Project 2: Biodegradable Nanomaterial-based Non-GMO RNAi Delivery for Controlling FHB Disease

The work conducted since July 2023 during NCE is reported under relevant sections in blue print

---

1. What are the major goals and objectives of the research project?

Spray-Induced Gene Silencing (SIGS) can be used as a safe and robust method of plant protection for both pre-harvest (Koch et al., 2016; Mitter et al., 2017) and post-harvest strategy (Wang et al., 2016), which is especially also useful for FHB disease with continuous mycotoxin production after harvest. In this research, which is a collaboration between plant-fungal scientists and a nanotechnologist, the goal is to use principles of RNA interference (RNAi) (Agrawal et al., 2003) and host-induced gene silencing (HIGS) (Huang et al., 2006) to develop non-transgenic approaches for wheat resistance to FHB. In particular, this research aims to test the hypothesis that whether a spray product, containing double-stranded RNA tool(s), can control the FHB disease. Because the lifetime of RNA molecules in the environment is short, and they are degraded rapidly, we aim to leverage nanotechnology and incorporate it into silencing RNA tools for slow-release and possibly the penetration of RNA molecules to plant or fungal cells.

2. What was accomplished under these goals or objectives? *(For each major goal/objective, address these three items below.)*

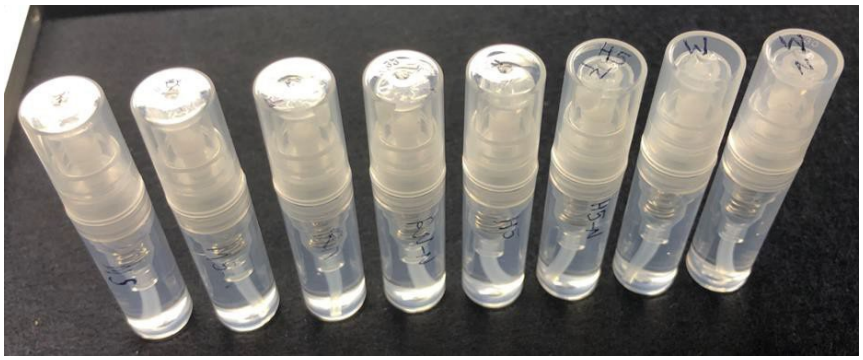
What were the major activities?

1a. Design and develop scalable production of core-shell dsRNA-chitosan nanostructures for controlled sustained release of dsRNA over the course of two weeks in the plant.

After the initial failure with Chitosan as nanocarrier, the team continued the project with the synthesis of graphene quantum dots (GQDs) nanoparticles as an effective alternative that has no anti-fungal properties. The Graphene Quantum Dots (GQDs) used in this study were prepared (**Figure 2a**) using the previously reported method with minor modifications through a bottom-up approach by direct pyrolysis of citric acid (CA). The size and shape of GQDs were confirmed using Transmission Electron Microscopy (TEM) and the corresponding TEM image is shown in **Figure 2b**. The presence of several nanosized black dots (indicated with yellow color arrows) in the TEM image confirms the successful synthesis of GQDs having a size of around 2-5 nm. Since the siRNA is a negatively charged molecule, to load the siRNA onto GQDs, it is necessary to understand the surface charge of as-synthesized GQDs. Therefore, the zeta potential of GQDs was collected using Zetasizer and observed that GQDs displayed (**Figure 2c**) a slightly negative charge (-1.71 mV). To make the surface charge of GQDs positive, branched Polyethyleneimine (bPEI) surface functionalization strategy was followed. The zeta potential collected (**Figure 2d**) after the surface functionalization of GQDs with bPEI exhibited a positive surface charge (+59.1 mV). These surface functionalized GQDs were further used for loading dsRNA through the ionic interaction mechanisms. Citrate-derived carbon dots functionalized with branched PEIs (bPEIs) have been used to deliver plasmid DNA and siRNA to animal cells (Liu et al., 2012; Pierrat et al., 2015).

