Optimization of Fast-Flowering Mini-Maize for Characterization of Ear Rots Caused by *Stenocarpella maydis* and *Fusarium graminearum*

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INTRODUCTION

There are several fungal pathogens that cause ear rot diseases of maize. In 2020, ear rots cost American farmers 53,560,642 bushels, worth $236,569,764[1]. This study focused on two of these ear rot fungi: *Fusarium graminearum* which causes Gibberella Ear Rot (GER); and *Stenocarpella maydis* which causes Diplodia Ear Rot (DER). Both *F. graminearum* and *S. maydis* are common in the United States. *Fusarium meridionale* was also utilized in a few experiments as it is a close cousin of *F. graminearum* that causes GER in Brazil but not in the United States.

Despite the major losses that these fungi cause to crop growers every year, there is relatively little research on pathogenicity mechanisms of ear rot fungi. This is partly due to the technical difficulty of doing experiments with mature maize under controlled conditions. To address this issue, my study focused on optimizing the use of Fast-Flowering Mini-Maize (FFMM) as a model for ear rots. FFMM produces mature ears in less than 60 days and could be very beneficial for the advancement of ear rot research. FFMM can also be grown in a greenhouse throughout the year, so research would not need to depend on growing seasons.

The objectives of this experiment were:

- To optimize protocols for infecting FFMM ears with *S. maydis* and *F. graminearum.*
- To create a green-fluorescent strain of *S. maydis* and a red-fluorescent strain of *F. graminearum.*
- To determine if co-inoculations of *S. maydis* and *F. graminearum* affect disease severity and mycotoxin accumulation.
- To develop methods for quantifying *S. maydis* and *F. graminearum* and GER and DER in infected FFMM ears.

MATERIALS AND METHODS

Growing and Pollinating the Fast-Flowering Mini-Maize (FFMM)

Pots were filled with a soil mix consisting of 2/3 Pro Mix and 1/3 steam-sterilized field soil. Three 2-inch holes were created, and dried FFMM kernels were placed in each and then covered. The seeds were left to grow, watering as necessary and fertilized every other day. Any plants that failed to thrive were removed. Once tassels and silks were visible, the silks were bagged. Once tassels began to release pollen, they were also bagged to collect the pollen. Silks were trimmed and then 24 hours later they were hand-pollinated with fresh pollen (less than 24 hours old) collected from the tassels. They were pollinated a second time 24 hours later[2].

Inoculating the FFMM

Five days after the second pollination, the plants were inoculated with fungi either at the top or the bottom of the ears in a randomized treatment. The treatments were: *S. maydis* strain DM6.001 top and bottom; *F. graminearum* strain PH1-GFP top and bottom; a co-inoculation of both fungi top and bottom; and a water control. The top inoculations utilized a [1 x 10^6] spore suspension of the fungi, recovered after two weeks of growth at 23°C and continuous light on agar plates [Potato Dextrose Agar (PDA) for *F. graminearum* and Oatmeal Agar (OA) for *S. maydis*]. The spore suspension was pipetted directly onto the silks and the very top of the corn cob. The bottom inoculations utilized a 3 mm plug taken directly from the agar plates of the fungi. A lesion was created with a scalpel, and the agar was placed directly on the lesion and secured with parafilm. Because *S. maydis* infected the plants very rapidly and extensively in earlier experiments, the inoculum was reduced by half for the later experiments (Plants 41-62). Corn ears and stalks were harvested fourteen days after inoculation.

Quantitative Evaluation of Visible Disease on Corn Ears

After harvesting, corn ears and stalks were photographed in a variety of ways. Corn ears with very few kernels were simply photographed horizontally. Corn ears with sufficient kernels were placed on a rotating apparatus that would present the corn ear vertically while spinning through 360^o. Videos were taken while the corn ear rotated. Once the data had been gathered, the videos were then flattened into a 2D image using ImageJ and FFmpeg. The flattened images could then be used for quantification also through ImageJ (Warman *et al.* 2021). The overall area of kernels and cob was measured, along with the area of visible disease. The percentage of diseased area was used as a quantitative measure of disease severity.

Qualitative Evaluation of Visible Disease on Corn Ears

Along with a quantitative method, a qualitative method was also used for this experiment. A disease severity scale (Figure 1) was developed for both *F. graminearum* and *S. maydis* and used to evaluate disease severity on corn ears with sufficient kernels.

