalfa fields (B) in 2022. Markers represent means, bars are ±1 SEM, arrows point to alfalfa cut dates. Asterisks denote the level of statistical significance (*$P = 0.05$, ***$P = 0.001$). Aphids were collected weekly using 18 yellow pan traps filled with propylene glycol (A) and weekly to every 10 days in five alfalfa fields using sweep nets (B). *Aphis sp.* were the most abundant species of aphids in the pan traps on

all sampling dates, whereas *T. maculata* was the most abundant species of aphids collected in alfalfa fields.

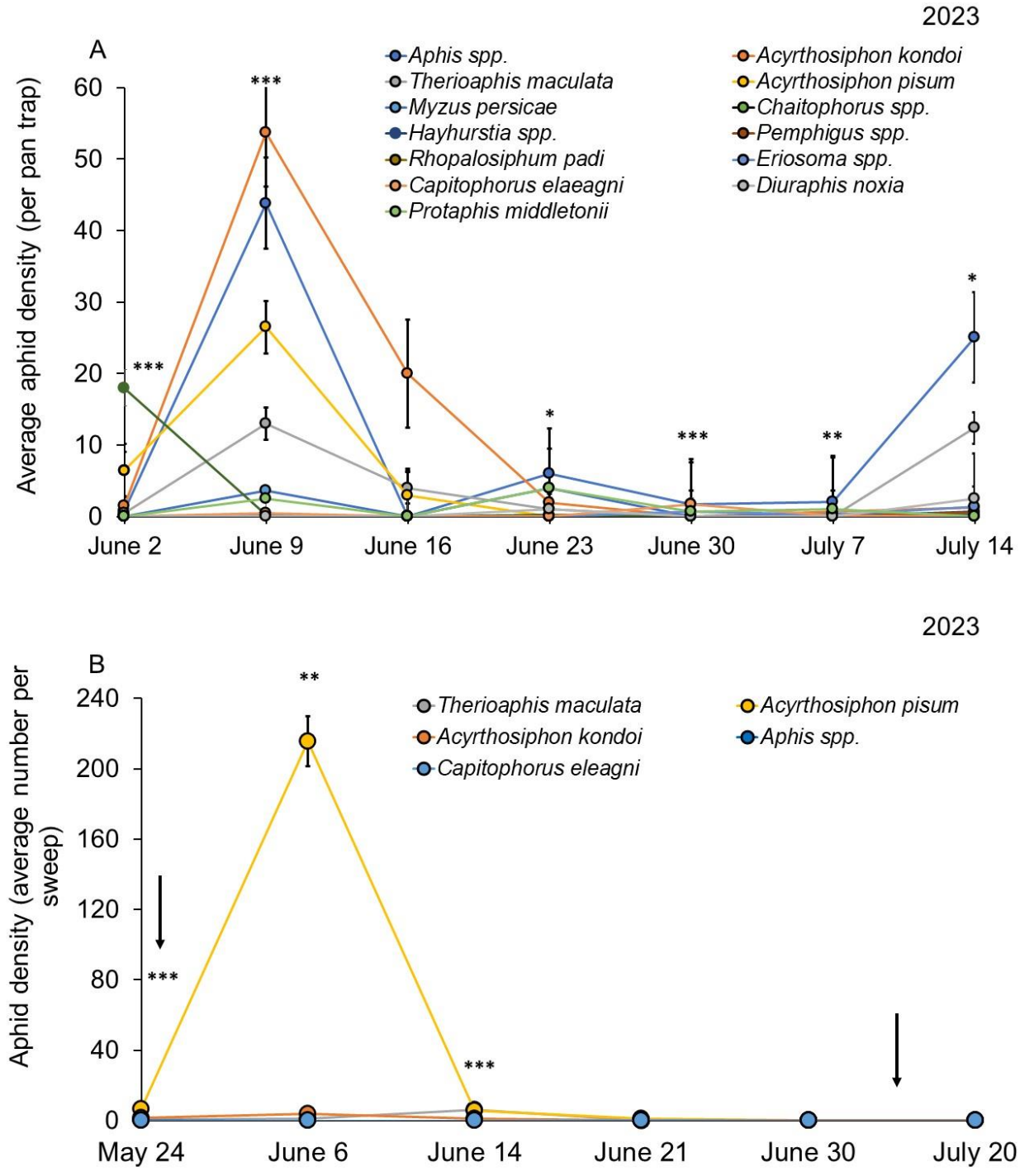


Figure 13. Densities of the most abundant aphid species in chile pepper plots (A) and alfalfa fields (B) in 2023. Markers represent means, bars are ±1 SEM, arrows point to alfalfa cut dates. Asterisks denote the level of statistical significance (**P = 0.01 ***P = 0.001). Aphids were collected weekly using 9 yellow pan traps filled with propylene glycol (A) and weekly to every 10 days in five alfalfa fields using sweep nets (B). *A. kondoi* was the most abundant species of aphids in the pan traps early in the season, whereas *A. pisum* was the most abundant species of aphids collected in alfalfa fields

and averaged over 200 aphids per sweep on 6 June 2023.