1b. Integration of designed dsRNA into core shell nanoparticles with uniform spray application process.

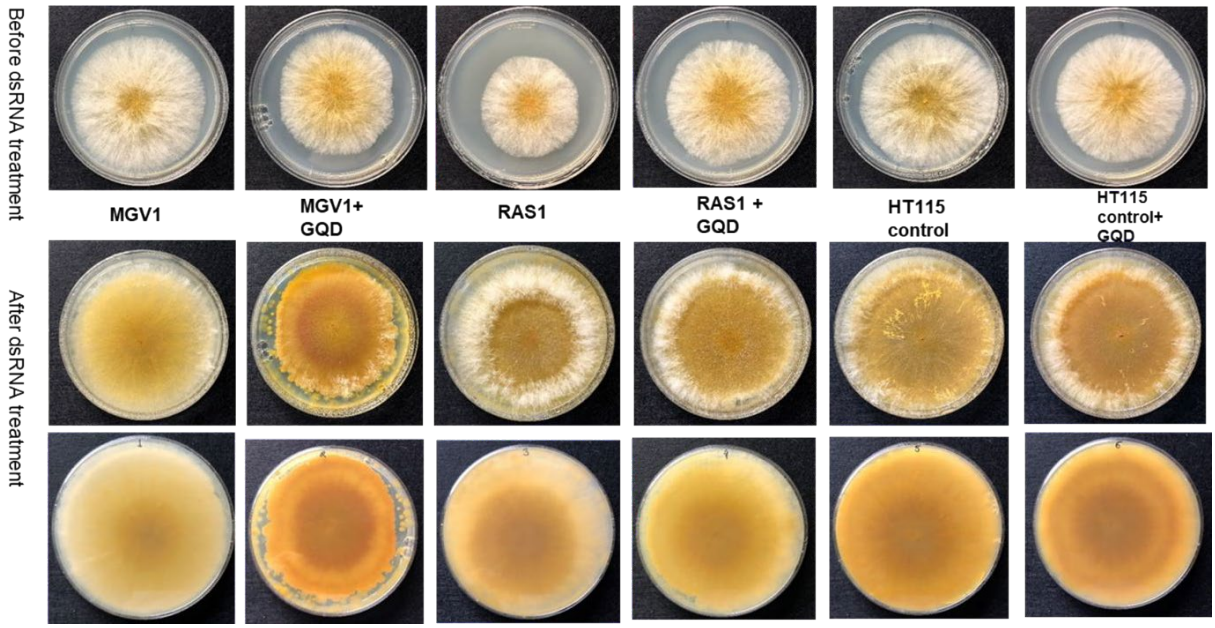
We have developed a protocol for the mixing of GQDs with dsRNAs along with the non-ionic surfactants silwet L-77, which allows successfully administering the dsRNA with functionalized GQDs onto plants using spray application using commercially available sprayer bottles.



Objective 2. Evaluate silencing of candidate genes in *F. graminearum* with siRNA oligos delivered by nanoparticles