Verification of Pathogen Presence via PCR

Harvested inoculated ears were dried in a grain dryer and a coffee mill was used to grind them to a fine powder. DNA was extracted from the ground corn tissue with a fungal DNA extraction method (Thon *et al.*, 2000) as follows: lysis buffer (0.5 M NaCl, 1% SDS, 10 mM Tris-HCl pH 7.5, 10 mM EDTA) was added to the ground tissue and incubated in a 37°C hot water bath. An equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1) was then added and the sample was spun in a centrifuge at 13,000 rpm for 15 minutes to create an aqueous and organic layer. The aqueous layer was removed, and the PCI step was repeated to further separate the DNA from any remaining protein contamination. The DNA was then precipitated by using 0.1 volume 3 M sodium acetate and 0.7 volume isopropanol and washed using 70%

ethanol. After drying, the DNA pellet was resuspended in diethyl pyrocarbonate (DEPC) water and RNase A. Qubit was utilized to quantify the amount of DNA extracted from each sample.

Three different primer pairs were used to amplify corn actin as a DNA quality control, and also *F. graminearum* (Tri 5), and *S. maydis* (ITS) (Table 1).

	Forward Sequence (5'-3')	**Reverse Sequence (5'-3')**	**Primer Melting Temperature**
Actin	CGT TGC TGC ATC GAA CCT GTT TCA	ACC TCA GGG CAC CTA AAC CTT TCT	F – 60.3 R – 60.1
Tri 5	AGC GAC TAC AGG CTT CCC CT	AAA CCA TCC AGT TCT CCA TCT G	F – 58.9 R – 54.7
S. maydis	CCT GCT ATG CAT AGG TCG	CAC CAG GCC GTT AAG CCT TA	F – 52.6 R – 57.4

Table 1. The three primer pairs used in the experiment. Forward and reverse sequences are shown, as well as the primer melting temperature (Tm). Actin primers were found in Sharma *et al.* 2011. Tri5 primers were found in Doohan *et al.* 1999. S. maydis primers were found in Romero & Wise 2015.

For all reactions, PCR were conducted in a 25 - µL reaction volume, containing template DNA at 10 ng/µL, 2.5 mM each TakaRa deoxyribonucleotide triphosphate, TakaRa Extaq DNA polymerase at 5 U/µL, TakaRa 10 x PCR buffer, 10 µM each forward and reverse primer (Table 1), and 17.3 µL of Invitrogen nuclease-free sterile water. Corn actin was amplified with the following program: an initial heating cycle of 94°C for 3 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 15 s, and extension at 72°C for 30 s; and a 7 min final extension at 72°C. The *F. graminearum* Tri 5 sequence was amplified under the following program: an initial heating cycle of 94°C for 3 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 11 s, and extension at 72°C for 30 s; and a 7 min final extension at 72°C. The *S. maydis* ITS sequence was amplified using the following program: an initial heating cycle of 95°C for 2 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min; and a 10 min final extension at 72°C.

The amplicons were separated on a 1% agarose gel at 90 V for 1 hour and 30 min. Each sample included 7 µL of nuclease-free DNA water, 5 µL stop dye, and 3 µL amplicons/Invitrogen 1 Kb Plus DNA Ladder (Cat. No. 10787018/10787026). Each agarose gel was stained with 1% Ethidium Bromide in 1 x TAE for 20 minutes. Gels were then visualized under Trans UV light.

Recovering Fungi from Ground Corn Tissue

A single colony dilution method was also tested to determine the quantity and identity of fungi causing disease symptoms in corn tissue. One gram of ground inoculated ear tissue (see above) was placed into 100 mls of sterile water to suspend the particles. Ten-fold serial dilutions were produced and six mL of each serial dilution was placed in six mL of 0.8% agarose and poured over a cooled Potato Dextrose Agar (PDA) plate. Ampicillin and chloramphenicol were added to the agarose gel to prevent grow of bacteria during the experiment. The plates

were then incubated at 23°C with continuous light for approximately four days, and subcultures of the single colonies on the 1:100,000 were taken for verification.

Agrobacterium-mediated Transformation of *Stenocarpella maydis* and *Fusarium meridionale*

A long-term goal is to use fluorescent strains of fungi to visualize fungal infection patterns in intact inoculated or co-inoculated corn ear tissues. In this project I tested a convenient method for transforming fungi involving the bacterium *Agrobacterium tumefaciens* (Flowers *et al.* 2005). This method had not been used before for *S. maydis* or *F. meridionale*.

