2023-2024 CALIFORNIA LEAFY GREENS RESEARCH PROGRAM
ANNUAL REPORT

Project Title: Towards developing an efficient, topical application of RNAi-based biopesticides for managing viruses in lettuce

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Abstract:
Western flower thrips (*Frankliniella occidentalis*) is an insect pest that creates cosmetic issues in lettuce and is the primary insect vector for impatiens necrotic spot virus (INSV). INSV has become an increasing problem in lettuce production on the Central Coast of California. Only limited strategies exist for controlling thrips due to the lack of efficacious chemistries, and increasingly tighter regulations will create new challenges in managing the pest. There are also no direct methods for managing INSV, due to the lack of genetic-based resistant varieties available to the industry. This has created a need for new management strategies. RNA interference (RNAi) technology is an emerging strategy that has potential for the agriculture sector and has been demonstrated to be effective as a biopesticide for crop protection against insect pests and pathogens. RNAi is a natural process that results in gene silencing in insects, plants, and humans, and it's use as a biopesticide can be applied using methods that avoids genetic engineering of plants. Furthermore, RNAi-based biopesticides can be tailored to be species-specific and are completely degradable in the environment, providing unique advantages over many conventional insecticides. With previous support from the California Leafy Greens Research Program, we have successfully designed and synthesized double stranded RNAs

(dsRNAs; the active triggers of RNAi) to target western flower thrips and INSV. This support also enabled our capacity to test larger volumes of dsRNA. We have demonstrated that dsRNA targeting INSV can be absorbed through the roots, move systemically throughout the plant, and persist for at least 30 days. We have also generated preliminary results that suggest the dsRNAs have suppressive effects on INSV development. The current work describes our work over the past year, including 1) a field trial to test the efficacy of dsRNAs applied to the roots of lettuce, and 2) foliar applications using nanoparticle-based formulations. Both applications have strong implications for managing INSV in CA lettuce.

Immediate Objectives:

1. Test dsRNA root delivery strategies for managing INSV.
   Deliverables: Reports on efficiency of delivering dsRNAs through the roots of lettuce, systemic persistence, and suppression of INSV.
2. Test bio-based formulations of dsRNA as a foliar application for managing INSV.
   Deliverables: Reports on a proof-of-concept for nanoparticle-based dsRNA formulations and its potential as a foliar application for managing INSV.

Procedures:

Objective 1: Test dsRNA root delivery strategies for managing INSV. A field trial at the Spence Research Farm was conducted from 7/19/23 to 9/27/23. Romaine (var. Parris Island Cos) was direct seeded in 40-inch beds, sprinkler irrigated for 21 days, and drip irrigated for the remainder of the trial. Synthesized dsRNAs targeting INSV were manually applied to the crown of individual plants beginning at 23 days and continued once a week for four consecutive weeks. DsRNA was applied at a rate of 500 ug per plant in 1 ml of water. The dsRNA treatment and untreated blocks were replicated eight times in the field. Evaluations were conducted on alternating weeks to assess, 1) visual symptoms of INSV infection was performed using a 0-5 symptom severity scale (0=no symptoms, 5=dead) on five plants per replicate per week, 2) testing a single plant (leaves only) per replicate per week for INSV infection using TAS-ELISA, 3) testing a single plant (leaves, crown, and roots) per replicate per week for presence of dsRNA using RT-PCR. A total of four evaluations were made, with a final evaluation at 65 days to calculate the above ground wet biomass of three lettuce plants from each treatment and replicate. All field activities and evaluations are included in **Table 1.**

Objective 2. Test bio-based formulations of dsRNA as a topical application for managing INSV. Mesoporous Silica Nanoparticles (MSNs) were synthesized using tetraethyl orthosilicate (TEOS) as a silica precursor and cetyltrimethylammonium bromide (CTAB) as a pore generating agent (**Figure 2**). MSNs were functionalized by combining Toluene, 3-aminopropyl triethoxysilane (ATPS) and stirring for 16 hours. Functionalized MSNs were dissolved in ethanol and sonicated to conjugate with dsRNAs targeting the N gene or the NSs gene. All solutions were incubated overnight. Preparation of MSNs, inoculation procedures, RT-PCR and qPCR protocols have been previously optimized in Dr. Pappu's lab at Washington State University.