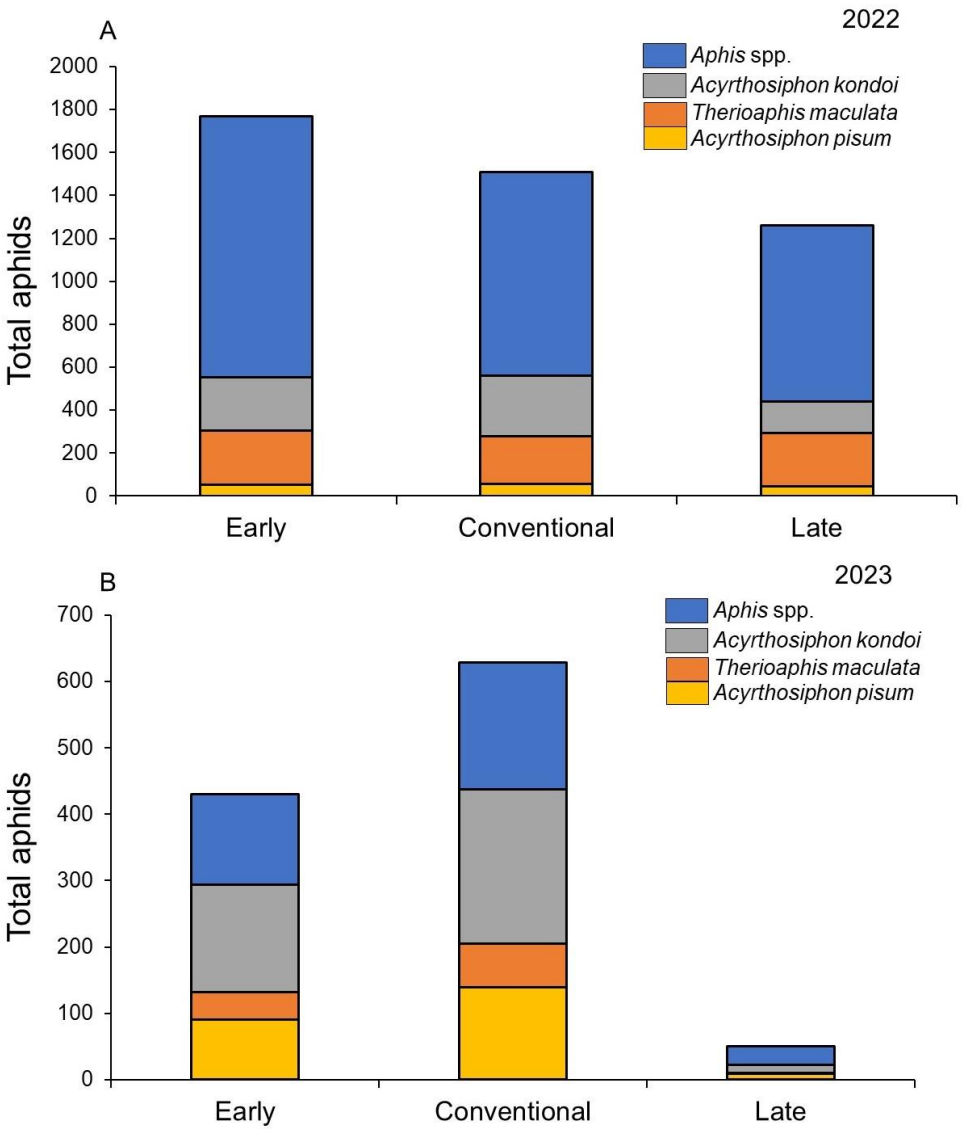

A
2022
Aphis spp.
Acyrthosiphon kondoi
Therioaphis maculata
Acyrthosiphon pisum
Total aphids
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Early
Conventional
Late
B
2023
Aphis spp.
Acyrthosiphon kondoi
Therioaphis maculata
Acyrthosiphon pisum
Total aphids
700
600
500
400
300
200
100
0
Early
Conventional
Late

Figure 14. Total number of the most common species of aphids collected in chile pepper plots by species in 2022 (A) and 2023 (B). Plots with late planted peppers had substantially lower total number of aphids collected from pan traps compared to conventional and early plantings.

CONCLUSIONS

The goal of this research was to explore management strategies to suppress the virus given its devastating impact on the yield and quality of chile peppers. I found that innate host plant resistance had the most powerful effect on decreasing incidence of AMV and improving plant fitness (Figure 1A). Furthermore, peppers planted early appeared to have higher symptoms and titers of the virus, which was likely related to higher abundance of the aphid vectors early in the season (Fig. 1A). Aphid abundance was markedly lower later in the season, which likely lowered the risk of infection. Early planted peppers with resistance to AMV are likely to suffer the lowest yield losses without any additional inputs (Figure 1B). This research contributed to formulating integrated pest management tactics that chile pepper producers can implement in their production to suppress the impact of AMV on the crop. Finally, this is the first report of AMV transmission through seed in peppers and is the first study describing this pathosystem in Colorado.