Agrobacterium containing the plasmids pJF1 (Figure 2) or pRAN (Figure 3) was streaked onto Luria-Bertani (LB) agar augmented with 50 µL/mL of Kanamycin. The plates were incubated at 28°C for 48 hours. *Stenocarpella maydis* strain DM6.001 and *F. meridionale* strain 004 were grown on OA or PDA media, respectively, and spores were collected after 3 weeks of growth under continuous light and 23°C and used to produce spore suspensions of [1 x 10^6]. The fungus and bacteria were then co-cultivated on solid induction media (Flowers et al., 2005) amended with acetosyringone and thiamine . After approximately one week, hygromycin-resistant colonies were transferred to PDA plates amended with hygromycin 50 µg/mL.

Verification of Fluorescence via Microscope

After approximately three weeks of growth, strains that grew on the PDA plates and also on the PDA plates amended with hygromycin were visualized under an epifluorescent microscope. Blue light (to visualize green fluorescence) and green light (to visualize red fluorescence) were both used. Green light was utilized to determine any auto-fluorescence from agar that may have been included in the sample.

Verification of Transformation via PCR

Mycelia from PDA plates was placed into approximately 8 mL of yeast extract peptone dextrose liquid medium (YEPD) and allowed to grow for six days. The mycelia were then harvested from each tube, flash frozen in liquid nitrogen, and lyophilized. DNA was extracted from the ground lyophilized mycelia by using the DNA extraction method described above.

Three different primer pairs were used to detect the gene encoding green fluorescent protein (GFP) and the Hygromycin Phosphotransferase gene (HYG) in the putative transformed strains (Table 2).

	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Primer Melting Temperature
GFP	CGA TAT CTA GAA TGG TGG CAA GGG CGA GGA	TGA CTT CTA GAT TAC TTG TAC AGC TCG TCC A	F – 64.1 R – 59.9
HYG 1	GCT GCG CCG ATG GTT TCT ACA	GCG CGT CTG CTG CTC CAT	F – 60.3 R – 61.5
HYG 2	GCA CAG GGT GTC ACG TTG CAA	GCG GCC ATT GTC CGT CAG GAC	F – 60.9 R – 63.3

Table 2. The three primer pairs used in the experiment. The forward and reverse sequences are shown, as well as the primer melting temperature (Tm).

For all reactions, PCR were conducted in a 25 - µL reaction volume, containing template DNA at 10 ng/µL, 2.5 mM each TakaRa deoxyribonucleotide triphosphate, TakaRa Extaq DNA polymerase at 5 U/µL, TakaRa 10 x PCR buffer, 10 µM each forward and reverse primer (Table 2), and 17.3 µL of Invitrogen nuclease-free sterile water. Fragments were amplified using the GFP and HYG 2 primers (Table 2) under the following program: an initial heating cycle of 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min; and a 7 min final extension at 72°C. Fragments were amplified using the HYG 1 primers (Table 2) under the following program: an initial heating cycle of 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min; and a 7 min final extension at 72°C.

The amplicons were separated on a 1% agarose gel at 90 V for 1 hour and 30 min. Each well had 7 µL of nuclease-free DNA water, 5 µL stop dye, and 3 µL amplicons/Invitrogen 1 Kb Plus DNA Ladder (Cat. No. 10787018/10787026). Each agarose gel was stained with 1% Ethidium Bromide in 1 x TAE for 20 minutes. Gels were then visualized under Trans UV light.

Characterization of Fluorescent strains of *Stenocarpella maydis* and *Fusarium meridionale*

Agrobacterium-transformed strains of *S. maydis* and *F. meridionale* were grown on PDA plates and PDA plates amended with Hygromycin. Once grown, spore suspensions of [1 x 10^4] were collected and spread onto 2% water agar plates. After the plates were incubated overnight at 23°C with continuous light, germinating spores were cut out of the agar and placed onto mung bean agar (MBA) plates (40 g mung beans and 20 g Agar in 1 L of water), PDA plates, and carrot agar plates (150 g carrot in 200 mL water). MBA was utilized to determine the number of conidia per mL from each strain. PDA plates were utilized to determine daily growth rate of each transformed strain. Carrot agar plates were utilized to describe mycelial growth and the production of perithecia.

RESULTS

Development of GER and DER on FFMM inoculated in the greenhouse

Corn ears collected from this experiment were shucked and photographed, as shown in Figure 4. There were some pollination issues, possibly due to environmental factors e.g. the high temperatures in the greenhouse, or recovery of insufficient pollen from the tassels. Ears that were poorly pollinated had very few kernels. The corn ears exhibited very few noticeable symptoms of GER, but DER was evident and severe in nearly all inoculated corn ears. Corn ears inoculated with both fungi only exhibited symptoms of DER. There were a few ears that were inoculated with *S. maydis* that displayed no symptoms ("escapes"), possibly due to inoculation failures.