For experiments, the inoculum mixture was prepared by combining the conjugated MSNs-dsRNAs with INSV plant sap and used to manually inoculate tobacco (*Nicotiana*

benthamiana). For controls, plants were inoculated with 1) only MSNs and INSV inoculum, or 2) only INSV inoculum. Inoculated plants were kept in a growth chamber and INSV symptom development was monitored. At regular intervals of 3, 7, and 14 days post inoculation (dpi), leaves were harvested for total RNA extraction using Trizol followed by RT-PCR using INSV-specific primers. To quantify the viral RNA levels, qPCR was performed using the endogenous housekeeping gene, *Phosphatase 2A* to normalize expression of the INSV target gene. To calculate the abundance of INSV RNAs in the treatments, the delta-delta Ct method was used. Three technical replicates were performed.

2023		Activity	Days	Notes	Data collected*
Wed	7/19	Planting (direct-seeded romaine)	0		
Fri	7/21	First water	2		
Thu	8/10	Thinning	22		
Fri	8/11	RNAi drench (1)	23	500 ug dsRNA / plant in 442 ul	
Wed	8/16	Drip tape	28		
Fri	8/18	First Evaluation	30		1, 2, 3
Fri	8/18	RNAi drench (2)	30	500 ug dsRNA / plant in 1 ml	
Fri	8/25	Second Evaluation	37		1, 2, 3
Fri	8/25	RNAi drench (3)	37	500 ug dsRNA / plant in 1 ml	
Fri	9/1	Third Evaluation	44		1, 2, 3
Fri	9/1	RNAi drench (4)	44	500 ug dsRNA / plant in 1 ml	
Fri	9/8	Fourth Evaluation	51		1, 2, 3
Fri	9/22	Fifth Evaluation	65		1, 2, 3, 4
	9/27	Crop terminated	70		

Table 1. Schedule of activities for RNAi field trial. The trial was conducted at the Spence Research farm at USDA-ARS. *Data collected: 1 = In-field INSV symptom severity, 2 = Plant collections for INSV testing using TAS-ELISA, 3 = Plant collections for dsRNA detection using RT-qPCR, 4 = Plant collections for above ground wet biomass.

Results:

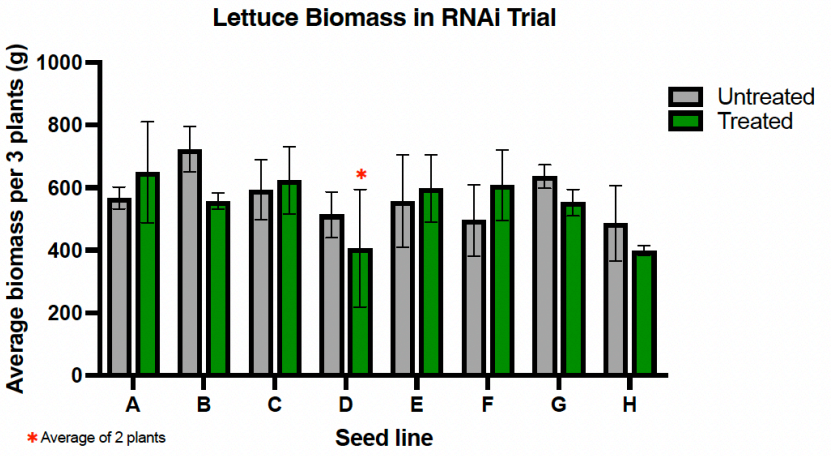
Field trial testing root delivery methods of dsRNA

The first application of dsRNA targeting INSV was applied one day after thinning (23 days after planting) to the base of the plant. Each plant received a total of 500 ug of dsRNA (**Figure 1**). Seven days after dsRNA treatment (30 days after planting), plants were collected, RNA was extracted from the roots, inner leaves (youngest leaves), and outer leaves (oldest leaves), and PCR amplified for the detection of the administered dsRNA. The dsRNA was detected in 72% of the roots that were sampled, 69% of the inner leaves, and 75% of the outer leaves. Additional applications of dsRNAs in subsequent weeks confirmed the presence of dsRNA. However, since dsRNA was applied to the same plants, we could not distinguish the

persistence of the dsRNA that was applied at 23 days versus the dsRNA that was applied in subsequent weeks.

The overall INSV disease incidence was very low in the field trial, which was estimated to be ~1% at the end of the trial. Visual evaluation data did not reveal any differences in INSV symptom severity between the dsRNA treated and untreated plots at 30, 37, 44, 51, and 65 days after planting. Additionally, INSV was not detected in the dsRNA treated or untreated plots when tested using ELISA (data not shown). At the end of the trial (65 days after planting), the aboveground wet biomass was calculated for each plant, which determined that repeated applications of dsRNA did not affect the growth of the plants when compared to those that were untreated (**Figure 2**).

