CALIFORNIA MELON RESEARCH BOARD
Final Report, December 2, 2019

PROJECT: Discovery and validation of elicitor products for control of aphid and whitefly-transmitted viruses in melons

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Background

Melons in California routinely suffer losses from viral pathogens transmitted by aphids and whiteflies. These insect vectors can also reach extremely high population sizes, both within melon crops and at a landscape level across multiple crop species that serve as suitable hosts. Because the virus-vector association poses a dual threat, management of these pests presents a unique challenge for growers. Cultivars with resistance to both viruses and vectors are not available. Insecticides often do not act fast enough to prevent inoculation of viral pathogens, particularly in the case of aphid-virus associations. Many aphid-transmitted viruses of melons, such as *Cucumber mosaic virus* (CMV) and various species within the *Potyvirus* genus, are acquired and inoculated from plant tissue during brief probes that last only a few seconds. These exposures are not sufficient to ingest lethal doses of insecticides, particularly if active ingredients are only taken up by aphids during feeding from the plant vascular tissue (phloem). Insecticides can also exacerbate aphid dispersal and plant-to-plant movement, leading to greater virus spread rather than the intended effect of virus control.

In contrast to most aphid viruses that are problematic in melons, whitefly-transmitted viruses, such as *Cucurbit yellow stunting disorder virus* (CYSDV), are acquired and inoculated from and to plant tissues

during longer term feeding (hours) that requires ingestion of plant sap from phloem. Some insecticides can prevent this acquisition and limit virus spread (Castle *et al.*, 2017a; Castle *et al.*, 2017b), but if the viruses infection is severe and virions are in high titers (as often occurs for CYSDV), even brief periods of feeding on the phloem may be sufficient for transmission before the vector succumbs to insecticidal effects. Whiteflies reach massive population sizes in the Imperial Valley growing region and growers recently reported unexpected surges in whitefly activity in the Central Valley during the 2018 growing season, which may be associated with invasion of a new biotype. Under this kind of vector pressure, insecticides are not a reliable option for virus control because inoculum will regularly enter melon fields due to whiteflies from other crops acquiring CYSDV from infected weeds, crop reservoirs, and volunteer melons (Carrière et al., 2014; Carrière *et al.*, 2017; Wintermantel *et al.*, 2016; Wintermantel *et al.*, 2009).

In our prior work with CMV infection in melons, we found evidence that application of a commercially available plant immune elicitor - acibenzolar-S-methyl (ASM), can attenuate virus symptoms and reduce likelihood of vectors being differentially attracted to infected plants. These effects are linked to other positive benefits, such as reduced virus titers and attenuated negative effects on host growth. We further documented that the label rate for the ASM product (Actigard, Syngenta) is not suitable for effective disease control in melons (instead having phytotoxic effects) and we determined the correct application rate based on plant growth experiments. The ASM product also deters aphid vector feeding directly, which will help reduce vector populations by impeding ingestion of plant resources. Based on these activities, we expect that ASM applied through drip irrigation or foliar sprays at the newly tested rate established in our experiments could be a viable treatment option for protecting melon plants against aphid-transmitted CMV. **In 2019, we expanded on these results by performing additional experiments to validate protective effects against CYSDV, validate preliminary experiments demonstrating disruption of vector attraction, and test dosage rates and protective effects in the field. We also performed preliminary evaluations of several other elicitor products currently available or in development.**

<u>Objective 1</u>: Validate use of ASM (Actigard) as a tool for mitigating negative effects of viruses on melons.

Summary of activities

We performed additional experiments to determine efficacy of ASM against *Cucurbit yellow stunting disorder virus* (CYSDV) in the laboratory, as our initial experiments during the prior project period were not conclusive. These experiments included effects of ASM treatment on symptom progression, virus titer, and vector attraction to infected and healthy plants with and without ASM pre-treatment. To confirm protective effects against CMV, we also repeated a subset of these experiments. We then performed a field experiment to determine the efficacy of ASM against CMV in a realistic context using local CMV isolates and controlled inoculations. During this field trial, we further tested the effects of multiple applications on plant growth and virus infection.

Obj. 1A: greenhouse experiments

All experiments were carried out with melon, *Cucumis melo* var. Gold Express (Syngenta Seeds Inc.), germinated in seed flats in a climate-controlled growth chamber. For all experiments, plants were treated with a foliar spray of 20 ml of 25 ppm (25 mg/L) Actigard (Syngenta) at the one true leaf stage, approximately 1.5 weeks after sowing and one to three days after transplanting and moving to the greenhouse. Control plants were treated with a foliar spray of 20 ml of distilled water. Plants were inoculated with CMV (mechanical inoculation) or CYSDV (whitefly inoculation) 3-4 days following ASM

application (Fig. 1A). Over the subsequent 3-5 weeks, we tracked appearance and severity of symptoms, virus titers, plant health metrics, and insect vector preferences.