The corn stalks were also qualitatively analyzed in this experiment (Figure 5) since disease was evident at the time of harvesting. All corn stalks were healthy except for plants inoculated with *S. maydis* on the bottom. Since both *F. graminearum* and *S. maydis* are known to cause stalk rot in field, it is unclear why only one treatment produced stalk rot in the FFMM. It is possible that *S. maydis* was more efficient at moving into the stalk via the ear shank when inoculated from the bottom, but more testing will be needed to establish that.

Disease severity was evaluated on all corn ears that had sufficient kernels to be measured. For quantitative analysis, only corn ears that could be flattened via the rotating apparatus were utilized. Examples of the flattened photos used are shown in Figure 6. The percentage of disease severity was higher on ears inoculated with *S. maydis* (Figure 7). Corn ears inoculated with *F. graminearum* exhibited much smaller percentages of disease. Qualitative analysis was performed on corn ears that didn't display obvious pollination issues. As shown by Figure 8, the disease severity ratings were consistently higher on corn ears inoculated with *S. maydis*. *F. graminearum* produced consistently low disease ratings on inoculated corn ears.

Verification and Quantification of *S. maydis* and *F. graminearum* in inoculated ears

PCR performed on the ground tissue produced amplicons of the expected size for actin, Tri5, and *S.maydis* ITS. Co-inoculations exhibited bands for both *F. graminearum* and *S. maydis*. The method of serial dilutions produced single fungal colonies on the plates with a dilution of 1:100,000, as shown by Figure 9. However, the single colonies did not appear to be either *S. maydis* or *F. graminearum* (which would be expected to express GFP). The water control also displayed a large number of colonies, which provides evidence that the colonies are some sort of contaminant or endophyte from the ears themselves. Further testing is needed to understand why the inoculated fungi are not recovered from the ears. It is possible that the drying and/or grinding process has resulted in their destruction.

Development of Fluorescent Strains of *S. maydis* and *F. meridionale*

The growth of pRAN versus pJF1 colonies is shown in Figures 10 and 11. Since pRAN-transformed fungi grew faster than pJF1-transformed fungi, pRAN strains were utilized for the subsequent experiments. Approximately 20 subcultures of both the *S. maydis* and *F. meridionale* strains were taken and grown on secondary plates of PDA and PDA augmented with Hygromycin. Only four *S. maydis* strains grew on the Hygromycin-augmented secondary PDA plates, and only six *F. meridionale* strains. Those sub-cultures were the likely transformant

strains, and other subcultures were probably false positives. To further confirm fluorescence in the likely transformants, mycelia and spores were examined under a fluorescent microscope. As shown in Figures 12 and 13, green fluorescence was observed in the transformed strains of each fungi.

Characterization of the transformed strains of *Stenocarpella maydis* and *Fusarium meridionale* is ongoing, but Figure 14 shows the daily growth rate of each transformed strain over a period of seven days. Only *F. meridionale* strains were utilized for the characterization because *S. maydis* strains had not produced sufficient spores for experimentation at that time. There were no major differences between the growth rate of each transformed strain as shown in the graph.

Future Work

The long-term goals of the project are to use the FFMM system to study the process of ear colonization by ear rot fungi, and the process of ear rot disease development. Protocols for inoculation and quantification of disease and pathogen colonization are necessary to achieve this goal, and this project established the viability of some of these methods. There is interest in evaluating co-inoculations of different ear rot fungi including *S. maydis*, *F. graminearum* and *F. meridionale* to better understand the competitive interactions between these fungi in corn ears. For this work, strains expressing different fluorescent proteins will be extremely useful since they can be directly visualized and identified among strains recovered from ears. Further analysis into the stalk rot caused by *S. maydis* is ongoing. Optimization of quantitative and qualitative methods for measuring disease severity is in progress. Further characterization of transformed strains of *S. maydis* and *F. meridionale* is underway. Additional verifications, transformations, and DNA and mycotoxin extractions are also in progress.

Acknowledgements

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This work would not be possible without the assistance of Gabdiel Yulfo-Soto, Renata Belisario, Caleb Mathias, Etta Nuckles, Kiersten Wise, and Lisa Vaillancourt.

Figures

Figure 1. (a) Examples of qualitative scale for disease severity of *Fusarium graminearum* taken for corn ears with sufficient kernels. Scale only goes from one to three as disease severity never classified higher. (b) Examples of qualitative scale for disease severity of *Stenocarpella maydis* taken for corn ears with sufficient kernels. Scale was determined based on mycelial growth, kernel viability, and overall ear visual condition.

