Project title: Evaluating plant immunity priming agents for protection against virus infection in lettuce.

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Abstract

California leads the U.S. in lettuce production, supplying the country with over 65% of all lettuce, with an evaluation at over $2 billion each year. Unfortunately, lettuce crops in CA are increasingly subject to infections by plant viruses, particularly those vectored by thrips. For example, in lettuce grown in Monterey County (60% of CA production), managing thrips and the thrips-transmitted virus, Impatiens necrotic spot virus (INSV) has become one of the top challenges facing growers, resulting in 100% losses in fields in 2019 and 2020. From our prior work on these recent outbreaks, we have determined that economically damaging levels of infection routinely occur at vector densities well below those causing damage to crops through direct feeding; a small number of insects (often from concurrent plantings in different stages of growth) can initiate widespread infection, often before succumbing to chemical controls. Despite this, virus issues are driving increases in prophylactic applications of insecticides that pose high risks to human, aquatic, and terrestrial animal health (e.g., methomyl). Therefore, new integrated disease management strategies are needed to reduce the frequency of virus infections and/or attenuate virus impacts on yields without increasing use of high-risk insecticides. One way to achieve this is by improving the innate virus immune response of existing lettuce cultivars. Immunity priming agents that activate different defense systems are commercially available as labelled fungicides for various specialty crops, but remain underexplored as tools for virus management, especially in lettuce. However, our recent work has shown that immunity priming can have dramatic improvements in virus tolerance in specialty crops. The proposed project will build on this work by testing the hypothesis that immunity priming can be used to improve virus control and reduce high-risk insecticide use. To test this hypothesis, we will first evaluate the overall effects on growth and appearance of four commercially available immunity priming agents (elicitors) labeled for use in lettuce. After identifying products and doses that do not induce trade-offs in growth or quality, we will evaluate the protective effects of specific product x dose combinations against inoculation and infection by INSV. This work will provide broad recommendations for safe use of elicitor products acting on diverse pathways, which will benefit

overall integrated disease management efforts, as well as specific recommendations for targeted use of elicitors for controlling INSV impacts and spread.

Procedures

<u>Objective 1. Evaluate four elicitor products for effects on the growth and appearance of iceberg and romaine lettuces.</u>

Based on our observations that elicitor dose is critical, especially when used in early stages of plant growth, we performed a suite of experiments to evaluate the off-target effects of elicitors with diverse modes of action. The specific products are described below:

1. Actigard (Active Ingredient [AI] = acibenzolar-*S*-methyl [ASM]). Activates plant defenses by mimicking the structure of salicylic acid, the master hormone regulator (activator) of canonical anti-pathogen defenses in all vascular plants.

2. Regalia (AI = extract of Giant Knotweed *Reynoutria sachalinensis*). Mechanism not fully known. Boosts plant vigor and growth while stimulating the plant's ability to develop resistance to plant pathogens (induced resistance).

3. Cabrio EG (AI = pyraclostrobin). Strobilurin compounds have strong immunity priming activity that functions independent of the salicylic acid pathway (which Actigard mimics), thus providing a priming option that is quite unique relative to Actigard (ASM) and other products. Pyraclostrobin has demonstrated priming activity against viruses from three different families in solanaceous crops, but is untested for this application in lettuce.

4. DEsect (AI = silicon dioxide). The AI is absorbed by plant roots and distributed throughout various tissues, where it primes inducible plant defenses operating over several pathways. Silicon-treated plants respond to pathogen challenge by more strongly activating jasmonic acid, salicylic acid, and ethylene defense pathways. This product, which is very inexpensive and applied as a soil additive, may therefore synergize well with other agents.

Using greenhouse experiments, we evaluated each product for effects on growth and appearance relative to an application of water alone. Using romaine lettuce (Parris Island Cos) we measured survival, growth progression (plant size rating relative to controls), appearance (phytotoxicity symptoms that may affect marketability), and final biomass at 3 weeks post-application. Plants were grown in pots in a greenhouse at 73 ± 5°C. Treatments for each product x variety combination initially included a dose at the label rate, a dose at one half the label rate, and a water control. Products were applied at the 2-3 leaf stage to soil or foliage as recommended by label instructions. This stage is the target for protection because, 1) Prior results indicate that viruliferous thrips migrate into new plantings from adjacent older fields, 2) Infections at this point are guaranteed to lead to reductions in marketability, and 3) Early inoculations will establish inoculum sources that may attract vectors and fuel secondary spread within a field. We included at least 10 plants per variety x dose treatment combination (typically 16-18). Additionally, we performed an experiment to evaluate effects of elicitors on metabolite composition (sugars and amino acids) in leaf tissues. Treatment effects on plant growth and marketability features were evaluated using general linear models. Each response variable constituted a separate analysis with treatment (3 doses) as a fixed factor.