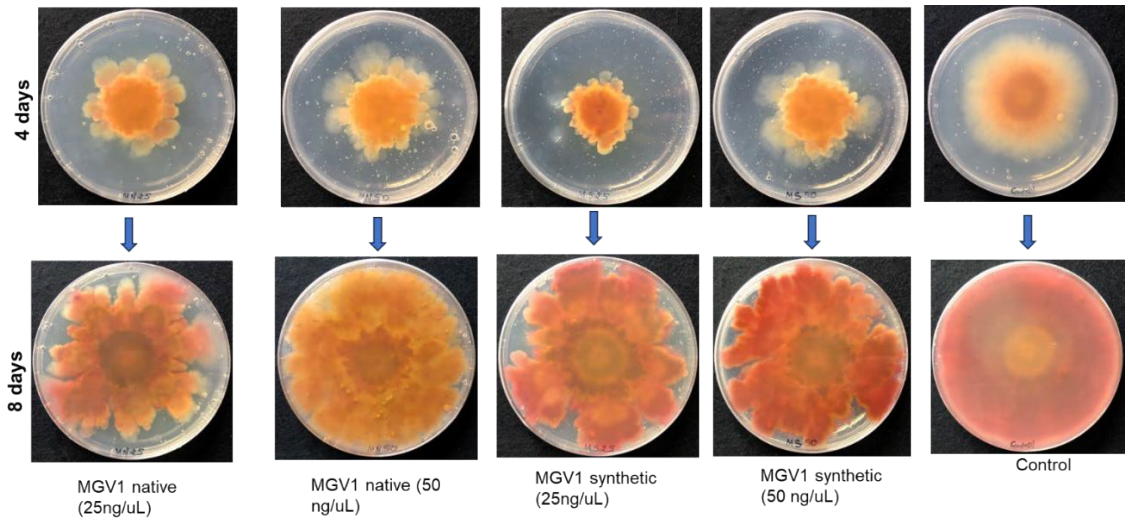
2a. Silencing of candidate genes with nanoparticle-coated siRNA oligos.

We designed an assay for the application of dsRNA rather than siRNA for the silencing of the Fusarium genes. For the confirmation of the effectiveness of the in-vivo produced dsRNAs while coating with GQDs, Fusarium were grown on PDA plates for four days. dsRNA for *MGV1* and *RAS1* (both native and synthetic dsRNAs) molecules were sprayed after four days. PDA plates were further incubated at 28 °C for four days. The resulting phenotypes were compared with the HT115 as well as water control.



Results show that the there is the inhibition of the mycelium growth in the plates while dsRNAs were mixed with the GQDs as compared to the control plates.

In the next step, Fungal mycelia were grown on potato dextrose agar (PDA) plates using the initial mycelium of 0.5 mm in length from the fresh plate culture. PDA plates were prepared by mixing two different concentrations (25 ng/uL as well as 50 ng/uL) of the respective dsRNA produced to see their effectiveness. The fusarium strain was incubated on potato dextrose agar (PDA) medium for 8 days (Lee et al. 2014). Results show that 25 ng/uL was effective as compared to the 50 ng/uL which also resonates with the previous studies. At higher concentrations of dsRNA, aggregation is likely to occur which decreases the silencing efficacy of formulations (Pierrat et al., 2015; Schwartz et al. 2020).



2b. Assays for siRNA delivery and effects of two or more siRNA oligos.

The co-PI JRX just received the backorder siRNAs materials. The work will be resumed immediately.

2c. Determine accumulative effects of simultaneously silencing of two or more genes.

The co-PI JRX just received the backorder siRNAs materials. The work will be resumed immediately.

Objective 3. Target gene prioritization, dsRNA design with cell-penetrating facilitation, and spraying and phenotyping FHB inoculated susceptible varieties by nanomaterial coated dsRNAs

3a. Prioritzation of *Fg* genes, development and dsRNA design with cell-penetrating facilitation for applications on wheat.