Figure 1. Romaine lettuce 23 days after germination when the first dsRNA application was made. For each plant, 500 ug dsRNA was applied to the soil at the base of the plant.



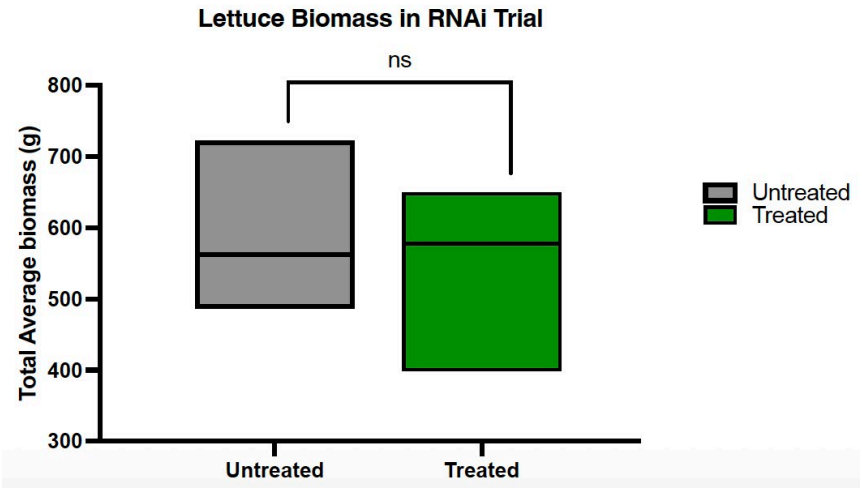


Figure 2. Wet biomass of aboveground tissue of lettuce. The weight of 24 plants was averaged per treatment; $p < 0.05$.

Bio-based formulations for topical applications of dsRNA

Mesoporous silica nanoparticles (MSNs) were first synthesized (**Figure 3**) and sonicated with dsRNAs targeting the N or NSs genes of the INSV genome. The conjugated MSNs and dsRNAs were then mixed with INSV-infected plant sap and used to manually inoculate tobacco leaves. At all three evaluation time points of 3, 7, and 14 dpi, INSV RNA levels were suppressed in the dsRNA treated, with the greatest suppression at the 14 dpi time point (**Figure 4**). DsRNAs targeting the N gene or the NSs gene showed an equal level of suppression of INSV. Furthermore, INSV was expressed at a high and similar level in the nanoparticle treatment and untreated controls, suggesting that the application of the MSNs alone did not influence INSV. Furthermore, expression of the housekeeping gene, *Phosphatase 2A,* did not change over time, which suggested that the quality of RNA and preparation for RT-qPCR analysis at each of the time points were comparable. Overall, the work provides preliminary evidence of INSV suppression using an MSN topical delivery strategy.

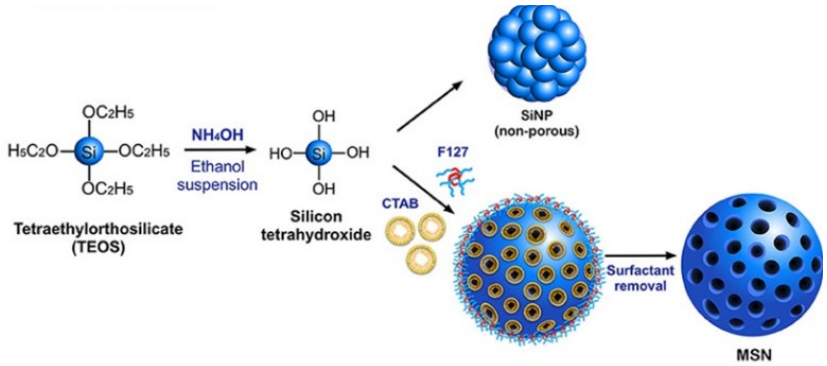


Figure 3. Synthesis of mesoporous silica nanoparticles (MSNs). Tetraethyl orthosilicate (TEOS) is used as a silica precursor and cetyltrimethylammonium bromide (CTAB) as a pore generating agent. The resulting MSNs were then further functionalized and conjugated with dsRNAs.