2. Evaluate protective effects of select products identified in Objective 1 against INSV inoculation and disease progression.

This objective evaluated hypothesized protective effects of elicitor products against INSV and allowed us to relate the degree of protection to activation of different defense pathways. We tested all four products in this objective with repeated experiments, as well as select combinations of products based on the results of individual product performance tests. Products were applied to plants prior to virus challenge (2-3 leaf stage) in the appropriate doses, as suggested by results of Objective 1. We also evaluated multiple doses of select products applied before and after virus challenge. Each experiment included a water control applied in the same manner as the product being tested. Two days following product application, lettuce plants were mechanically inoculated with INSV, using current methods used by D. Hasegawa. Plants were observed weekly for symptom development for 3 weeks, prior to assessing plant biomass and infection severity. Biomass data were analyzed using statistical approaches described in K. Mauck's prior work on elicitor treatments in melons (Kenney et al. 2020, Viruses, 12, 257; doi:10.3390/v12030257). Tissue was collected to confirm infections and estimate virus levels using a semi-quantitative enzyme-linked immunosorbent assay (ELISA) protocol. For a subset of samples, we will perform a more accurate assessment of virus replication/titer by extracting RNA and performing RT-qPCR (in progress, as in Kenney et al. 2020).

Results

Initial pilot experiments were run in Summer 2021 to optimize growing conditions for lettuce in the greenhouse. Pilot results indicated some negative effects of Actigard at full and half doses, but this was only evident under sub-optimal growing conditions (Fig. 1). Once growing conditions were optimized, we did not see this effect. Based on these pilot experiments, we proceeded with testing full doses of the selected products under the optimized conditions. However, these initial experiments suggest that plants must be in good health to tolerate applications of some elicitor products, and that abiotic stress factors may contribute to sensitivity to full elicitor doses.

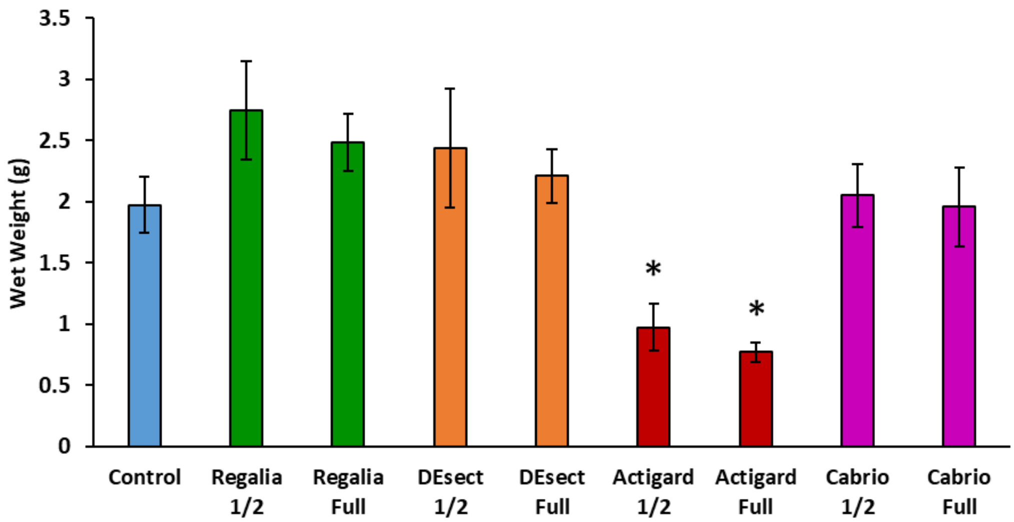

*Figure 1. Results of the pilot experiment to evaluate full and half doses of elicitors for effects on plant growth. * indicates treatments significantly different from control (P<0.05). Plants showed signs of stress throughout the experiment in all treatments, likely due to sub-optimal lighting in the greenhouse. Future experiments took place in a different growth chamber and greenhouse location. Similar results were seen with a pilot experiment on iceberg lettuce.*

Following pilot experiments and short experiments to test lettuce growth in different greenhouse/growth chamber locations, we repeated growth experiments and proceeded to INSV challenge experiments. Two replications each of the growth experiment and INSV challenge experiment were run. In growth experiments, none of the full dose elicitors significantly reduced plant size relative to the control, although Regalia and Actigard treatments were significantly different from each other in multiple comparison post-hoc testing for replication two of the experiment (Fig. 2) and Actigard did reduce growth in the pilot experiment. This suggests that Regalia and Actigard may be a good combination for simultaneous or sequential applications.