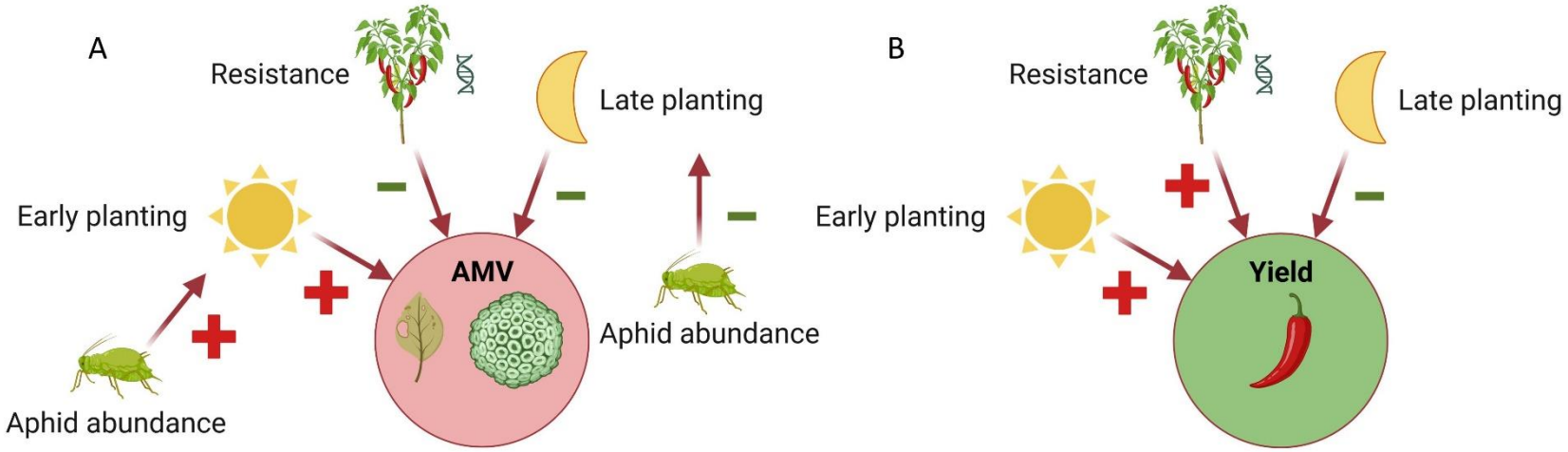


Figure 15. Conceptual model summarizing the outcomes of research on integrative tactics to suppress AMV in chile peppers. Severity and titers of AMV were positively correlated with earlier planting date, which was likely related to higher aphid densities early in the season (A). Yield of resistant peppers planted early was significantly greater than that of peppers planted later in the season and lacking resistance (B).

APPENDIX A: CHAPTER 1 SUPPLEMENTARY MATERIAL

Table 5. Mean separation using Tukey's HSD for proportion of leaf discoloration in greenhouse experiment.

Variety Comparison	*P* value
CSU-243-12-Pueblo Primrose	$P < 0.001$
CSU-243-12-Yellow Bardo	$P < 0.01$
CSU-243-12-Charger	$P < 0.01$
CSU-432-20-Pueblo Primrose	$P < 0.01$
Sonora-Pueblo Primrose	$P < 0.05$
Joe Parker-Mira Sol	$P < 0.05$
CSU-243-12-Desperado	$P < 0.05$
CSU-243-12-Giant Marconi	$P < 0.05$
Karisma-Pueblo Primrose	$P < 0.05$
CSU-432-20-Yellow Bardo	$P < 0.05$
CSU-432-20-Yellow Bardo	$P < 0.05$
CSU-243-12-Pueblo Popper	$P < 0.05$
CSU-432-20-Charger	0.053
Sonora-Yellow Bardo	0.073