The genes we selected for targeting in wheat-Fusarium interaction included *MGV1*, *RAS1*, *COT1*, *YCK1*, and *CAK1* genes that are important or essential for growth (Hou et al. 2002, Bluhm et al. 2007, Wang et al. 2011), *CID1* (Zhou et al., 2010), *Fgpal1* (Yin et al., 2020) *FgPp2A* & *FgPpg1* (Yu et al., 2014), and *FTL1* (Ding et al., 2009), that were all previously published by Xu laboratory. We started designing and producing dsRNA tools targeting five genes *MGV1*, *RAS1, COT1, YCK1 and FgPp2A.* To design RNAi constructs that could silence each target gene, we used the plant-specific dsRNA design pssRNAit server (Ahmed et al., 2020). A 500-700 bp fragment from the coding region was used for prediction of siRNA sites. Parameters were set to maximize the number of siRNA sites in 100-300 predicted region and minimize any off-target region. After identification of the most suitable region, we used the native stretch of DNA predicted pssRNAit to have greatest number of predicted siRNAs, and called it a “native” segment. In addition, we used each of the predicted siRNA in tandem one after another in a hypothetical manner that does not exist in nature. We named this hypothetical tandem a “synthetic” segment. We then synthesized both native and synthetic segments for each gene by commercial DNA synthesis providers. For each of the Fusarium genes *MGV1* and *RAS1* and the wheat *phytoene desaturase* (*PDS*) gene we produced a native and a synthetic segment and cloned them in the double T7 promoter vector L4440 (Addgene 1654). The recombinant L4440 was transformed into the mutant strain HT115 (DE3) by a standard transformation procedure. This strain lacks RNase III, which is a dsRNA degradation enzyme. Therefore, the dsRNA produced in this bacterium will not be degraded. The L4440 also has T7 RNA polymerase-mediated transcription promoter which is induced by isopropyl β-d-1-thiogalactopyranoside (IPTG). For induction and large-scale production of dsRNA, the procedure developed by Lisa Timmons (Carnegie Institution of Washington), with some modifications based on Zhang et al (2019) and Ahn et al., (2019) was followed. Recent production resulted in a bulk of native and synthetic dsRNA with high yields.

We have optimized an in-vivo dsRNA production by using HT115 strain and published our results in Agrotechnology (See Section ***Publications, Conference Papers, and Presentations*** Page 9).

Previously, we reported design and production of dsRNA against two Fusarium genes *MGV1* and *RAS1*. Since July 2023 until now, we extended our effort to include dsRNA against six additional Fusarium genes including *COT1*, *FgPp2A*, *YCK1*, *TRI5*, *GMK1*, and *CAK1*. Unlike MGV1 and RAS1 where we designed native and synthetic constructs, for these additional genes, we only designed and produced native dsRNA by simple cloning approach. We also included in-vitro assessment of dsRNAs against fungal growth in comparison with fungicide, to learn at what concentrations they prohibit fungal growth. Our in-vitro assays indicated that different dsRNA shows different patterns in controlling the mycelium growth and conidia formation in the *Fusarium graminearum*. Currently, we are using microscopy facility to study absorption of the dsRNA by fungi and plants by dye incorporation.

3b. Treating FHB inoculated susceptible varieties by nanomaterial coated dsRNA design, and performing FHB phenotyping to characterize the efficacy of non-GMO gene silencing strategy.

We were able to develop the same strategy using a marker gene that is commonly used for the most silencing applications in wheat. Similar to the approach taken for *MGV1* and *RAS1*, we used phytoene desaturase (*PDS*) and designed native and synthetic constructs. We are also collaborating with co-PI RR group to test the efficacy of dsRNAs coated with graphene quantum dots (GQD) nanoparticles. A Graduate student in RR lab and Binod Gyawali in MM lab are working on the preparation of formulation for the graphene quantum dots (GQD). Various concentrations of GQD in response to the concentrations of produced dsRNA are yet be investigated. It will help to decipher the best combination of dsRNA with the GQDs for the phenotypic study.