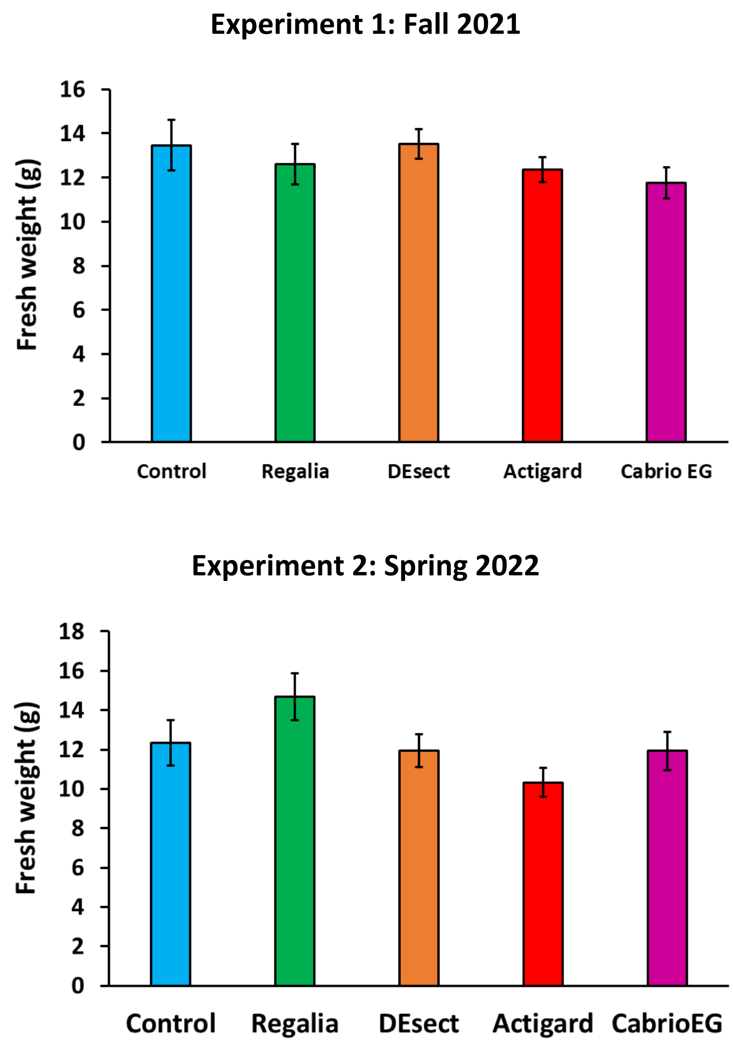

*Figure 2: Summary of elicitor effects on plant size (fresh weight). Bars represent the mean of 16-18 replicate plants per treatment. The graph on the top is the first replication of the experiment (Fall 2021), and the graph on the bottom is the second replication (Spring 2022). Data were analyzed using a general linear model. The * designates treatments that are significantly different from the control (water) as determined by Dunnett's test ($P<0.05$). In multiple comparisons (Tukey's test), Regalia was significantly higher than Actigard.*

In addition to plant growth, we assessed the effects of elicitor treatments on primary metabolite composition of lettuce at two time points post application (7 days and 14 days). In each time point, eight plants per treatment (five treatments total) were sampled to evaluate levels of amino acids and sugars. At present, we have processed and quantified sugar levels in half of the first time point samples. Data analysis of amino acid measurements in these samples is ongoing.

Remaining samples have been extracted and are in the process of being derivatized for metabolite measurements. Initial measurements of half of the samples from time point one demonstrates no significant effects of elicitor treatments on sugar levels (Fig. 3). However, we saw a trend of Regalia increasing concentrations of all sugars (glucose, fructose, and sucrose). This is consistent with overall positive effects of Regalia on plant growth, as seen in the previous growth experiment.