Sonora-Charger	0.084
CSU-243-12-Mira Sol	0.090
Carne Duro-Pueblo Primrose	0.098
Joe Parker-Yellow Bardo	0.105
Joe Parker-Charger	0.117
Karisma-Yellow Bardo	0.155
Big Jim-Pueblo Primrose	0.160
Karisma-Charger	0.177
Aristotle-Pueblo Primrose	0.198

CSU-420-19-Pueblo Primrose	0.203
CSU-442-20-Pueblo Primrose	0.219
CSU-432-20-Desperado	0.242
CSU-432-20-Giant Marconi	0.253
Carne Duro-Yellow Bardo	0.253
Lexus-Pueblo Primrose	0.263
Carne Duro-Charger	0.265
CSU-RLC-Pueblo Primrose	0.289
CSU-432-20-Pueblo Popper	0.305
Sonora-Desperado	0.326
Sonora-Giant Marconi	0.338
Mosco-Pueblo Primrose	0.355
Abay-Pueblo Primrose	0.385
Big Jim-Yellow Bardo	0.388
Big Jim-Charger	0.402
Sonora-Pueblo Popper	0.405
Milena-Pueblo Primrose	0.411
Joe Parker-Desperado	0.413
CSU-243-12-Masivo	0.416
Joe Parker-Giant Marconi	0.427
Aristotle-Yellow Bardo	0.449
Aristotle-Charger	0.460
CSU-420-19-Yellow Bardo	0.474
Tam Vera Cruz-Pueblo Primrose	0.486
CSU-420-19-Charger	0.490
CSU-442-20-Yellow Bardo	0.499
CSU-432-20-Mira Sol	0.500
Joe Parker-Pueblo Popper	0.503
CSU-442-20-Charger	0.514
Lexus-Yellow Bardo	0.540
Lexus-Charger	0.547
CSU-RLC-Yellow Bardo	0.574