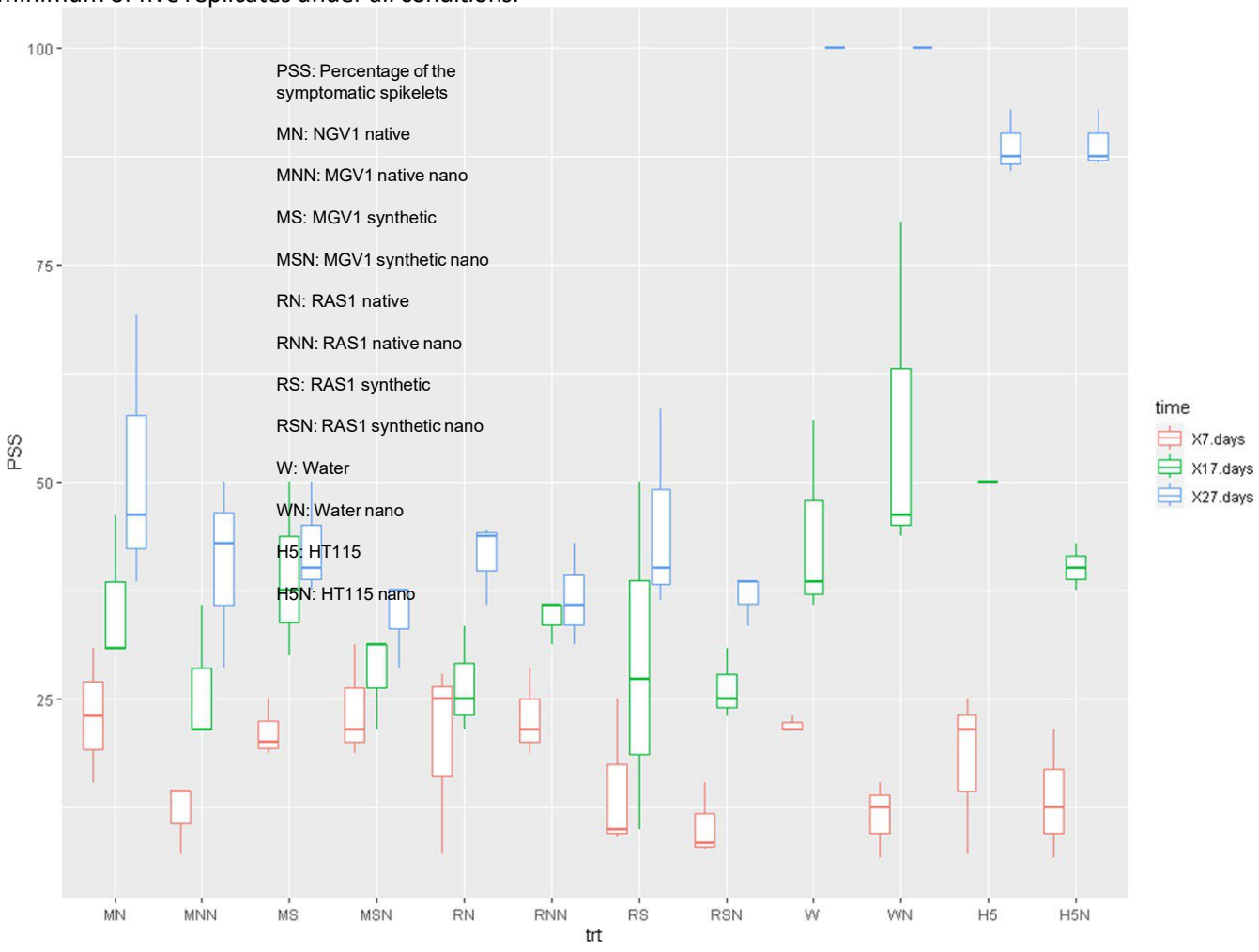
Inoculation of healthy plants with fusarium followed by dsRNA spray for the control of fusarium spread within the spikes

We also tested the effectiveness of the GQDs coated dsRNAs on the wheat heads.

Photos were taken 21 days after the first fusarium inoculation. Results show that *RAS1* dsRNAs-GQDs reduced the spread of fusarium in the wheat spikes as compared to control.

Experiments were done in the greenhouse under controlled conditions. We used two susceptible spring growth habit wheat varieties 'Gilat' (An Israeli accession) and 'AL105' (which is a line derived from the cross of parents 'Yecora' and 'Penny') to examine whether dsRNA-GQD conjugate application is able to control the disease establishment or spread. The field collected *F. graminearum* strains from Indiana was used for the preparation of inoculum. For greenhouse assay, spores were diluted to (5×10^4 spores/mL) (Lai et al. 2020).

