Project FY22-TS-009: Biodegradable Nanomaterial-based Non-GMO RNAi Delivery for Controlling FHB Disease

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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.

Currently, chemical fungicides remain the major component of FHB disease control, which carries risks to human and environmental health. Use of exogenous dsRNA to induce RNAi against pathogen's genes, is an effective and sustainable approach for the control of FHB.

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

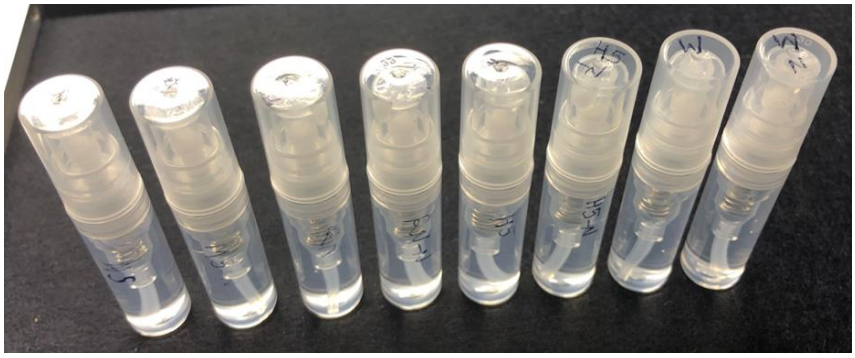
exhibited a positive surface charge (+59.1 mV). These surface functionalized GQDs were further used for loading dsRNA through the ionic interaction mechanisms. Citrate-derived carbon dots functionalized with branched PEIs (bPEIs) have been used to deliver plasmid DNA and siRNA to animal cells (Liu et al., 2012; Pierrat et al., 2015).

In this study, we used this approach to investigate the in-vitro and in-vivo effects of dsRNA applications on growth and pathogenicity of Fusarium graminearum by using Graphene Quantum Dots (GQDs) as nanocarrier.

For the design and production of dsRNA, we selected eight F. graminearum genes (FgMGV1, FgRAS1, FgCOT1, FgPp2A, FgCAK1, FgTRI5, FgGMK1, and FgYCK1), which have previously been shown to have a functional role in fungal growth or pathogenicity. Supplementation of dsRNA on fungal growth within SNA liquid culture media showed reductions in fungal biomass, measured by optical density, by almost half.

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.

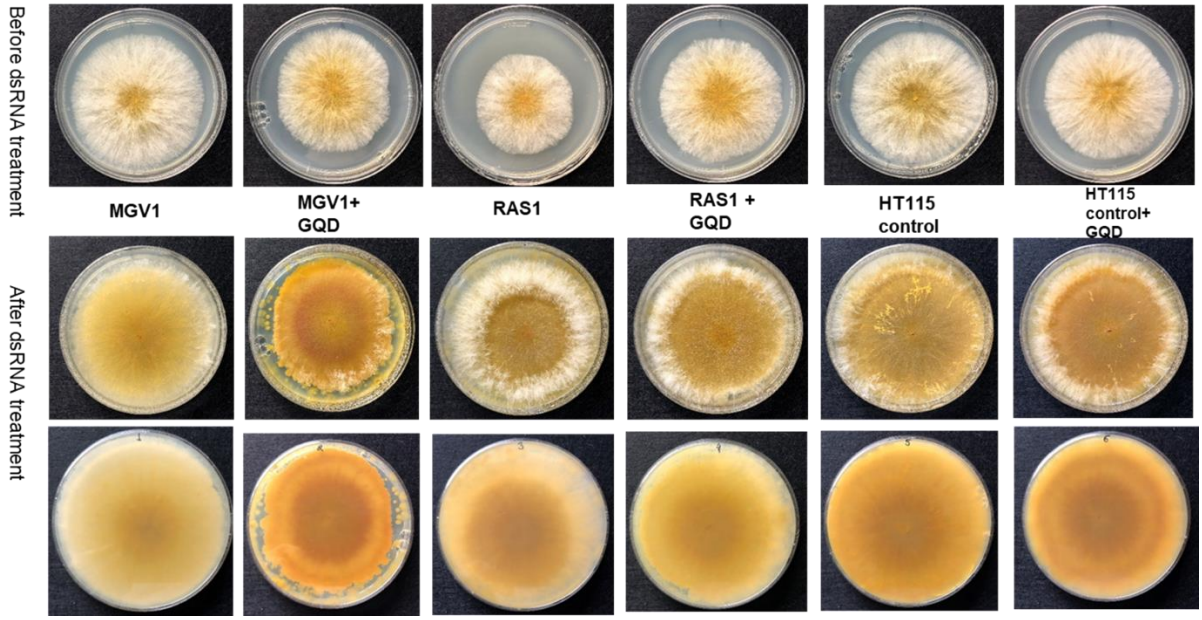


Objective 2. Evaluate silencing of candidate genes in *F. graminearum* with siRNA oligos delivered by nanoparticles