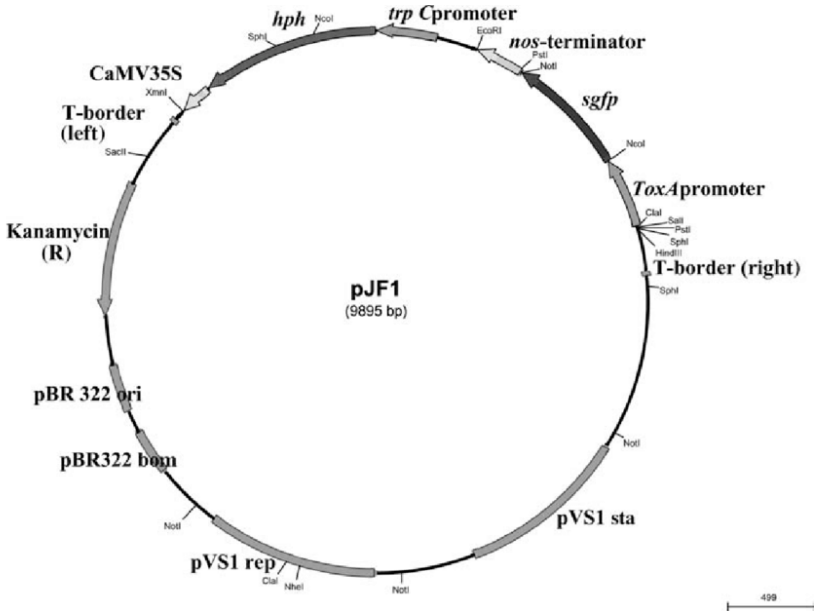

Figure 2. pJF1 vector construct[5].

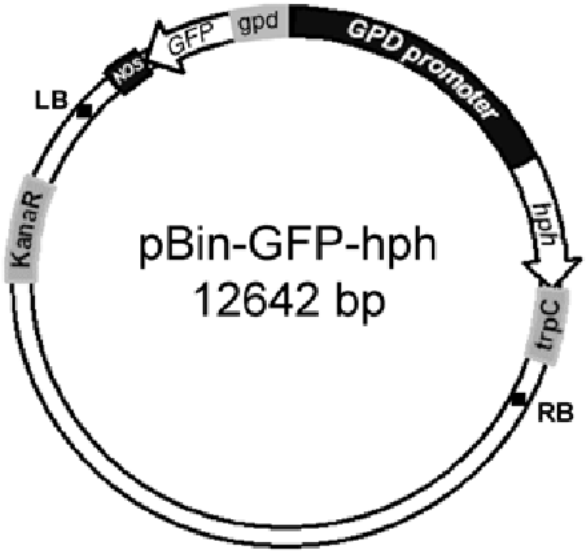

Figure 3. pRAN vector construct[6].