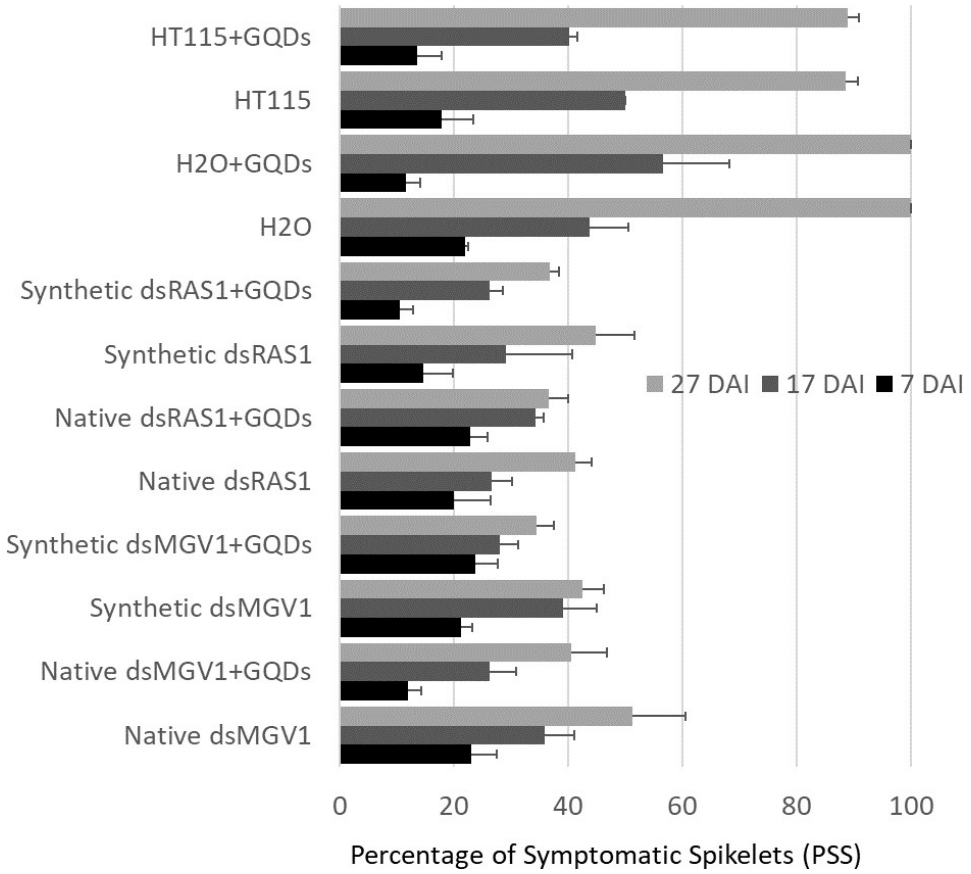
The pathogenicity assay of Fg strain was carried out with wheat cultivars 'Gilat' and 'AL105'. Wheat spikes at flowering stages were inoculated with 10 µL droplet of macroconidia suspension (5×10^4 spores/mL) (Lai et al. 2020). The inoculated spikes were covered with sealed polyethylene bags for 3 days. Visually infected spikelets were scored at 7, 17 and 27 days after inoculation (dai). The percentages of infected spikelets were calculated as percentage of symptomatic spikelets (PSS) (Su et al. 2019). For each variable, two major treatments were used as naked dsRNA as well as nanomaterials coated dsRNA and will be used as plant spraying (Wang and Jin 2017; Koch et al. 2019). Each treatment includes a minimum of five replicates under all conditions.



Boxplot showing the percentage of symptomatic spikelets (y-axis) in response to the dsRNA treatment for two genes *MGV1* and *RAS1* having both native and synthetic fragments. Two treatments,

dsRNA with or without GQDs were used in all the conditions. Data showed a reduction in FHB severity in GQDs-mediated dsRNA treatment compared to control treatments. The percentage of infected spikelets (PSS) were notably lower in GQDs-mediated dsRNA-treated plants, indicating effective suppression of *F. graminearum* pathogenicity.