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

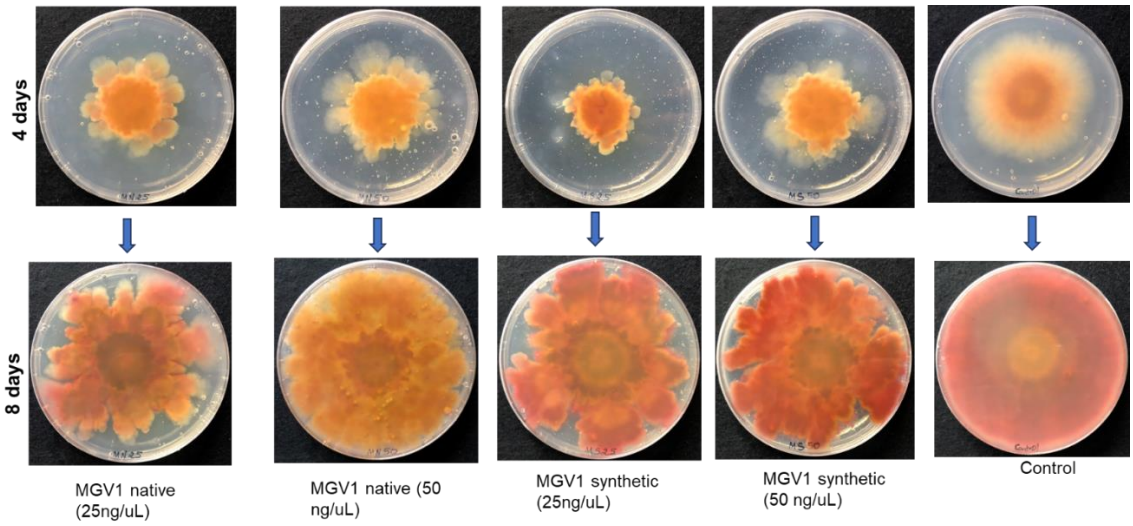
We designed an assay for the application of dsRNA rather than siRNA for the silencing of the Fusarium genes. For the confirmation of the effectiveness of the in-vivo produced dsRNAs while

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).

Similarly, mycelial growth of Fg on plate was also highly inhibited with supplementation of dsRNA. The dsRNA spray on Fusarium-inoculated plants showed significant reductions in the percentage of symptomatic spikelets (PSS) by 20-25% as compared to control.



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

This objective, which was with co-PI JRX delayed because of siRNAs materials are on backorders.

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

This objective, which was with co-PI JRX delayed because of siRNAs materials are on backorders.

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

3a. Prioritization 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.