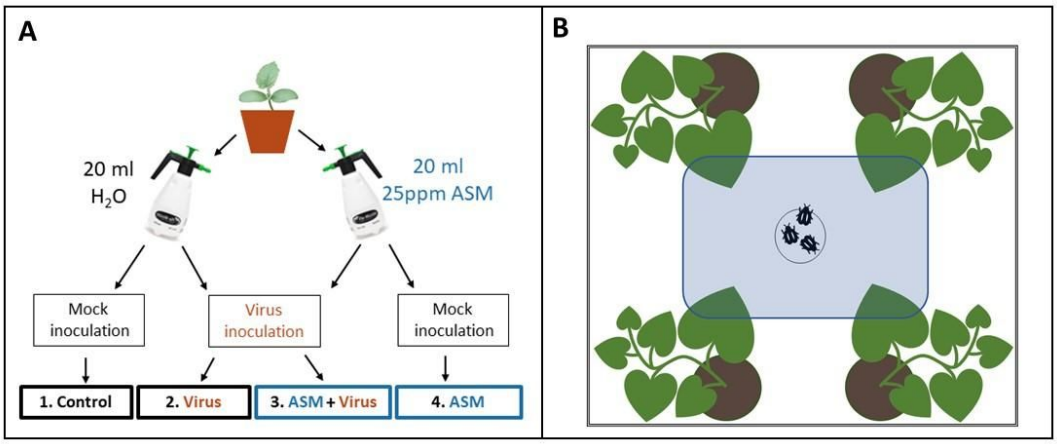
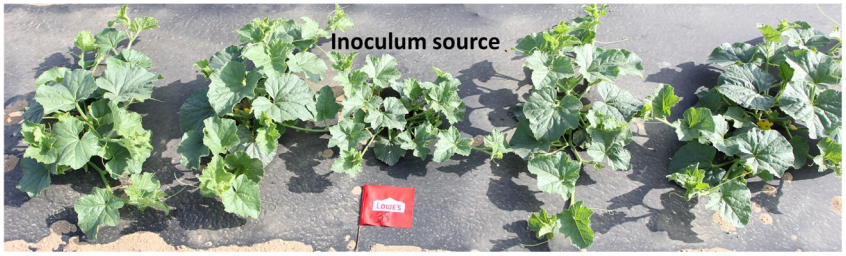


Figure 1: [A] Diagram showing the treatment groups for greenhouse experiments with CMV and CYSDV (each virus performed separately). [B] Behavioral assay set up aerial top down view. This assay was used to determine whitefly and winged aphid preferences among the four treatment groups indicated in part [A]. The double black line represents white poster board, the blue box represents a clear, sealed plastic arena with slits for single leaves to pass through the sides. The small black circle represents a hole in the middle of the bottom of the arena where insects were allowed to enter from a small holding area below at the beginning of each test.

Obj. 1B: field experiments with CMV

At the UC Riverside Agricultural Operations facility, we performed an experiment to evaluate ASM efficacy under field conditions (Spring 2019). Six beds were prepared according to standard practices and outfitted with drip irrigation. In each bed, we planted four focal melon plants on which observations would be made and one central melon plant infected with a local strain of CMV (see image below). Beds were partitioned into plots with one of three treatments (untreated, ASM treated 25ppm, ASM treated 12.5ppm). All other plot management activities were performed according to standard agronomic practices (fertilizer, weed management, and irrigation by drip tape). Pesticides were not applied.



During the growing season, we collected samples at several intervals to determine infection prevalence among the focal plants. We also collected samples at later stages of growth to determine differences in the virus community of ASM-treated and untreated plants using next-generation sequencing techniques, which do not require prior knowledge of virus identity. Plant health and growth were monitored at each tissue sampling point and yield was quantified at the end of the season (honeybee colonies are on site to ensure pollination).

Results: Objective 1A – Greenhouse experiments

With further repetitions, we established that ASM has clear protective effects against both CMV and CYSDV, delaying infection symptoms by up to 2 weeks with a single application (Fig. 2). However, ASM did not significantly reduce overall infection rates (Fig. 3). Effects against CYSDV are most evident when conditions are conducive to symptom development – i.e., long day length of natural sunlight available in the greenhouse (Fig. 2). Attenuation of symptoms is associated with titer reductions, especially at earlier stages of infection (Figs. 4 and 5). This may reduce the chances of vectors acquiring virus from the plants. Behavioral assays indicate that whitefly attraction to CYSDV-infected plants is disrupted by ASM treatment (Fig. 7). ASM itself also tends to make plants less palatable and attractive for whiteflies. Protective effects may therefore extend to repellency as well as direct attenuation of symptoms following inoculation. Similar assays with CMV did not show a strong effect on vector preferences between non-infected and infected plants, as CMV infection does not have strong effects on vector preferences even in the absence of treatment (Fig. 6). However, ASM treatment still attenuates symptoms and improves plant health under CMV infection (Fig. 4). Future experiments are testing the effects of multiple applications on symptom development and the effects of ASM on leaf morphology and toughness as a measure of possible off-target effects on plant resistance to other pests (e.g., cucumber beetles).