Karisma-Desperado	0.575
CSU-243-12-Anaheim118	0.578
CSU-RLC-Charger	0.579
Karisma-Giant Marconi	0.591
CSU-243-12-Grenada	0.607
Sonora-Mira Sol	0.616
Mosco-Charger	0.622
Mosco-Yellow Bardo	0.627
Karisma-Pueblo Popper	0.674
Abay-Charger	0.679
Abay-Yellow Bardo	0.680
Carne Duro-Desperado	0.685
Giadone-Pueblo Primrose	0.691
Carne Duro-Giant Marconi	0.698
Milena-Charger	0.704
Milena-Yellow Bardo	0.706
G76-Pueblo Primrose	0.712
Joe Parker-Mira Sol	0.720
CSU-243-12-Sweet Delilah	0.735
Carne Duro-Pueblo Popper	0.778
Tam Vera Cruz-Charger	0.799
Tam Vera Cruz-Yellow Bardo	0.800
CSU-243-12-Tam Vera Cruz	0.813
Big Jim-Desperado	0.847
CSU-432-20-Masivo	0.851
Big Jim-Giant Marconi	0.857
Karisma-Mira Sol	0.873
Giadone-Charger	0.885
CSU-243-12-G76	0.885
Aristotle-Desperado	0.888
Sonora-Masivo	0.890
Aristotle-Giant Marconi	0.896

Giadone-Yellow Bardo	0.897
G76-Charger	0.909
CSU-420-19-Desperado	0.914
Big Jim-Pueblo Popper	0.914
G76-Yellow Bardo	0.918
CSU-420-19-Giant Marconi	0.921
CSU-442-20-Desperado	0.925
Carne Duro-Mira Sol	0.929
CSU-442-20-Giant Marconi	0.932
Lexus-Desperado	0.933
Joe Parker-Masivo	0.935
CSU-243-12-California Wonder	0.936
Grenada-Pueblo Primrose	0.937
Lexus-Giant Marconi	0.939
Aristotle-Pueblo Popper	0.943
CSU-RLC-Desperado	0.946
Anaheim118-Pueblo Primrose	0.948
CSU-432-20-Anaheim118	0.949
CSU-RLC-Giant Marconi	0.951
Mosco-Desperado	0.954
CSU-432-20-Grenada	0.957
Mosco-Giant Marconi	0.958
CSU-420-19-Pueblo Popper	0.960
Sonora-Anaheim118	0.966
CSU-442-20-Pueblo Popper	0.967
Sweet Delilah-Pueblo Primrose	0.969
California Wonder-Pueblo	0.970
Primrose	0.971
Lexus-Pueblo Popper	0.972
Sonora-Grenada	0.975
Abay-Desperado	0.978
CSU-RLC-Pueblo Popper	0.978

Abay-Giant Marconi	0.978
CSU-243-12-Giadone	0.979
CSU-432-20-Sweet Delilah	0.980
Milena-Desperado	0.982
Mosco-Pueblo Popper	0.983
Milena-Giant Marconi	0.985
Joe Parker-Anaheim118	0.986
Sonora-Sweet Delilah	0.986
CSU-243-12-Milena	0.987
Big Jim-Mira Sol	0.988
Joe Parker-Grenada	0.989
Karisma-Masivo	0.989
CSU-243-12-Abay	0.990
Grenada-Charger	0.991
Mira Sol-Pueblo Primrose	0.992
CSU-243-12-CSU-442-20	0.992
Carne Duro-Masivo	0.992
Abay-Pueblo Popper	0.992
Anaheim118-Charger	0.993
Grenada-Yellow Bardo	0.993
Aristotle-Mira Sol	0.994
CSU-243-12-CSU-420-19	0.994
California Wonder-Charger	0.994
Milena-Pueblo Popper	0.994
Joe Parker-Sweet Delilah	0.995
Anaheim118-Yellow Bardo	0.995
Tam Vera Cruz-Desperado	0.995
Sweet Delilah-Charger	0.996
CSU-432-20-Tam Vera Cruz	0.996
Tam Vera Cruz-Giant Marconi	0.996
California Wonder-Yellow Bardo	0.997
CSU-243-12-CSU-RLC	0.997