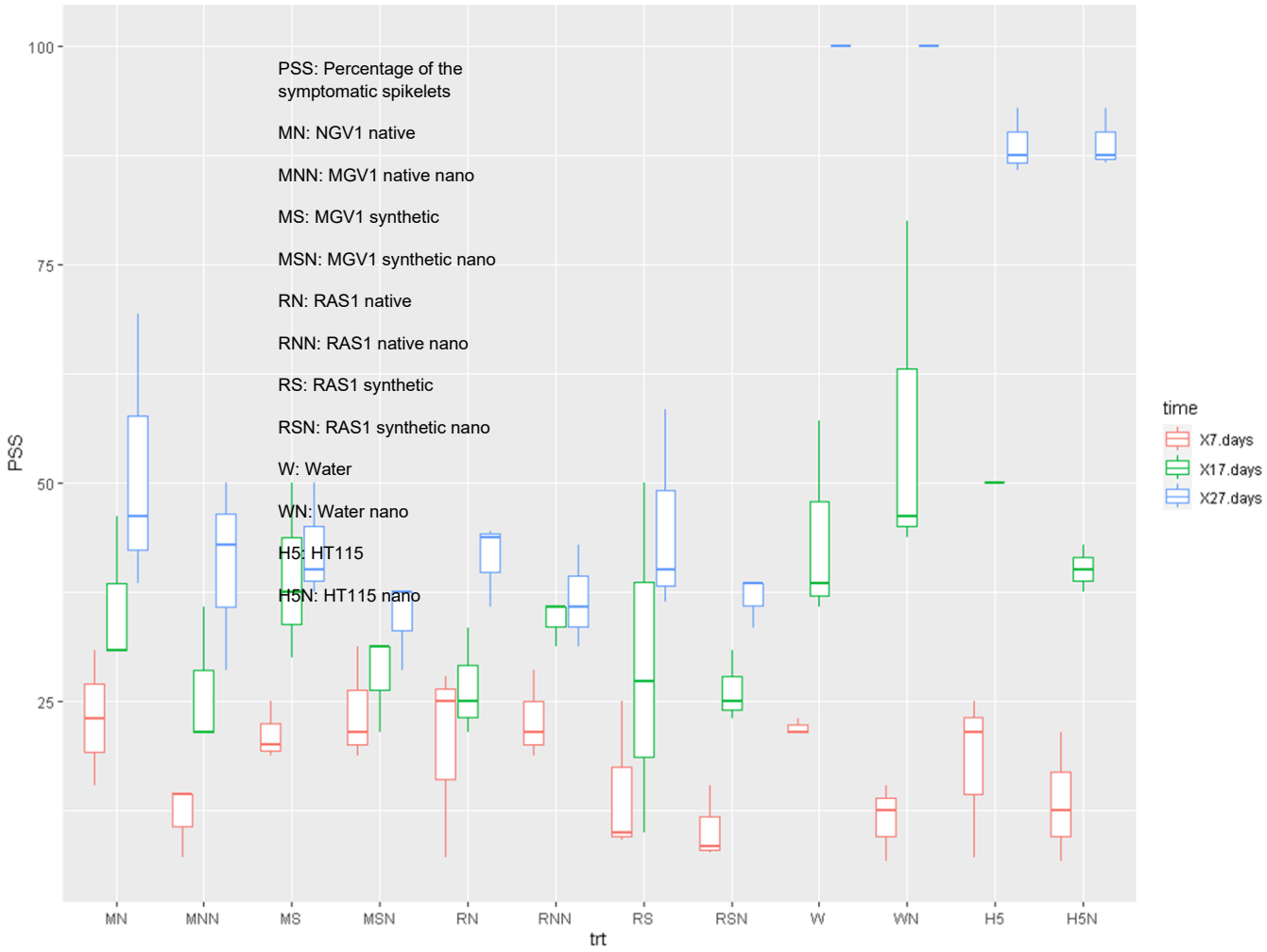
3b. Treating FHB inoculated susceptible varieties by nanomaterial coated dsRNA design, and performing FHB phenotyping to characterize the efficacy of non-GMO gene silencing strategy.

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.

We also evaluated the effect of dsRNA on lowering the accumulation of mycotoxin deoxynivalenol (DON) post-infection. Furthermore, analysis of transcript abundance for two genes FgRAS1 and FgPP2A after the application of dsRNA in the liquid culture showed the gene expression reduction in treated Fg cultures by one-third as compared to untreated cultures.

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.

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

Growth inhibition of Fusarium by supplementation of dsRNA in SNA medium. In this study, we performed the in-vitro liquid culture, plate assay, and in-planta assay to examine the effectiveness of the applied dsRNA in controlling the growth and pathogenicity of the Fg. To optimize the concentration suited for growth inhibition assay experiments with dsRNAs, we only tested the dsRNA against FgMGV1 and conducted assays with increasing concentrations of dsMGV1 ranging from 0.5, 5, 12.5, 25, to 50 µg/mL, and compared the growth of Fg in liquid culture, indicated by OD, with the growth obtained from cultures treated with water or crude RNA extract from HT115 (simply denoted as 'HT115 control') as control. Concentrations of 25 or 50 µg/mL were equally sufficient to suppress the growth by the 6th day (Suppl. Fig. 2a). For the recovery test, 1.5 ml subculture from each of the dsRNA concentrations was subjected to the 10-fold dilution with fresh liquid culture (1X SNA) in the final volume of 15 ml was further incubated for regrowth in fresh medium. Fusarium cultures treated with lower concentrations i.e., 0.5 µg/mL and 5 µg/mL, were able to resume growth when sub-cultured in fresh liquid medium. However, the culture treated with 12.5 µg/mL was partially able to resume growth more slowly, while cultures treated with high concentrations i.e., 25 and 50 µg/mL were not able to resume growth when sub-cultured in fresh medium (Suppl. Fig. 2b), indicating that Fusarium growth is effectively restricted after the treatment of Fusarium with dsRNA concentrations beyond a threshold.