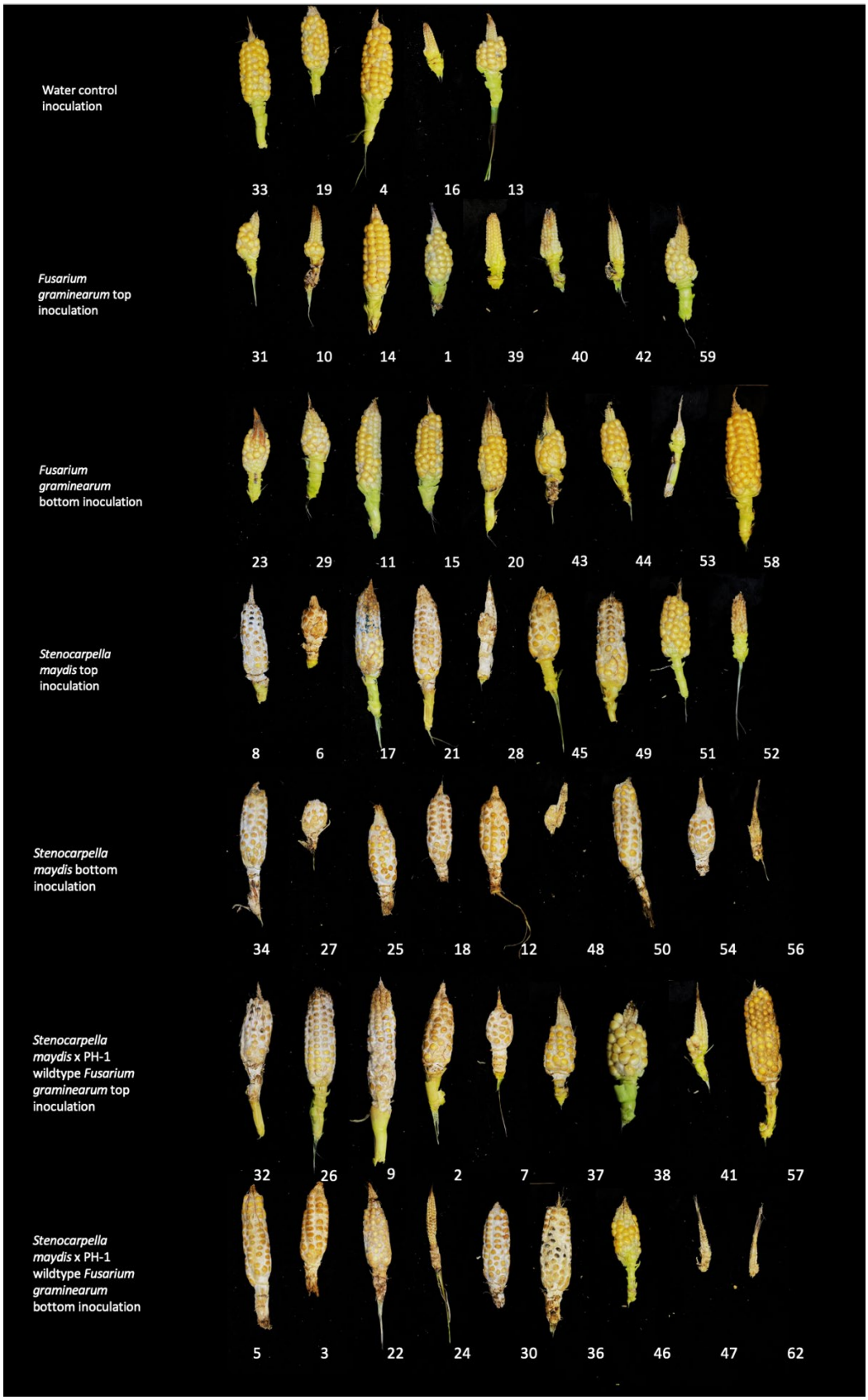

Figure 4. Pictures of shucked corn ears from experiment.

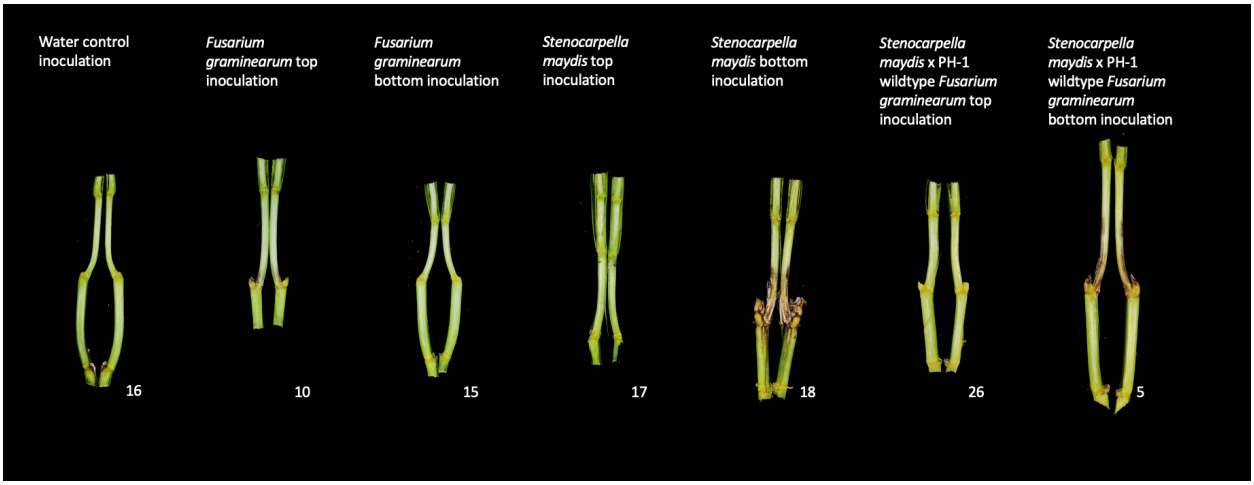

Figure 5. Example pictures of corn stalks from experiment. Stalk rot was only found on corn stalks inoculated with a bottom inoculation of *Stenocarpella maydis*, as shown by stalks 18 and 5.