CSU-420-19-Mira Sol	0.997
Sweet Delilah-Yellow Bardo	0.998
CSU-442-20-Mira Sol	0.998
Sonora-Tam Vera Cruz	0.998
CSU-243-12-Lexus	0.998
CSU-432-20-G76	0.998
Lexus-Mira Sol	0.998
Giadone-Desperado	0.998
Giadone-Giant Marconi	0.998
CSU-432-20-California Wonder	0.999
CSU-RLC-Mira Sol	0.999
Sonora-G76	0.999
Mosco-Mira Sol	0.999
Masivo-Pueblo Primrose	0.999
Sonora-California Wonder	0.999
Karisma-Anaheim118	0.999
Tam Vera Cruz-Pueblo Popper	0.999
G76-Desperado	0.999
Carne Duro-Anaheim118	0.999
G76-Giant Marconi	0.999
Karisma-Grenada	0.999
CSU-243-12-Aristotle	0.999
Big Jim-Masivo	0.999
Joe Parker-Tam Vera Cruz	1.000
Carne Duro-Grenada	1.000
CSU-243-12-Mosco	1.000
Mira Sol-Charger	1.000
Joe Parker-G76	1.000
Giadone-Pueblo Popper	1.000
Joe Parker-California Wonder	1.000
Abay-Mira Sol	1.000
Aristotle-Masivo	1.000

CSU-243-12-Big Jim	1.000
Karisma-Sweet Delilah	1.000
Mira Sol-Yellow Bardo	1.000
Carne Duro-Sweet Delilah	1.000
Milena-Mira Sol	1.000
CSU-432-20-Giadone	1.000
G76-Pueblo Popper	1.000
Masivo-Charger	1.000
CSU-420-19-Masivo	1.000
Sonora-Giadone	1.000
Lexus-Masivo	1.000
CSU-442-20-Masivo	1.000
Mosco-Masivo	1.000
CSU-RLC-Masivo	1.000
Masivo-Yellow Bardo	1.000
CSU-432-20-Milena	1.000
Sonora-Milena	1.000
CSU-432-20-Abay	1.000
Pueblo Popper-Pueblo Primrose	1.000
Joe Parker-Giadone	1.000
Big Jim-Anaheim118	1.000
CSU-243-12-Karisma	1.000
Sonora-Abay	1.000
Big Jim-Grenada	1.000
CSU-432-20-CSU-442-20	1.000
Tam Vera Cruz-Mira Sol	1.000
Carne Duro-California Wonder	1.000
Abay-Masivo	1.000
California Wonder-Desperado	1.000
Sonora-CSU-442-20	1.000
Grenada-Desperado	1.000
CSU-432-20-CSU-420-19	1.000

Aristotle-Anaheim118	1.000
Carne Duro-Tam Vera Cruz	1.000
California Wonder-Giant Marconi	1.000
Karisma-Tam Vera Cruz	1.000
Karisma-California Wonder	1.000
Milena-Masivo	1.000
Grenada-Giant Marconi	1.000
Carne Duro-G76	1.000
Joe Parker-Milena	1.000
Sonora-CSU-420-19	1.000
Big Jim-Sweet Delilah	1.000
Giadone-Mira Sol	1.000
Anaheim118-Desperado	1.000
Aristotle-Grenada	1.000
Karisma-G76	1.000
CSU-432-20-CSU-RLC	1.000
Anaheim118-Giant Marconi	1.000
Joe Parker-Abay	1.000
Sweet Delilah-Desperado	1.000
Sonora-CSU-RLC	1.000
Sweet Delilah-Giant Marconi	1.000
CSU-432-20-Lexus	1.000
Sonora-Lexus	1.000
Aristotle-Sweet Delilah	1.000
CSU-420-19-Anaheim118	1.000
Lexus-Anaheim118	1.000
Joe Parker-CSU-442-20	1.000
Mosco-Anaheim118	1.000
Pueblo Popper-Charger	1.000
CSU-442-20-Anaheim118	1.000
CSU-420-19-Grenada	1.000
G76-Mira Sol	1.000