Based on dsMGV1 concentration optimization results, the growth inhibition of Fusarium by supplementation of other dsRNAs in SNA medium was performed at a concentration of 25 µg/mL, and growth measurements indicated as OD values were taken over 10 consecutive days. We observed three types of responses in the growth curve (Fig. 1a). For control groups, water and HT115 treatments, the growth continued sharply and then plateaued at an OD of nearly 1.5 which is indicative of potential growth in a non-inhibiting (no dsRNA) growth environment as baseline control. For dsGMK1, dsCAK1, dsPP2A, and dsRAS1 growth continued to an OD of nearly 1 by the 5th day and then declined to around 0.4 OD, and then remained constant thereafter. Even for the first 5-6 days, the growth in this group was with considerably slower rates than what observed in control treatments water and HT115. For dsYCK1, dsTRI5, dsMGV1 and dsCOT1 the growth gradually continued to 0.4, with an even smaller rate than the second group, and then dropped to 0-0.2 OD values. Notably, in the third group, optical density values beyond 10 days treatments were remarkably low.

We rationalized that the area under the curve can be used to demonstrate the total capacity of growth or biomass produced over time (Fig. 1b). We took water treatment as 100% and normalized all other treatments based on water treatment. HT115 performed nearly similarly to water control by producing up to 90% of fugal biomass compared to water control. The growth based on approximation of area under the curve normalized relative to water treatment showed that when treated with dsYCK1 and dsTRI5, fungal growth was inhibited, and the cultures grew only to 10% levels of biomass produced in water treatment (Fig. 1b). The dsRAS1, dsMGV1 and dsCAK1 also inhibited fungal growth, and the cultures grew only to 40% levels of biomass produced in water treatment.

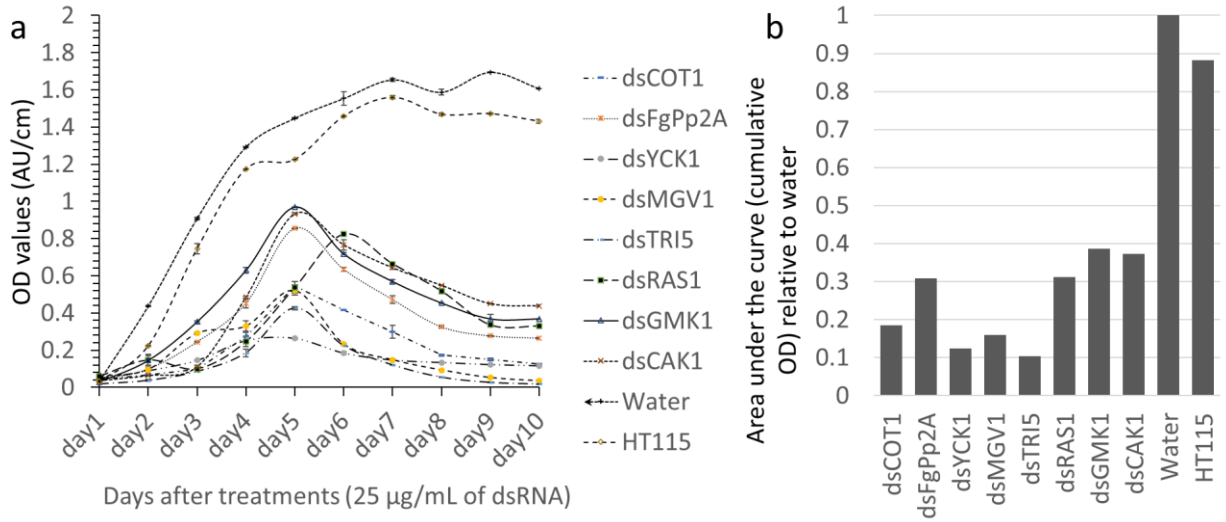


Figure 1. Results of feeding experiment of the dsRNA to the Fusarium culture. Treatment of the all dsRNA (25 µg/mL) with the fusarium culture over the 10 days with triplicates. a) Evaluation of the growth of the fusarium expressed as the OD values, where x-axis shows the days of treatment of the dsRNA (10-day range) while y-axis shows the average OD values of triplicates over the 10 consecutive days for each dsRNA. (b) Calculated area under the growth curve for the 8-dsRNA treatment for 10 days.

When growth of fungus mycelium in growth inhibition assays by dsRNA supplementation was visualized under microscope only for dsCOT1 and dsTRI5 treatments. We observed a clear reduction in the density of the mycelium in media supplemented by dsCOT1 and dsTRI5 compared with fungus mycelium growth observed in water and HT115 control (Fig. 2). In such cases, the mycelia appear sparse, and the structures are fragmented or thinned, indicating that the feeding with dsRNA has effectively inhibited normal fungal growth and development. In contrast, both the water control and HT115 control treatments show thick, dense, and more intact mycelia, which indicates normal, uninhibited growth, implying no interference with the growth of the Fusarium mycelium.