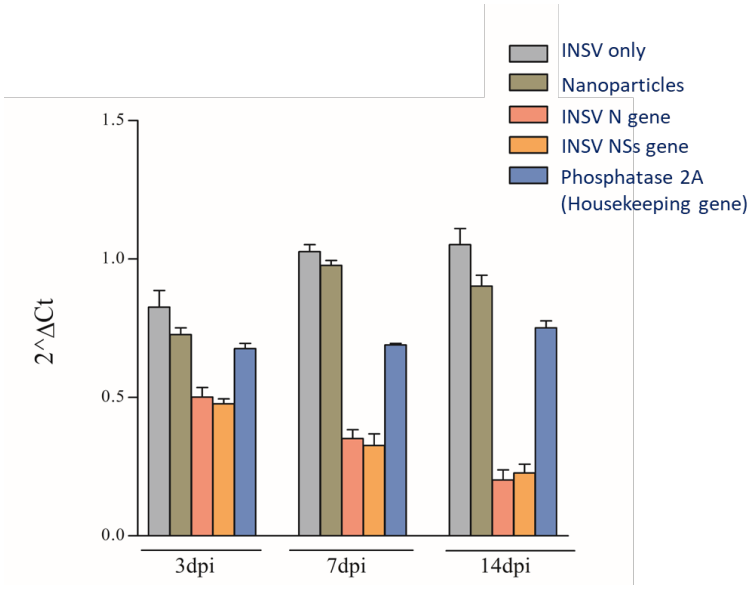


Figure 4. Effect of INSV-specific dsRNA on tobacco (*N. benthamiana*) plants inoculated with INSV. INSV only = untreated; Nanoparticles = MSNs only; INSV N gene = MSNs + dsRNA targeting the N gene of INSV; INSV NSs gene = MSNs + dsRNA targeting the NSs gene of INSV; Phosphatase 2A = endogenous gene of *N. bentamiana.*

Discussion:

RNA interference technology (RNAi) has numerous applications for crop protection and improvement. RNAi is a natural process in plants, insects, and humans that results in gene silencing. In this process, the formation of double stranded RNAs (dsRNAs) leads to the degradation of complementary mRNA, resulting in reduced expression for that gene. Identifying regions within a gene that are unique to a particular organism can be used to design specific dsRNAs. Using this knowledge, we can design dsRNAs to target key genes of the INSV genome. This proposal explored the development of RNAi technology for managing INSV affecting lettuce, and for the first time, was tested in a field trial at the Spence research farm.

Here, we demonstrated that dsRNAs can be successfully absorbed into the root systems of lettuce plants under field conditions, and furthermore, moves systemically throughout the plant and can be recovered from leaf tissue for at least 7 days after application. While natural infections of INSV in the field remained low for the trial, and we were not able to assess any differences in INSV infection between the dsRNA treated and untreated plots, we did determine that repeated applications of dsRNA at high concentrations did not affect the biomass of the lettuce plants. Further studies are required to fully understand if the dsRNAs have suppressive

effects on INSV infection and are currently being conducted with INSV-infected thrips populations in contained greenhouses and growth chambers at the USDA. If there is a level of protection that is conferred by the introduction of dsRNA, it would be important to understand how long protection against INSV lasts. It is expected that this may be limited by the stability and longevity of the introduced dsRNAs. Furthermore, we demonstrate that foliar delivery of dsRNAs targeting INSV using a silica-based nanoparticle delivery system had a suppressive effect on viral gene expression for at least 14 days. Additional studies will determine the effects of the dsRNA on INSV symptoms in both tobacco and lettuce, as well as characterization of the effects on symptom development. Combined, the two delivery strategies demonstrate a promising area of research to establish RNAi-based methods for managing INSV.

The long-term goal of this project is to establish an RNAi-based tool that can effectively manage thrips and INSV using non-GMO methods and fits into the model of lettuce production practices in the Salinas Valley. While this proposal specifically focuses on the goals that are equivalent to early Phase 1 development of a new product, there are subsequent objectives that will need to be addressed. If efficacy studies continue to show promise, one future objective will be to optimize a delivery strategy for the dsRNAs at the field level – a topic that is highly popular and constantly changing as new technologies are emerging to enhance the stability and delivery of dsRNAs. However, we have already begun to explore methods for delivering dsRNAs through the roots and as a foliar application. As these technologies continue to advance and the cost to produce dsRNAs at field-scale levels continue to become cheaper, the implementation of RNAi technologies for integrated pest management will arrive.

Several promising responses by the EPA have favored the use of RNAi in plant protection for the management of insect pests. In June 2017, the first RNAi product was registered for the management of corn rootworm, https://www.epa.gov/pesticide-registration/epa-registers-innovative-tool-control-corn-rootworm. Additionally, in December of 2023, the EPA registered the first RNAi pesticide for the management of Colorado potato beetle, https://www.epa.gov/pesticides/epa-registers-novel-pesticide-technology-potato-crops. Both actions are promising for the future of RNAi technology and its use for pest management across numerous cropping systems.