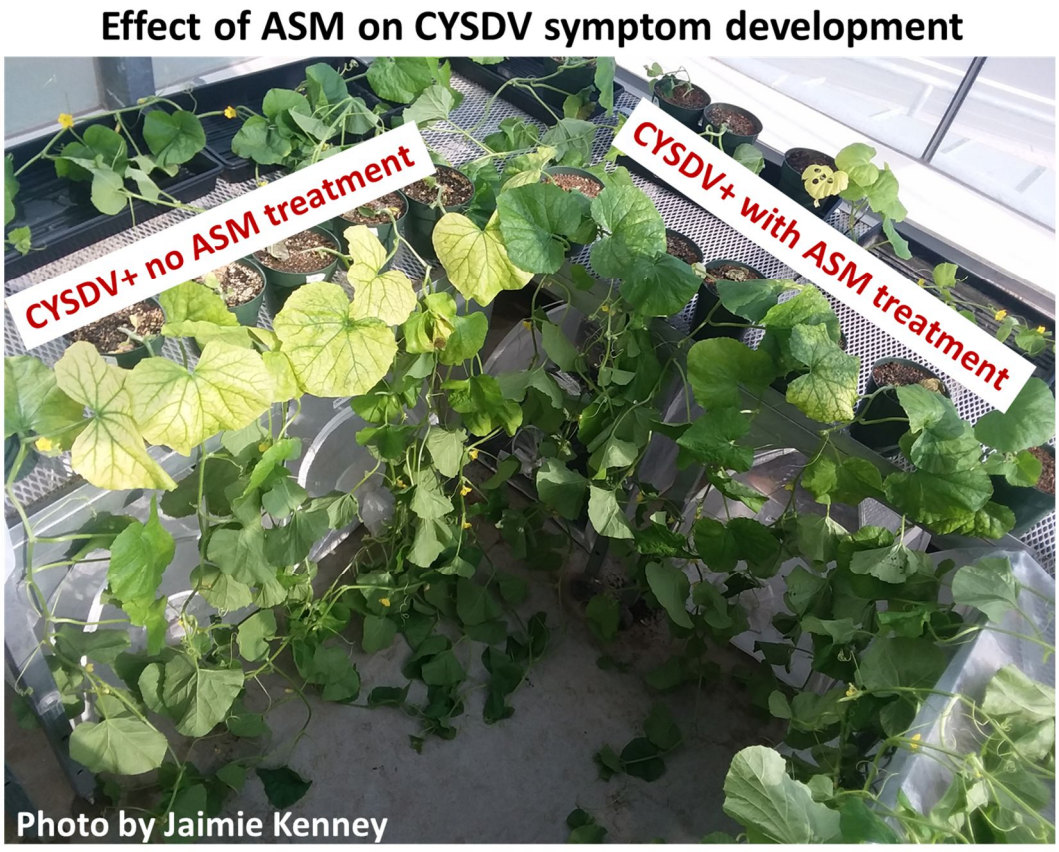


Figure 2: CYSDV symptom attenuation by ASM treatment in greenhouse experiments.

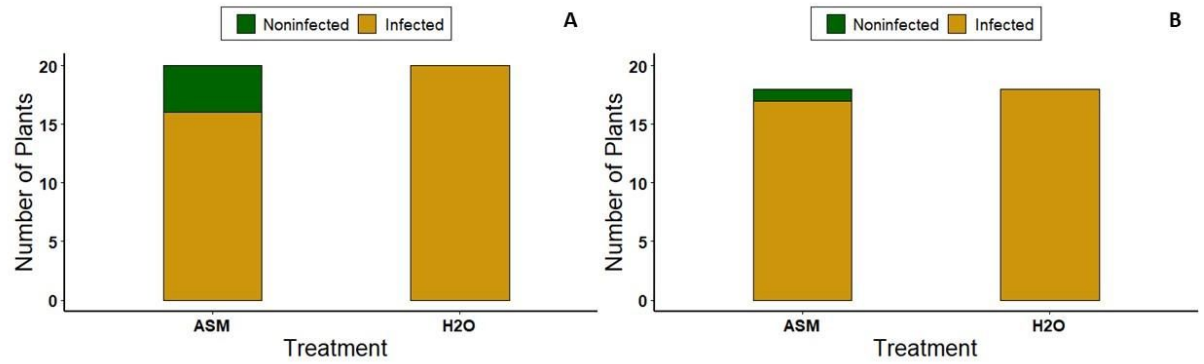


Figure 3: A) Rate of successful CYSDV infection in plants treated with either 25 ppm ASM or water and then inoculated with CYSDV via feeding by viruliferous *B. tabaci*. **B)** Rate of CMV infection in plants treated with either 25 ppm ASM or water and then mechanically inoculated with CMV. Differences are not significant by chi-square test.