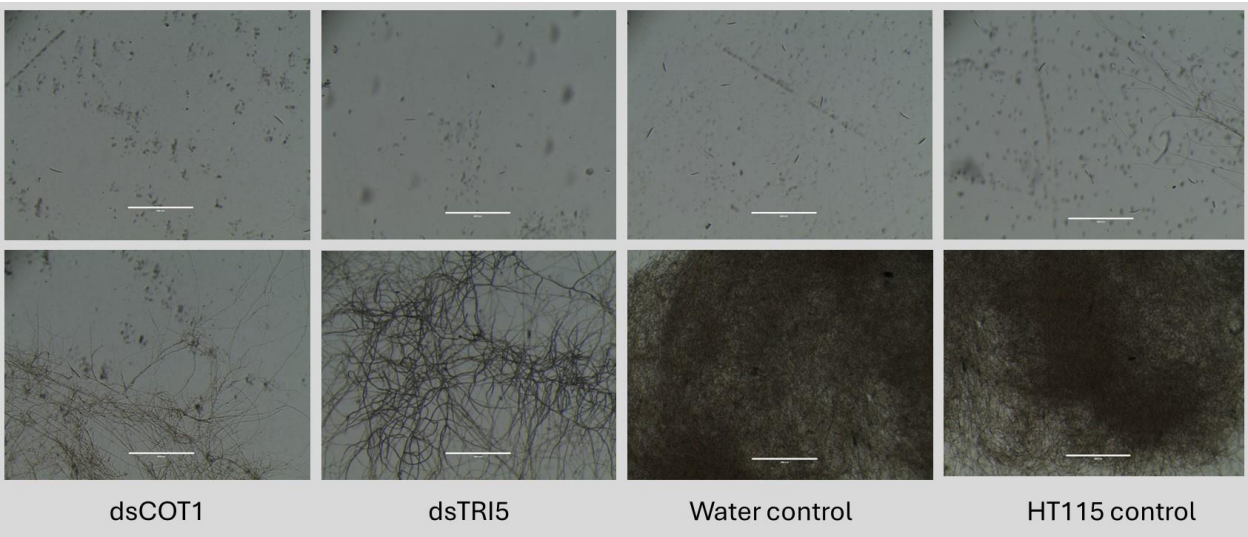


Figure 2. Representative phenotypes of the conidia number (upper panel) as well mycelium growth of the (lower panel) of ds*COT1* and ds*TRI5* as well as water and *HT115* control.

Growth inhibition of Fusarium by supplementation of dsRNA in PDA plates. Inhibitory activity of dsRNA on fungal growth was also assayed on solid media. In this experiment we included four treatment groups. The first two treatments were water and HT115 controls. The third treatment consists of the solid plate prepared with a mixture consisting of 'all' dsRNA that were produced against all eight genes. The fourth treatment was the solid plate prepared with mixture of a 'subset' (dsTRI5, dsGMK1 and dsFgPp2A) dsRNA against genes that are mainly responsible for the pathogenicity, conidia formation and reproduction. The data (Fig. 3) showed that in the subset treatment, the colony appears small, with a restricted growth pattern, showing less radial expansion compared to the water control.