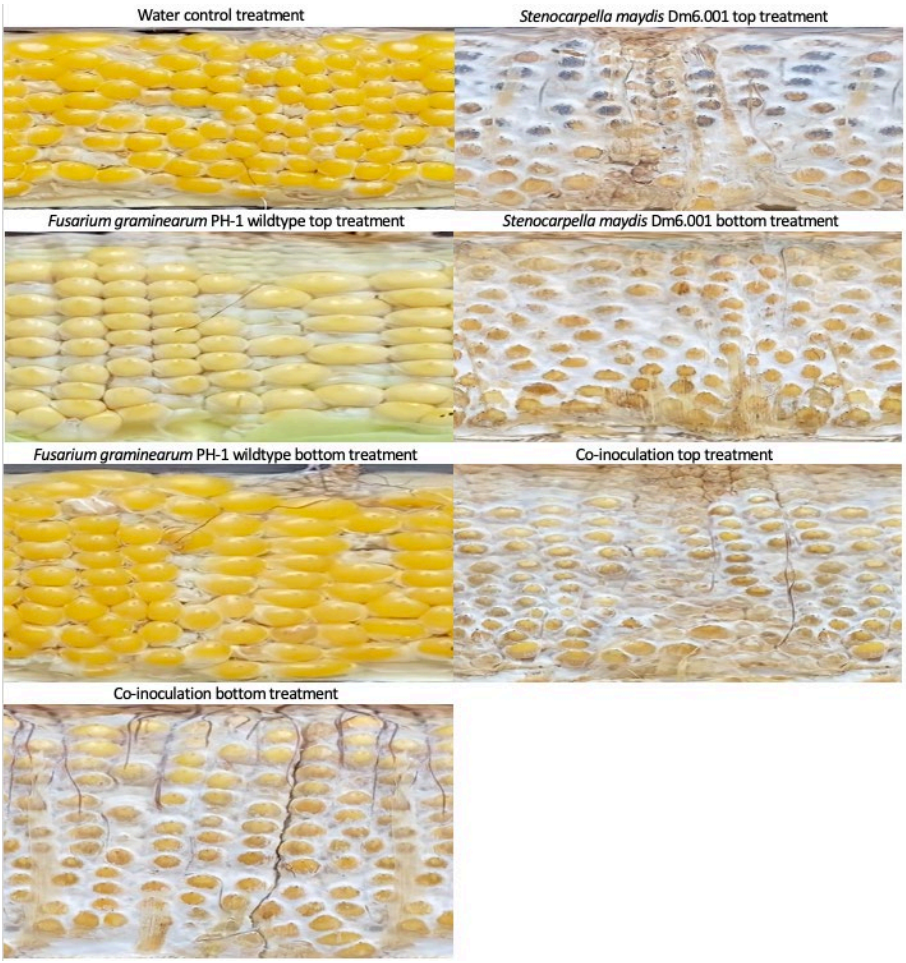

Figure 6. Example pictures of flattened corn ear photographs. Only ears with a sufficient number of kernels were videoed on the 360^{o} rotating apparatus for flattened photographs.