***NOTE**: In all subsequent figures in this section, dots represent individual data points. The lower and upper edges of boxes represent the first and third quartiles, with the horizontal line inside representing the median value. Whiskers extend to the highest and lowest data points within 1.5x the interquartile range. Outliers beyond this range are represented by an additional semi-transparent dot.*

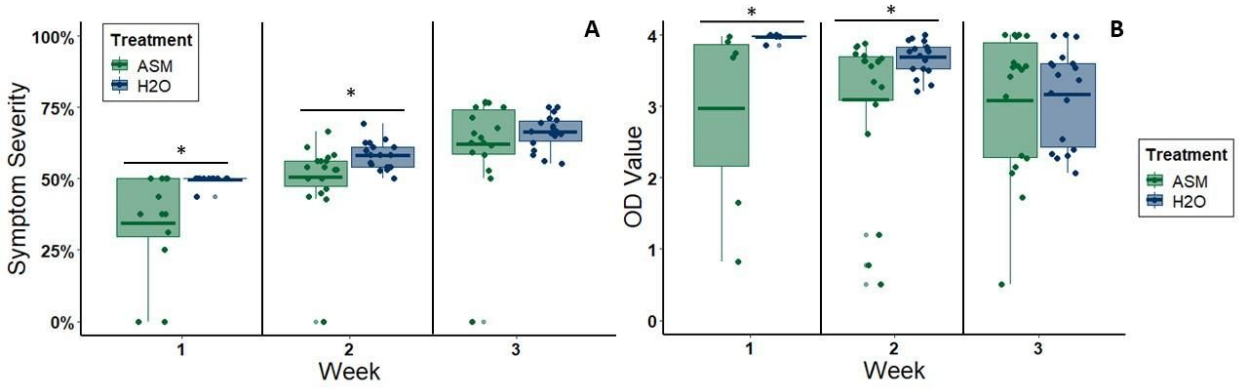


Figure 4: A) Symptom severity of CMV-inoculated melon plants (treated with ASM or water) at three timepoints during the same experiment: 1, 2, and 3 weeks post-inoculation (wpi). For 1 wpi *n* = 12 plants per treatment. For 2 and 3 wpi *n* = 18 plants per treatment. **B)** Standardized OD values of tissue samples from CMV-inoculated melon plants (treated with ASM or water) tested for CMV infection by DAS-ELISA at the same three timepoints: 1, 2, and 3 wpi. For 1 wpi *n* = 6 plants per treatment. For 2 and 3 wpi *n* = 18 plants per treatment. Bars with asterisks denote groups between which there is a significant difference at $p < 0.05$.

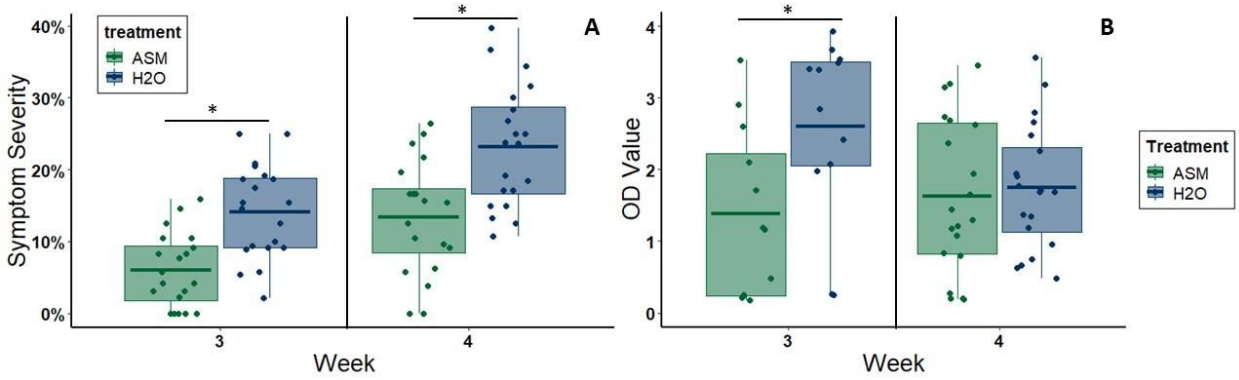


Figure 5: A) Symptom severity of CYSDV-inoculated melon plants (treated with ASM or water) at two timepoints during the same experiment: 3 wpi and 4 weeks post-inoculation (wpi) (*n* = 20 plants per treatment). **B)** Standardized OD values of tissue samples from CYSDV-inoculated melon plants (treated with ASM or water) tested for CYSDV infection by DAS-ELISA at the same two timepoints: 3 wpi and 4 wpi. For 3 wpi *n* = 12 plants per treatment. For 4 wpi *n* = 20 plants per treatment. Bars with asterisks denote groups between which there is a significant difference.