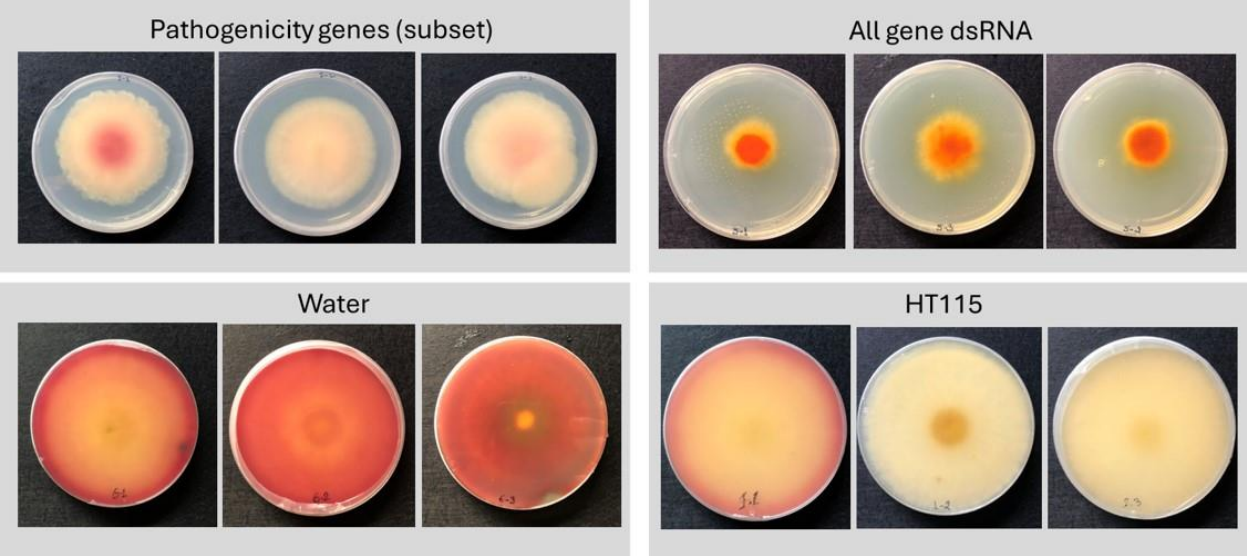


Figure 3. Results of the feeding assay of the dsRNA treatments against the fusarium growth in the plates are shown. Phenotypes with three replicates observed in 'subset dsRNA' as well as in 'all dsRNA' are in the upper panel while the phenotypes observed in two controls 'water' and 'HT115' treatments are in the lower panel. Images were taken after 8 days of culture.

In the treatment group with all eight dsRNA, the fungal colony is highly restricted, showing almost none to negligible radial growth having minimal hyphal density. For the water as well as HT115 application, the colony exhibits normal fungal growth, with clear radial expansion and a dense, free-growing fluffy mycelial appearance. The colony has fully expanded across the plate with a thick hyphal structure. One-way ANOVA results show that one or more treatments are significantly different ($F=190$, $P<0.001$). Further, Tukey comparison of means showed that all the treatments are significantly different from each other ($p<0.01$) except water and HT115 treatments. The results indicate the cumulative effects of a mixture of dsRNA (against subset, or all genes), can effectively restrict the horizontal spread of the fungus in the PDA medium (Fig. 3). The results indicate the cumulative effects of a mixture of dsRNA (against subset, or all genes), can effectively restrict the horizontal spread of the fungus in the PDA medium (Fig. 3).

Reduced disease symptoms when wheat was sprayed with dsRNA or dsRNA-GQDs conjugates. To measure the amount resistance attainable, we chose genotype 'AL105' that was previously shown susceptible phenotypes. To examine the effectiveness of the applied dsRNA against Fusarium

infection, we made a mixture of dsRNA from all eight constructs. For stability, delivery, and more effective usage, we used GQD as a nanocarrier. The mixture of dsRNA along with nanoparticle (GQD) conjugation was applied by spray on spikelets that were previously challenged by Fg point inoculation for 3 days to mimic SIGS in the greenhouse. Treatment of all dsRNA without and with GQD conjugation reduced the percentage of symptomatic spikelets to 50% and 25% of what observed in water control treatment, respectively (Fig. 4 and Fig. 5a). When treated with mixture of dsRNA, we observed more upward progression of bleaching across the spikelets. Treatment of GQD-conjugated dsRNA showed even stronger inhibitory effects on spread of disease compared to non-GQD treatment (Fig. 4). In control treatment, spread of the bleached spikelets were observed over the whole spike showing substantial fungal spread – a typical susceptible response type. In control treatments, the progression of the bleaching was bidirectional from the point of inoculation resulting in the severe effect of fungal infection showing dry and discolored spikes (Fig. 4).

Figure 4. Representative phenotypes observed in AL105 variety after 21 days of the dsRNA treatment as well as control. All of the dsRNA were mixed with GQD with 1:1 concentration and the wheat heads were detached and photographed after 27 days after inoculation (dai).

Reduced progression and accumulation of DON beyond the site of inoculation in spikes sprayed with dsRNA. To measure disease progression and DON beyond the site of inoculation in spike, we chose two genotypes 'AL105' and 'Gilat' that were previously shown susceptible phenotypes. We asked if dsRNA application can prevent or lower the accumulation of DON content. One treatment was testing the effect of a mixture of all dsRNAs. The other treatment was dsRNA against only FgTRI5. In this point-inoculation experiment we evaluated single kernels based on their relative position to the site of inoculation. We considered '0' for site of inoculation, +1 and +2 for spikelets above the site and -4, -3, -2, and -1 for spikelets below the site of inoculation. Results showed that treatment with all dsTRI5 reduced DON, aggregated across all grains, to below 47% and 48% of DON levels observed in 'AL105 and 'Gilat', respectively. Treatment with all dsRNAs reduced DON to below 14% and below 28% of DON levels observed in 'AL105 and 'Gilat', respectively (Fig. 5b).