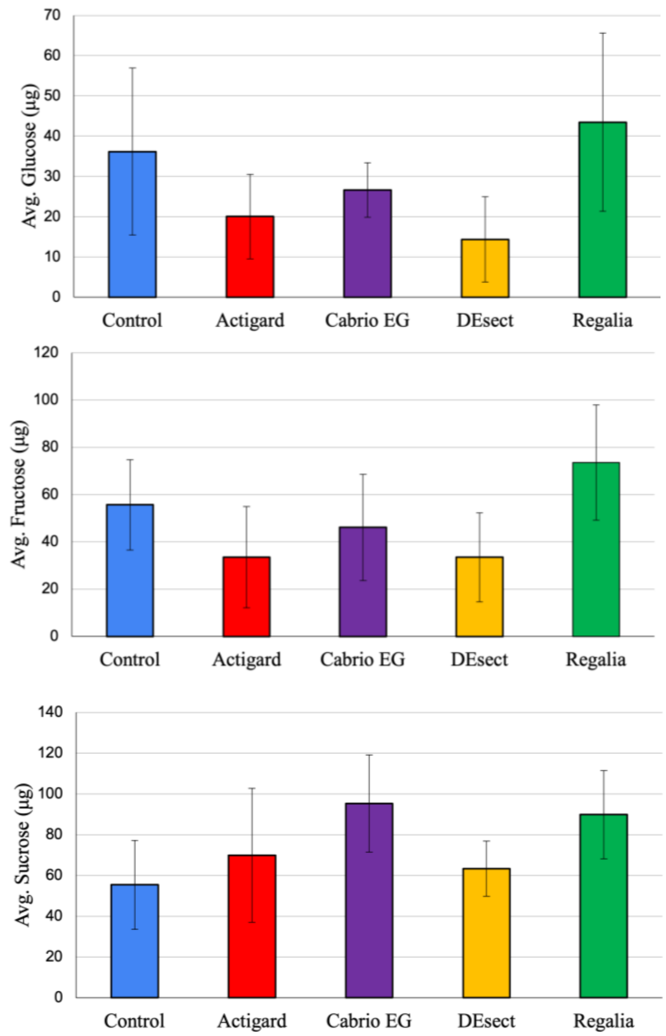

Figure 3: Effects of elicitors on levels of glucose (top), fructose (middle) and sucrose (bottom) at the first time point (7 days) after application. Graphs display means +/- standard deviations for each treatment, with a sample size of four plants per treatment. Four more samples for this time point will be processed and added to this data set for the final analysis.

For the INSV challenge experiments, INSV infection severity was overall low in replication one (Fall 2021) and was much higher in replication 2 (Spring 2022) (Figs 4-5). Under the low infection severity conditions of Fall 2021, elicitor activity was not very pronounced (Fig. 4). Actigard did reduce infection rate slightly, and there was a trend toward reduction of titer for

Actigard and Cabrio EG. Results of the Fall experiment lend further support to the idea that seasonality influences both infection and elicitor efficacy, as the same results were seen in experiments with cantaloupe run alongside experiments with lettuce. Seasonal influence on elicitor efficacy and infection severity will be explored in future experiments.

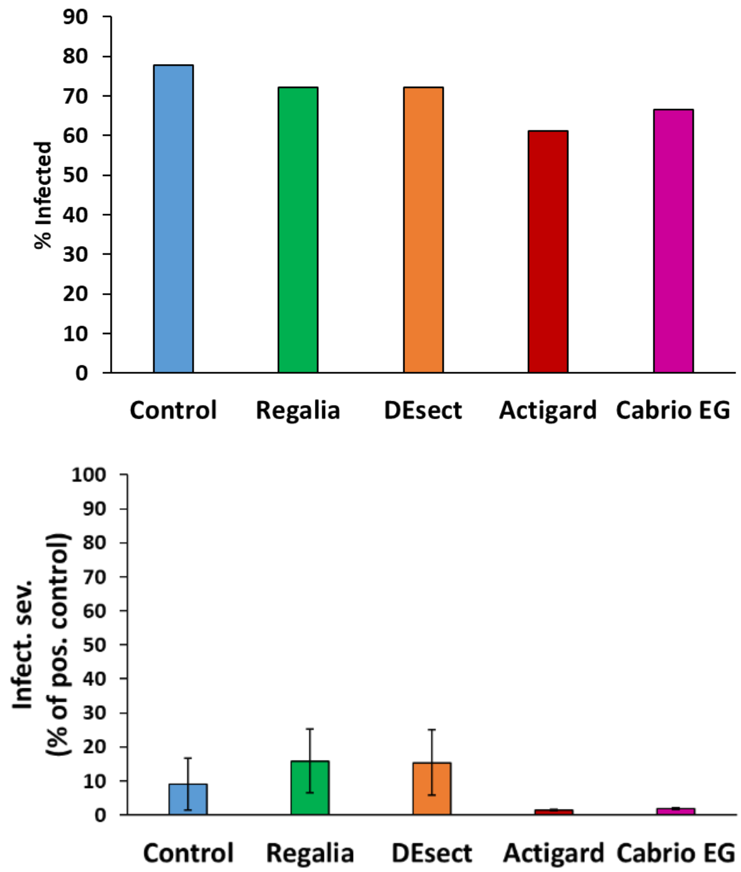

*Figure 4: Summary of elicitor effects INSV infection rates (left) and titers (right) for experiment replication 1 (Fall 2021). Bars represent the mean of 18 replicate plants per treatment. Data were analyzed using a general linear model. The * designates treatments that are significantly different from the control (water) as determined by Dunnett's test (P<0.05).*