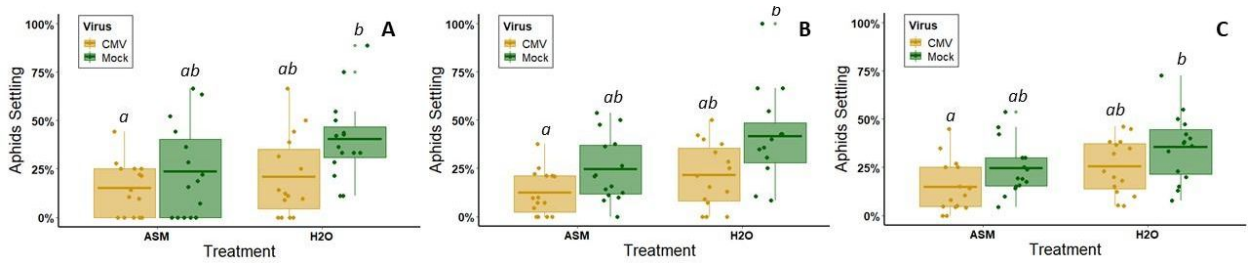


Figure 6: Results of alate aphid four-way choice tests between leaves from ASM+virus, ASM only, virus only, or healthy control plants (*n* = 15 iterations of the assay). **A)** Percentage of responding aphids on each of the four leaves 1 hour after release. **B)** Percentage of responding aphids on each of the four leaves 2 hours after release. **C)** Percentage of responding aphids on each of the four leaves 24 hours after release. Approximately 20 alate aphids were released per test. Lowercase letters above boxes denote treatment groups that did not have significantly different results.

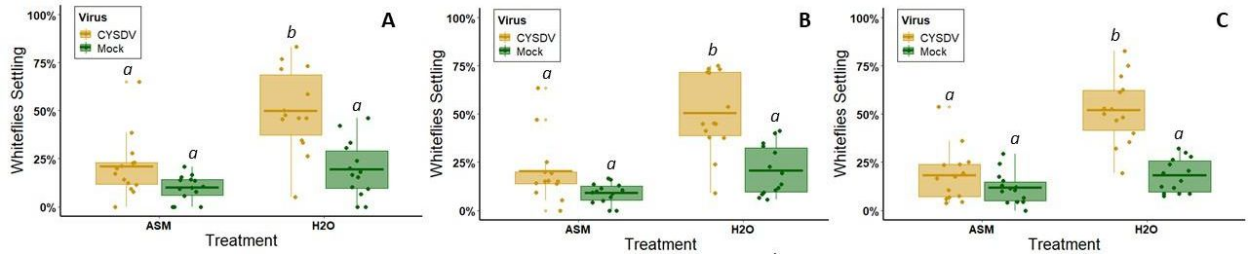


Figure 7: Results of whitefly four-way choice tests between leaves from ASM+virus, ASM only, virus only, or healthy control plants (*n* = 14 iterations of the assay). **A)** Percentage of responding whiteflies on each of the four leaves one hour after release. **B)** Percentage of responding whiteflies on each of the four leaves two hours after release. **C)** Percentage of responding whiteflies on each of the four leaves 24 hours after release. Approximately 25 whiteflies were released per test. Lowercase letters above boxes denote treatment groups that did not have significantly different results.

Results: Objective 1B – Field experiments

Overall, we did not detect a negative effect of ASM application to transplants (at either rate) on plant health or yield in the field experiment (Fig. 8). Plant growth was identical among ASM and control treatments. There were no differences in the number of melons falling into large, medium, and small size categories. Consumers of the melons indicated that all treatments were equally sweet and we received many positive comments about the crop being of excellent quality. In the greenhouse, we did detect slight negative effects on plant size when using the 25ppm dose applied as a foliar spray (Fig. 9). Additionally, a grower trial with seeded melons of several varieties suggests that later application is required. Some varieties (Cayucas, a unique ESL variety) were unusually susceptible to phytotoxic effects (stunting) which were worse in combination with Sandea herbicide. Overall, these trials indicate that later application (four leaf stage) is best, possibly in the absence of herbicide or well staggered from herbicide applications. Additionally, testing on more varieties is needed before recommending the product to growers.

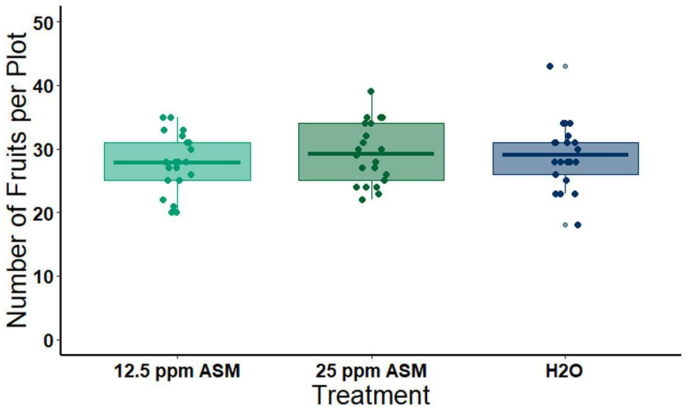


Figure 8: Melon yield per plot (four melon plants per plot) under standard field conditions over the course of a growing season. All plants in each plot were treated with a foliar spray of 20 ml of either 12.5 ppm ASM, 25 ppm ASM, or water one week after transplanting from the greenhouse to the field (*n*=21).