By using only dsRNA against RAS1, when we treated Fg point-inoculated wheat with dsRNA or dsRNA+GQDs, we observed suppression of disease spread when dsRNA was with GQDs.



When native and synthetic constructs of MGV1 and RAS1 genes were used with and without GQDs, we observed that, percent symptomatic spikelets (PSS) increased significantly after Fg-treatment, all

treatments containing dsRNA were able to reduce PSS significantly by the end of experiment compared with H2O and HT115 controls, and all treatments containing dsRNA+GQDs reduced PSS significantly when compared with HT115. We have optimized an in-vivo dsRNA production by using HT115 strain and published our results in American Chemical Society (ACS) Applied Bio Materials (See Section ***Publications, Conference Papers, and Presentations*** Page 9).

What were the significant results?

Obj1. We are continuously working on the synthesis of the GQDs which were found effective as compared to Chitosan. The process has shown successful results and we will further continue the efforts on using this nanomaterial on plants. **Obj2**. We tested GQDs to coat dsRNAs for *F. graminearum* treatments. We have seen successful results during the spray of the dsRNA or making the plate culture mixing with the dsRNA as part of the media. **Obj3**. A plant-specific prediction server (pssRNAit - Ahmed et al., 2020) was used to design native and synthetic dsRNA silencing tools. Both native and synthetic tools were synthesized. Compared to the high cost of in-vitro transcription, we were able to source L4440 vector coupled with the HT115 mutant to produce large scale sRNA in-vivo for three genes i.e., wheat PDS and Fg *MGV1* and *RAS1.* We were also able to reduce the Fusarium spray on the wheat heads after the application of the naked as well as GQDs coated dsRNAs. We extended in-vitro assays, optimized dsRNA production, and were able to show result for in-planta dsRNA application.

List key outcomes or other achievements.

Obj1. RR team is continuously synthesizing GQDs, surface modification and verification as per the requirement of the MM group.

Obj2. Graphene Quantum Dots (GQDs) were found to be effective as compared to other nanoparticles. Results showed that RAS1-dsRNA and MGV1-dsRNA were able to reduce disease spread on plate culture while simultaneously silencing the respective genes.

Obj3. The production of dsRNA targeting *MGV1* and *RAS1* was optimized in mutant strait HT115 by using inducible double T7 promoter that will be used on Fg-inoculated susceptible wheat plants in the fall greenhouse season. Results showed that RAS1-dsRNA and MGV1-dsRNA were able to reduce disease spread on plate culture as well as on wheat spikes. However, RAS1-dsRNA-GQDs and MGV1-dsRNA-GQDs treatments could effectively restrict the spread of disease on wheat rachis.

DON reporting of last year

Extending the approach to other genes and performing more in-planta greenhouse experiments to evaluate the efficacy of dsRNA method.

3. What opportunities for training and professional development has the project provided?

Obj1. Devendra Sarnaik is a graduate student and Sachin Kadian was a post-doctoral associate working on the project (RR research group). Venkat Kasi, a graduate student recently started working on this project.

Obj2. A MS student, Penelope Vu, was trained in this project (JRZ research group).

Obj3. Binod Gyawali, a graduate student, is working on his dissertation thesis on Obj3. He is actively involved in dsRNA design, cloning, and production of dsRNA for testing (MM research group).

Binod Gyawali is continuing his PhD dissertation on this project, during this NCE, PI covered BG assistantship by a variety of internal RA/TA. BG won a dissertation award from college of agriculture for the last semester of his dissertation. BG intends to publish one more paper from this project and complete his PhD degree program by December 2024.

4. How have the results been disseminated to communities of interest?

Two journal publications, one conference oral presentations, and one conference poster.

5. What do you plan to do during the next reporting period to accomplish the goals and objectives?

For the next reporting period we will continue to conduct the required activities to meet the outlined objectives.