In the Spring of 2022, we performed a second replication of this experiment. In this iteration, infection severity was much higher (overall higher titers) (Fig. 5). Actigard reduced infection success relative to the control by more than half and reduced INSV titer relative to the control treatment. Regalia also reduced INSV titer but did not reduce INSV infection rates. In contrast to the Fall experiment, the Spring experiment was more representative of typical INSV pressure and symptom expression.

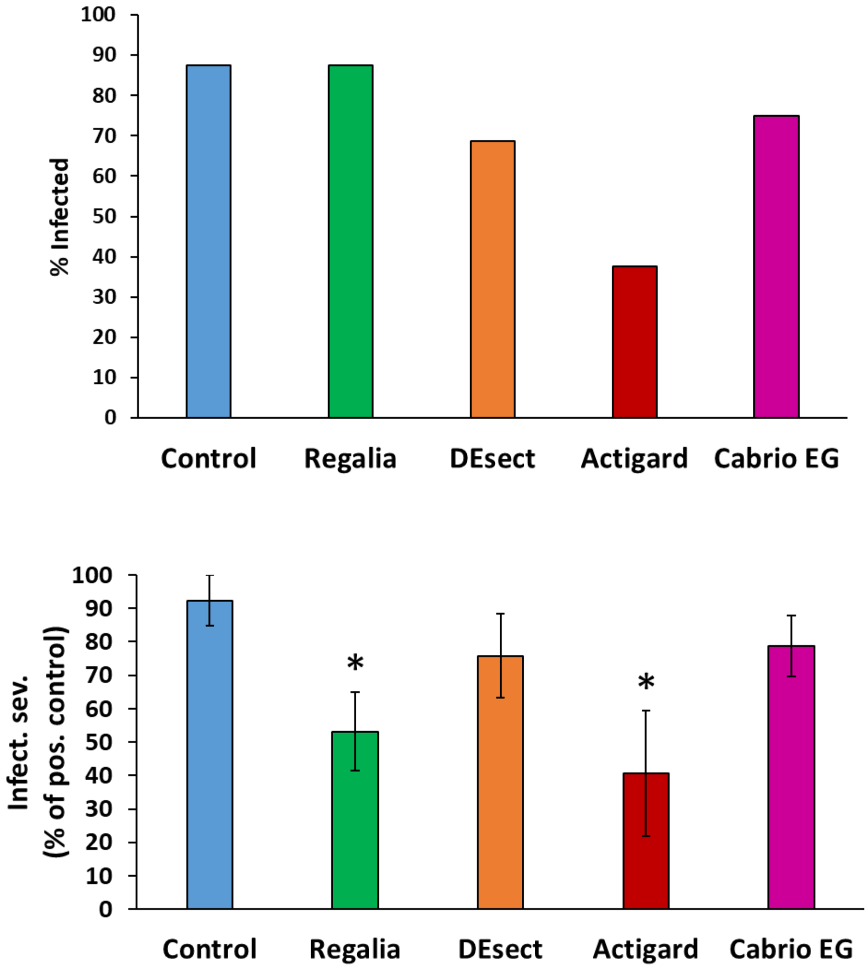

*Figure 5: Summary of elicitor effects INSV infection rates (left) and titers (right) for experiment replication 2 (Spring 2022). Bars represent the mean of 16 replicate plants per treatment. Data were analyzed using a general linear model. The * designates treatments that are significantly different from the control (water) as determined by Dunnett's test (P<0.05).*

Based on these results, we moved forward with testing multiple doses of Regalia and Actigard as well as combined applications of Regalia and Actigard. Samples for virus titer were collected on 5-27-22 and are being processed. Based on results of this experiment, we will make final selections for field testing of elicitors in the Salinas Valley in Summer 2022. Experiments will begin in June and will include testing on both romaine and iceberg varieties. Field experiments are funded by a grant from the Department of Pesticide Regulation, which was made possible by CLGRP support to begin this project.

Acknowledgements

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