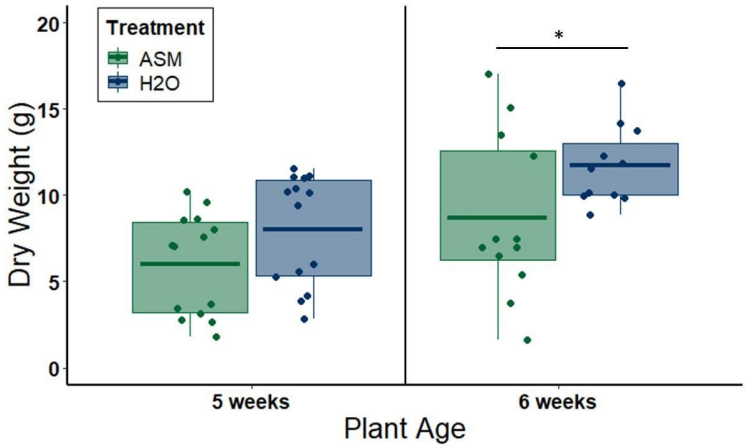


Figure 9: Dry weights of aboveground biomass of melon plants allowed to grow for a total of 5 or 6 weeks, respectively, following ASM applications. At 1.5 weeks old all plants had been treated with either a foliar application of 20 ml distilled water (H2O) or 25 ppm ASM solution (ASM). Bars with asterisks denote groups between which there is a significant difference at $p < 0.05$.

We collected tissue from treated plants at three time points throughout the experiment and this will be used to determine virus infection status of the plants. Overall, virus pressure was relatively low despite use of inoculum sources (a mild strain of cucumber mosaic virus collected locally). This may be due to aphid population fluctuations diverging from normal phenology as a result of an unusually wet winter and spring. Whiteflies were present in the field but only in low numbers. We collected tissue from a sub-set of plots for un-targeted virus discovery via next-generation sequencing to determine how ASM treatment affects the overall virus community in melons. Processing of these samples, as well as those taken at regular intervals, is still ongoing.

In addition to making good progress on establishing protective effects of ASM in melons, the data generated by Objective 1 of this project have been submitted for publication at the open-access journal *Viruses.* The manuscript cited below is currently under review.

Kenney, J.R., Grandmont, M-E., Mauck, K.E. (2019) Priming melon defenses using acibenzolar-S-methyl disrupts vector attraction to infected hosts in a virus-specific manner. *Submitted to Viruses on November 15, 2019. In review.*

Graduate student J. Kenney presented this work at the 2019 Annual Meeting of the Entomological Society of America and acknowledged CMRB support. She won second place for her oral presentation.

Objective 2: Quantify effects of alternative plant immunity modifiers on virus infection rates, disease progression, and symptom severity.

Summary of activities: ASM clearly shows promise as a priming agent, but our experiments, and testing by growers, indicates that finding the best dose is critical for avoiding phytotoxic effects. ASM is not the only product available for priming plants against pathogen attack. To determine the efficacy of alternatives to ASM, we tested three additional plant immunity modifiers: Regalia (Marrone Bio Innovations), Venerate (Marrone Bio Innovations), and a mixture of synthetic antimicrobial peptides (APs) currently under development as a tool for priming plants against pathogen infection.

Methods

Application of priming agents was performed as described in Objective 1A with some modifications. While ASM applications did not follow recommended doses on the label (due to these being phytotoxic), Regalia and Venerate applications were done at the mid-point of label recommendations for cucurbit crops. APs were applied at rates previously shown to prime defenses of *Citrus* plants and *Nicotiana benthamiana* (an annual tobacco species that is a laboratory model for studying plant pathogens). These included 10 micromolar and 1 micromolar concentrations in most experiments. We evaluated symptom expression and virus titer at one- and two-weeks post-inoculation for CMV and at 3-5 weeks post-inoculation for CYSDV, which has a longer period of disease progression. Experiments were repeated multiple times with 5-6 replications of each treatment per experiment. Preliminary trials with Regalia and Venerate were also performed in the field alongside the ASM trial described in Objective 1. At one-week post planting (2-3 leaf stage) we applied each product to approximately 16 plants per elicitor treatment and applied water to 16 control plants.

Active ingredient	Current uses	Mode of action	Potential role in virus defense	Commercial product name
Acibenzolar-S methyl (ASM)	Indirect fungicide	Mimics activity of SA	Primes salicylic acid regulated defenses	Actigard (Syngenta)
Reynoutria sachalinensis extract	Indirect fungicide	Elicits general defenses and phytoalexins	Primes multiple defense pathways	Regalia CG (Marrone)
Heat-killed *Burkholderia* spp. strain A396	Directly inhibits insect feeding	Produces insecticidal metabolites	Activates basal defenses via bacterial cell wall components	Venerate CG (Marrone)
Antimicrobial peptides	Indirect and direct defense against *Candidatus* Liberibacter pathogens	Direct action against bacteria, strong priming of plant defenses	Priming of basal plant defenses, delay/attenuation of symptoms	Not yet registered

Table 1: Products tested for efficacy as priming agents in melon. ASM was tested in Objective 1.