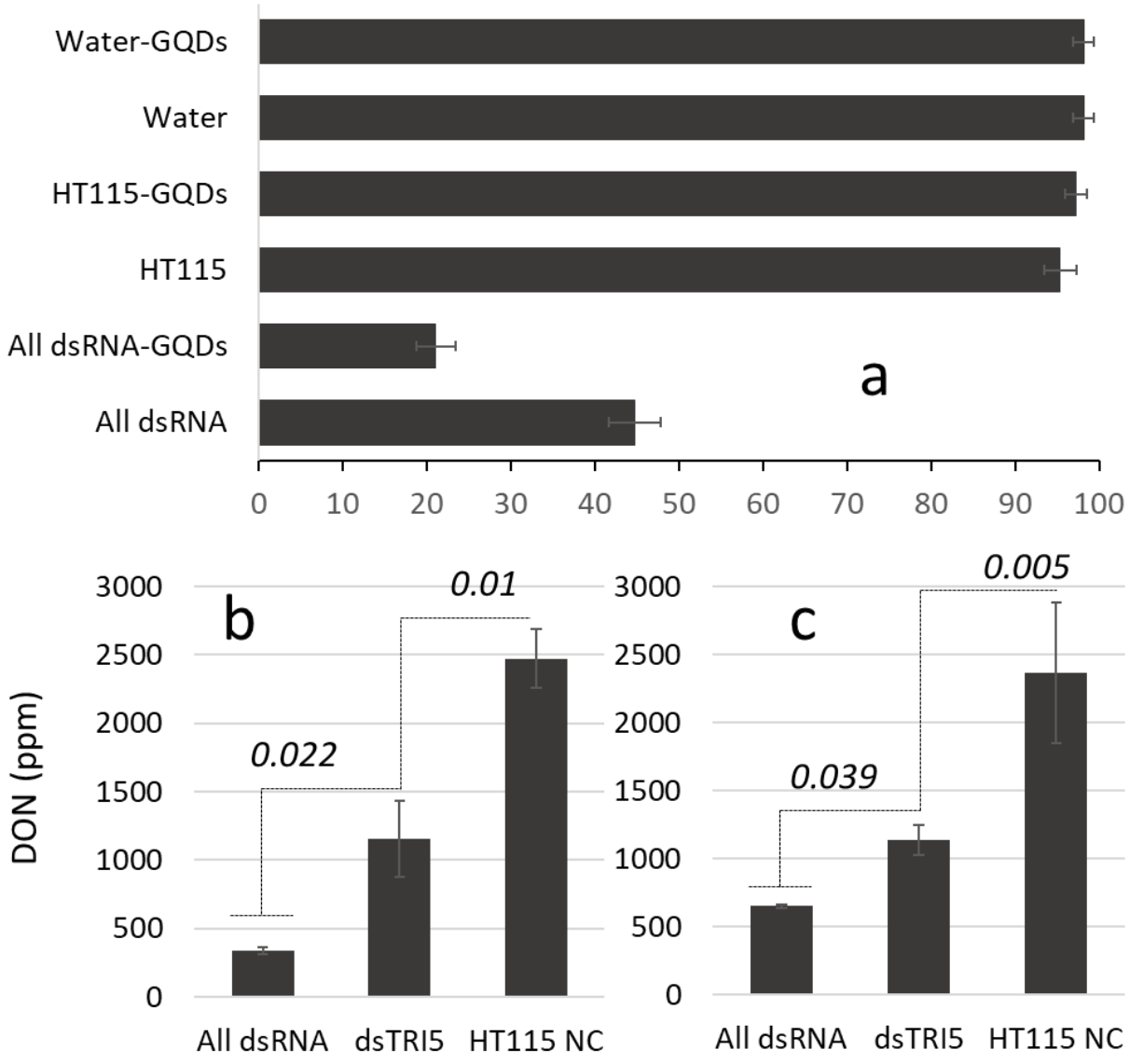


Figure 5. Percentage of symptomatic spikelets after the inoculation of the fusarium and treatment of all dsRNA along with GQD in AL105 variety (a). Deoxynivalenol observed after the treatment of the all the dsRNA as well as ds*TRI5* in the 'AL105' variety (b) as well as 'Gilat' variety (c). Student t-test was performed to find the p-values within the treatments.

DON analysis of single kernels showed that spray of mixture of all dsRNA also reduced the spread of DON to the neighboring spikelets significantly when compared to control group. This reduction was consistent in both wheat genotypes tested (Fig. 6). In control group, without any dsRNA intervention, the spread of disease was observed across the spike length. However, in all spread was contained only in the first two spikelets above and only one spikelet below the site of inoculation. Even with the application of single dsTRI5, DON content and its spread of infection to the neighboring spikelets was reduced significantly compared with control group although not to the levels observed in all dsRNA group (Fig. 5b and 6). The difference between the effect of all dsRNA group and dsTRI5 group on total DON and its spread was also significant (Fig. 5b and 6).