Lexus-Grenada	1.000
Joe Parker-CSU-420-19	1.000
CSU-RLC-Anaheim118	1.000
Mosco-Grenada	1.000
California Wonder-Pueblo Popper	1.000
CSU-442-20-Grenada	1.000
Joe Parker-CSU-RLC	1.000
CSU-243-12-Carne Duro	1.000
CSU-432-20-Mosco	1.000
CSU-RLC-Grenada	1.000
Grenada-Pueblo Popper	1.000
Giant Marconi-Pueblo Primrose	1.000
Sonora-Mosco	1.000
CSU-432-20-Aristotle	1.000
Sonora-Aristotle	1.000
Lexus-Sweet Delilah	1.000
CSU-420-19-Sweet Delilah	1.000
Mosco-Sweet Delilah	1.000
Desperado-Pueblo Primrose	1.000
Joe Parker-Lexus	1.000
Anaheim118-Pueblo Popper	1.000
Pueblo Popper-Yellow Bardo	1.000
Giadone-Masivo	1.000
CSU-442-20-Sweet Delilah	1.000
Carne Duro-Giadone	1.000
Sweet Delilah-Pueblo Popper	1.000
CSU-RLC-Sweet Delilah	1.000
Tam Vera Cruz-Masivo	1.000
Big Jim-California Wonder	1.000
CSU-432-20-Big Jim	1.000
Sonora-Big Jim	1.000
Karisma-Giadone	1.000

Abay-Anaheim118	1.000
Joe Parker-Mosco	1.000
Milena-Anaheim118	1.000
Abay-Grenada	1.000
Joe Parker-Aristotle	1.000
Aristotle-California Wonder	1.000
Big Jim-Tam Vera Cruz	1.000
Big Jim-G76	1.000
Milena-Grenada	1.000
G76-Masivo	1.000
Carne Duro-Milena	1.000
Giant Marconi-Charger	1.000
Abay-Sweet Delilah	1.000
Desperado-Charger	1.000
Carne Duro-Abay	1.000
Joe Parker-Big Jim	1.000
Milena-Sweet Delilah	1.000
Mira Sol-Desperado	1.000
Karisma-Milena	1.000
Mira Sol-Giant Marconi	1.000
Mosco-California Wonder	1.000
Aristotle-G76	1.000
Aristotle-Tam Vera Cruz	1.000
Lexus-California Wonder	1.000
Masivo-Desperado	1.000
CSU-420-19-California Wonder	1.000
Karisma-Abay	1.000
Masivo-Giant Marconi	1.000
CSU-RLC-California Wonder	1.000
Carne Duro-CSU-442-20	1.000
CSU-442-20-California Wonder	1.000
Giant Marconi-Yellow Bardo	1.000

Charger-Pueblo Primrose	1.000
Yellow Bardo-Pueblo Primrose	1.000
Yellow Bardo-Charger	1.000
Desperado-Yellow Bardo	1.000
Giant Marconi-Desperado	1.000
Pueblo Popper-Desperado	1.000
Pueblo Popper-Giant Marconi	1.000
Mira Sol-Pueblo Popper	1.000
Masivo-Pueblo Popper	1.000
Masivo-Mira Sol	1.000
Anaheim118-Mira Sol	1.000
Grenada-Mira Sol	1.000
Sweet Delilah-Mira Sol	1.000
California Wonder-Mira Sol	1.000
Anaheim118-Masivo	1.000
Grenada-Masivo	1.000
Sweet Delilah-Masivo	1.000
California Wonder-Masivo	1.000
Grenada-Anaheim118	1.000
Sweet Delilah-Anaheim118	1.000
California Wonder-Anaheim118	1.000
G76-Anaheim118	1.000
Tam Vera Cruz-Anaheim118	1.000
Giadone-Anaheim118	1.000
Sweet Delilah-Grenada	1.000
California Wonder-Grenada	1.000
G76-Grenada	1.000
Tam Vera Cruz-Grenada	1.000
Giadone-Grenada	1.000
California Wonder-Sweet Delilah	1.000
G76-Sweet Delilah	1.000
Tam Vera Cruz-Sweet Delilah	1.000