Results: Greenhouse trials

The 10 micromolar AP concentration and Regalia showed some suppressive effects on CMV symptom severity at 7 days post inoculation (Fig. 10). However, by 14-21 days post-inoculation, Regalia treatments no longer exhibited reduced symptom severity (Fig. 10). AP-treated plants continued to show some symptom attenuation until the conclusion of the experiment. There were no effects of priming agents on biomass of non-infected plants (Fig. 11). Overall, any protective effects were minor compared to the symptom suppression observed with ASM treatments (Objective 1). This suggests that these products are not viable alternatives as priming agents against CMV.

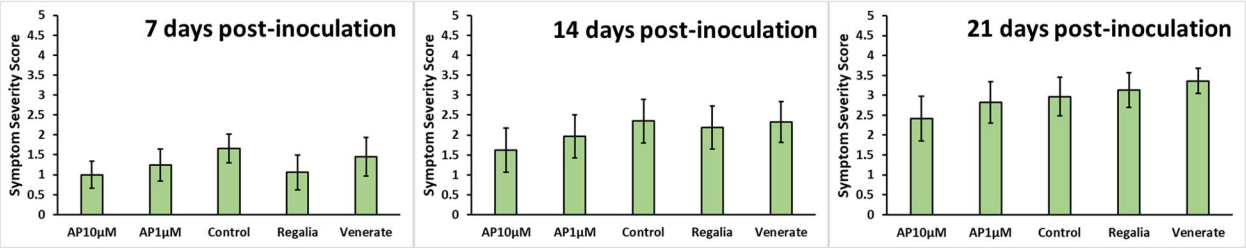


Figure 10: Symptom severity ratings (whole plant) for CMV-inoculated plants pre-treated with different plant immunity elicitors (priming agents). Bars are mean +/- standard error. Symptoms are evaluated on a per leaf basis with a rating between 1 (low/mild) to 5 (high/severe). Values for all leaves are averaged to get a total symptom rating for the entire plant. Results are from two experiments with N=5 plants per experiment (N=10 per treatment).

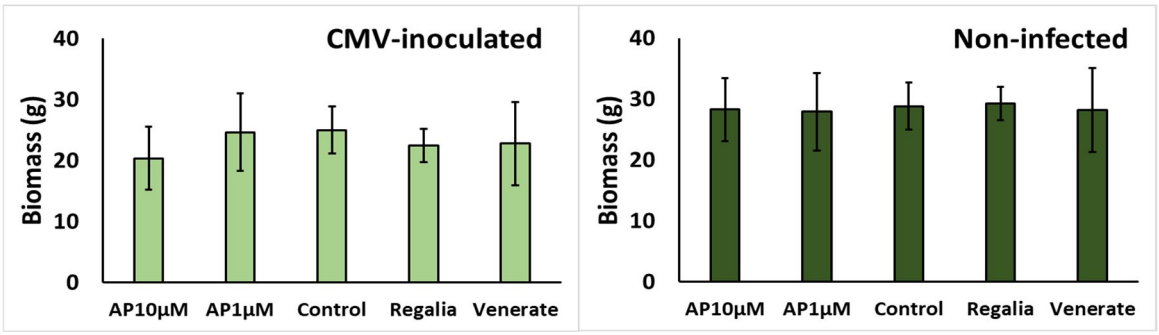


Figure 11: Biomass of plants treated with each elicitor product, or a mock treatment (control), with (left) and without (right) CMV infection. Bars are mean +/- standard deviation.

Results with CYSDV were similarly unimpressive. We did not see evidence of symptom suppression following priming by any of the tested products. Regalia was omitted based on lack of activity in CMV experiments, while Venerate was kept because it has insecticidal activity and may prevent virus inoculation as part of protective effects. We saw no evidence of protective effects or priming activity by any of the tested products. The 10 micromolar AP concentration had slight negative effects on plant growth. Virus titer measurements are ongoing for both CMV and CYSDV, but are unlikely to differ depending on priming treatment.