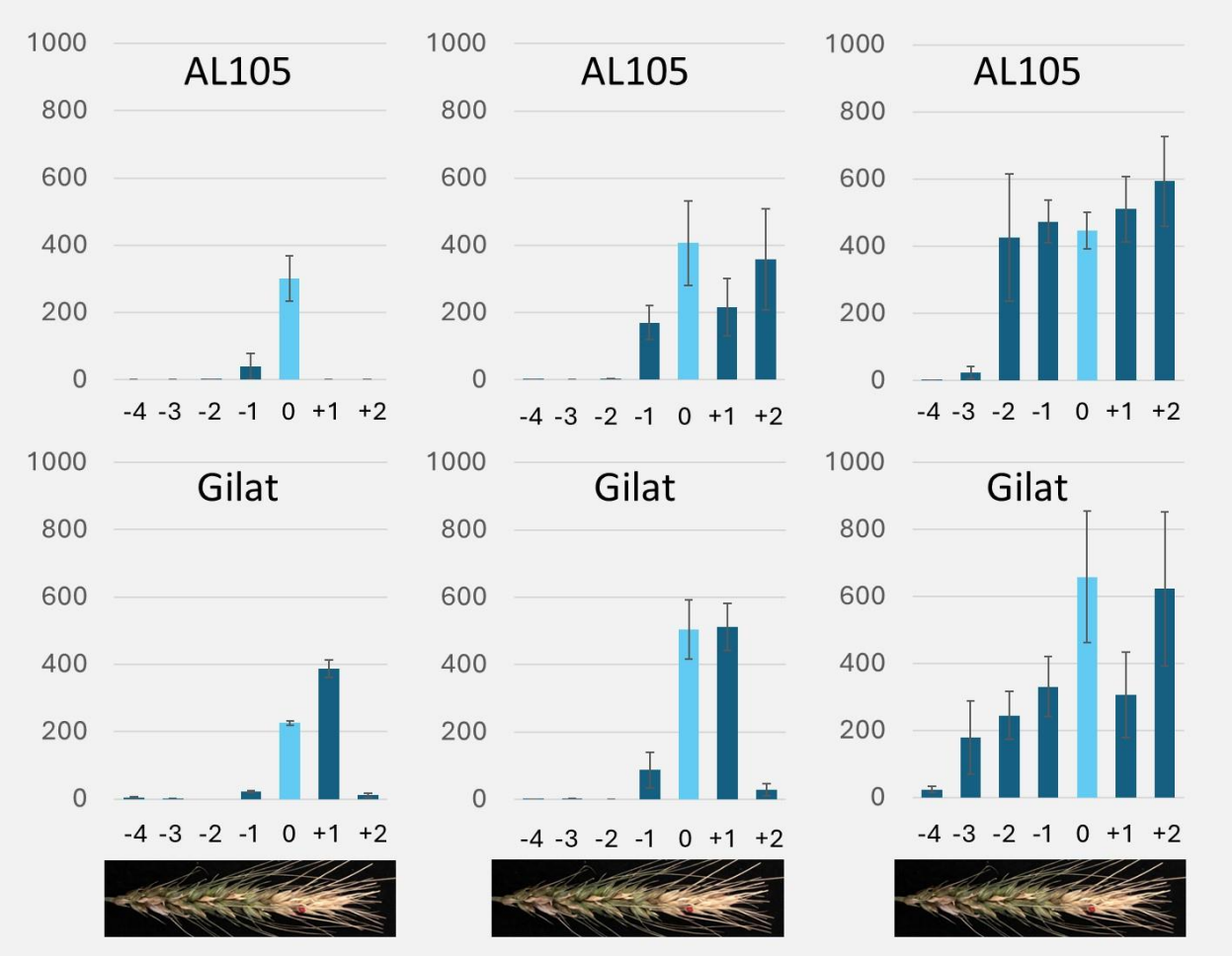


Figure 6. Representative values of the deoxynivalenol (DON) expressed in ppm after the treatment of the 'all dsRNA' (left panel), only ds*TRI5* (middle panel) as well as control treatment (right panel). For all cases, dsRNA was mixed with GQDs and sprayed on the wheat head. Upper three graphs are for the 'AL105' variety while the lower three graphs are for the 'Gilat' wheat variety. DON values were scored in the individual kernels (two kernels to the top of the inoculation '+1', '+2', and 4 kernels towards the base of the spike '-1', '-2', '-3', '4'), point of inoculation is represented with '0'. Top panel is for genotype 'AL105' and bottom panel is for genotype 'Gilat'.

List key outcomes or other achievements.

See the results provided.

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

One PhD student completed a doctoral dissertation in Dr. Mohammadi laboratory.

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

Two papers were published, one is under review, with three invited talks.