Giadone-Sweet Delilah	1.000
G76-California Wonder	1.000
Tam Vera Cruz-California Wonder	1.000
Giadone-California Wonder	1.000
Milena-California Wonder	1.000
Abay-California Wonder	1.000
Tam Vera Cruz-G76	1.000
Giadone-G76	1.000
Milena-G76	1.000
Abay-G76	1.000
CSU-442-20-G76	1.000
CSU-RLC-G76	1.000
CSU-420-19-G76	1.000
Lexus-G76	1.000
Mosco-G76	1.000
Giadone-Tam Vera Cruz	1.000
Milena-Tam Vera Cruz	1.000
Abay-Tam Vera Cruz	1.000
CSU-442-20-Tam Vera Cruz	1.000
CSU-RLC-Tam Vera Cruz	1.000
CSU-420-19-Tam Vera Cruz	1.000
Lexus-Tam Vera Cruz	1.000
Mosco-Tam Vera Cruz	1.000
Milena-Giadone	1.000
Abay-Giadone	1.000
CSU-442-20-Giadone	1.000
CSU-RLC-Giadone	1.000
CSU-420-19-Giadone	1.000
Lexus-Giadone	1.000
Mosco-Giadone	1.000
Aristotle-Giadone	1.000

Big Jim-Giadone	1.000
Abay-Milena	1.000
CSU-442-20-Milena	1.000
CSU-RLC-Milena	1.000
CSU-420-19-Milena	1.000
Lexus-Milena	1.000
Mosco-Milena	1.000
Aristotle-Milena	1.000
Big Jim-Milena	1.000
CSU-442-20-Abay	1.000
CSU-RLC-Abay	1.000
CSU-420-19-Abay	1.000
Lexus-Abay	1.000
Mosco-Abay	1.000
Aristotle-Abay	1.000
Big Jim-Abay	1.000
CSU-RLC-CSU-442-20	1.000
CSU-420-19-CSU-442-20	1.000
Lexus-CSU-442-20	1.000
Mosco-CSU-442-20	1.000
Aristotle-CSU-442-20	1.000
Big Jim-CSU-442-20	1.000
Karisma-CSU-442-20	1.000
CSU-420-19-CSU-RLC	1.000
Lexus-CSU-RLC	1.000
Mosco-CSU-RLC	1.000
Aristotle-CSU-RLC	1.000
Big Jim-CSU-RLC	1.000
Karisma-CSU-RLC	1.000
Carne Duro-CSU-RLC	1.000
Lexus-CSU-420-19	1.000
Mosco-CSU-420-19	1.000

Aristotle-CSU-420-19	1.000
Big Jim-CSU-420-19	1.000
Karisma-CSU-420-19	1.000
Carne Duro-CSU-420-19	1.000
Mosco-Lexus	1.000
Aristotle-Lexus	1.000
Big Jim-Lexus	1.000
Karisma-Lexus	1.000
Carne Duro-Lexus	1.000
Aristotle-Mosco	1.000
Big Jim-Mosco	1.000
Karisma-Mosco	1.000
Carne Duro-Mosco	1.000
Big Jim-Aristotle	1.000
Karisma-Aristotle	1.000
Carne Duro-Aristotle	1.000
Karisma-Big Jim	1.000
Carne Duro-Big Jim	1.000
Carne Duro-Karisma	1.000
Joe Parker-Karisma	1.000
CSU-432-20-Karisma	1.000
Sonora-Karisma	1.000
Joe Parker-Carne Duro	1.000
CSU-432-20-Carne Duro	1.000
Sonora-Carne Duro	1.000
CSU-432-20-Joe Parker	1.000
Sonora-Joe Parker	1.000
CSU-243-12-Joe Parker	1.000
Sonora-CSU-432-20	1.000
CSU-243-12-CSU-432-20	
CSU-243-12-Sonora	