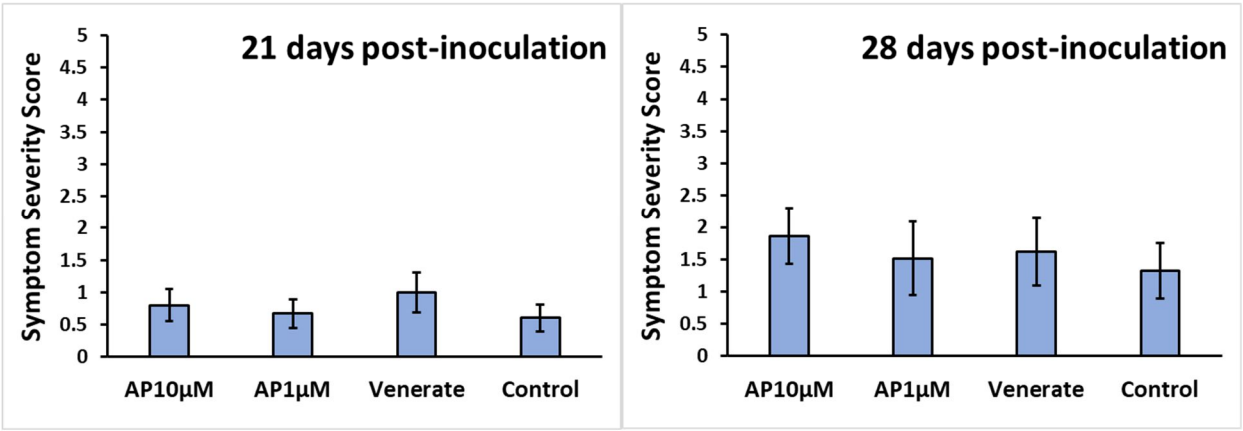


Figure 12: Symptom severity ratings (whole plant) for CYSDV-inoculated plants pre-treated with different plant immunity elicitors (priming agents). Bars are mean +/- standard error. Symptoms are evaluated on a per leaf basis with a rating between 1 (low/mild) to 5 (high/severe). Values for all leaves are averaged to get a total symptom rating for the entire plant. N=8-10 per treatment.

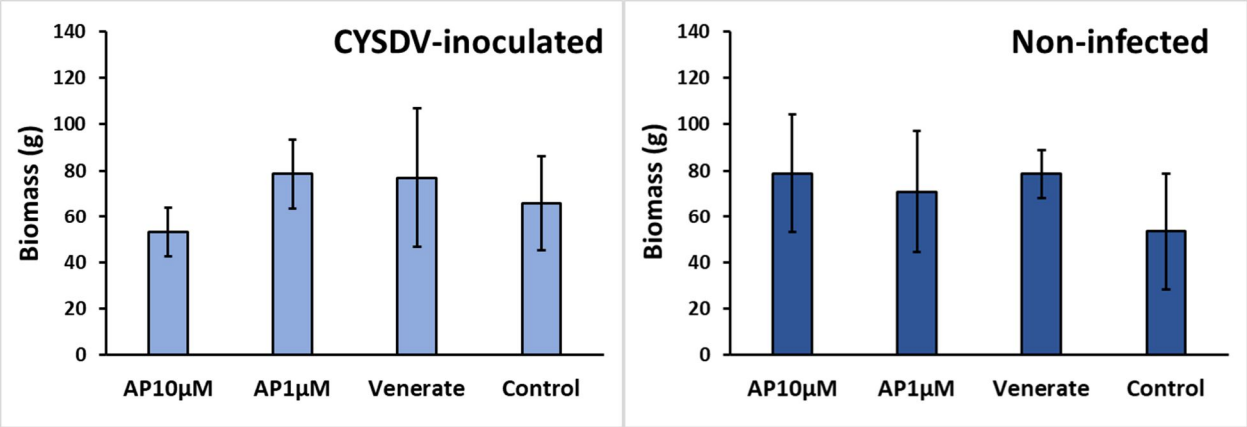


Figure 13: Biomass of plants treated with each elicitor product, or a mock treatment (control), with (left) and without (right) CYSDV infection. Bars are mean +/- standard deviation.

Field trials

Both Regalia and Venerate produced no phytotoxic effects when applied to Gold Express transplants. Regalia produced plants with darker leaves and a fuller canopy in the field, but these effects were not observed in the greenhouse. Regalia may be useful for improving plant health generally, or as a growth promoter. If these effects are substantial under field conditions, it may be useful to apply Regalia in tandem with ASM to elicit defenses and boost plant growth.

Future directions

Our results suggest that ASM is the best product for attenuating virus effects on plant health, despite the need for careful dosing. When used in tandem with an insecticide program, it may significantly improve virus management outcomes. Attenuation of symptoms also reduces negative effects on plant size and possibly physiology. In future work, we hope to evaluate ASM treatment under field conditions with more consistent virus and vector pressure, as Riverside has not been suitable for trials. For fiscal year 2020, we have proposed an experiment to be performed at the Westside Research and Extension Center in collaboration with Tom Turini. This area has good pressure from aphids and CMV. We will test different ASM dose regimes (one vs. two applications) in tandem with insecticide treatments. Along with this experiment, we will integrate testing of Ethephon (Florel) as a phytohormone mimic of ethylene. Ethylene can be used to modify set of female flowers within a defined window of time. And ethylene is also involved in defense against insects and plant pathogens (Casteel et al. 2015). We will test low doses of ethephon in combination with ASM to simultaneously evaluate viability of these hormone mimics for manipulating growth, defense, and fruit set window in melons.

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