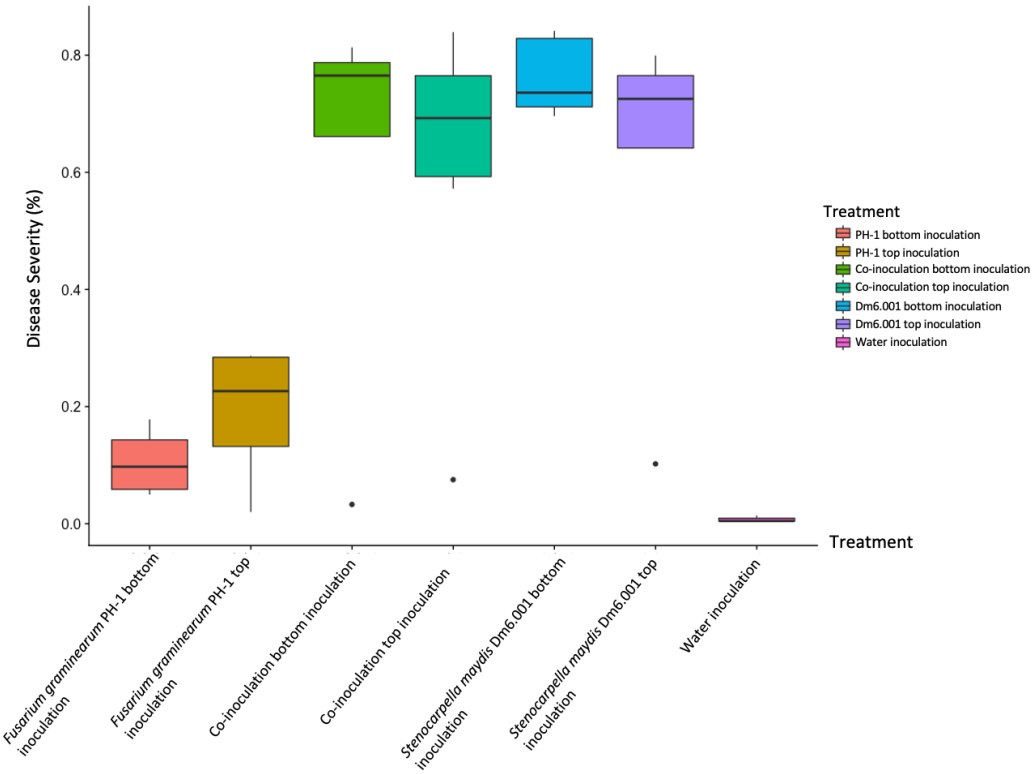

Figure 7. Graph of quantitative data taken from experiment. Flattened photographs (examples in Figure 3) were thresholded on ImageJ and a percentage of ear area was taken. Corn ears inoculated with *Stenocarpella maydis* all exhibited a larger percentage of disease severity than corn ears only inoculated with *Fusarium graminearum*.

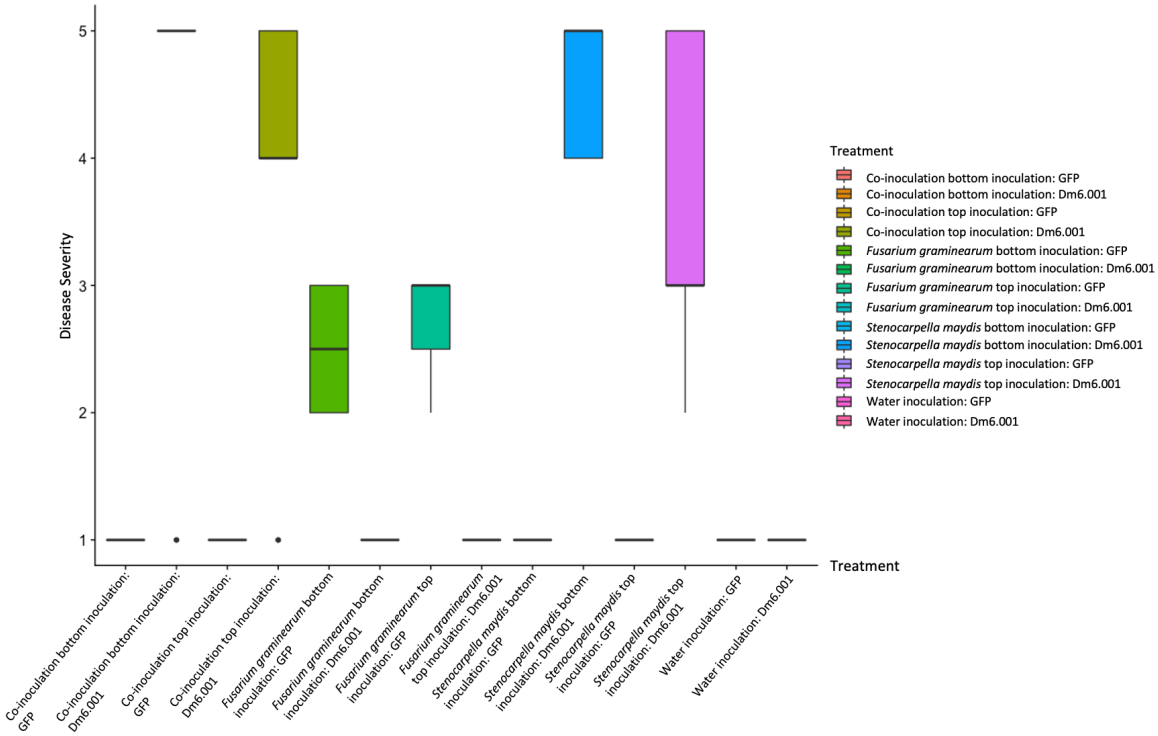

Figure 8. Graph of qualitative data taken from experiment. Each treatment was evaluated for both disease severity of *Stenocarpella maydis* and *Fusarium graminearum*.

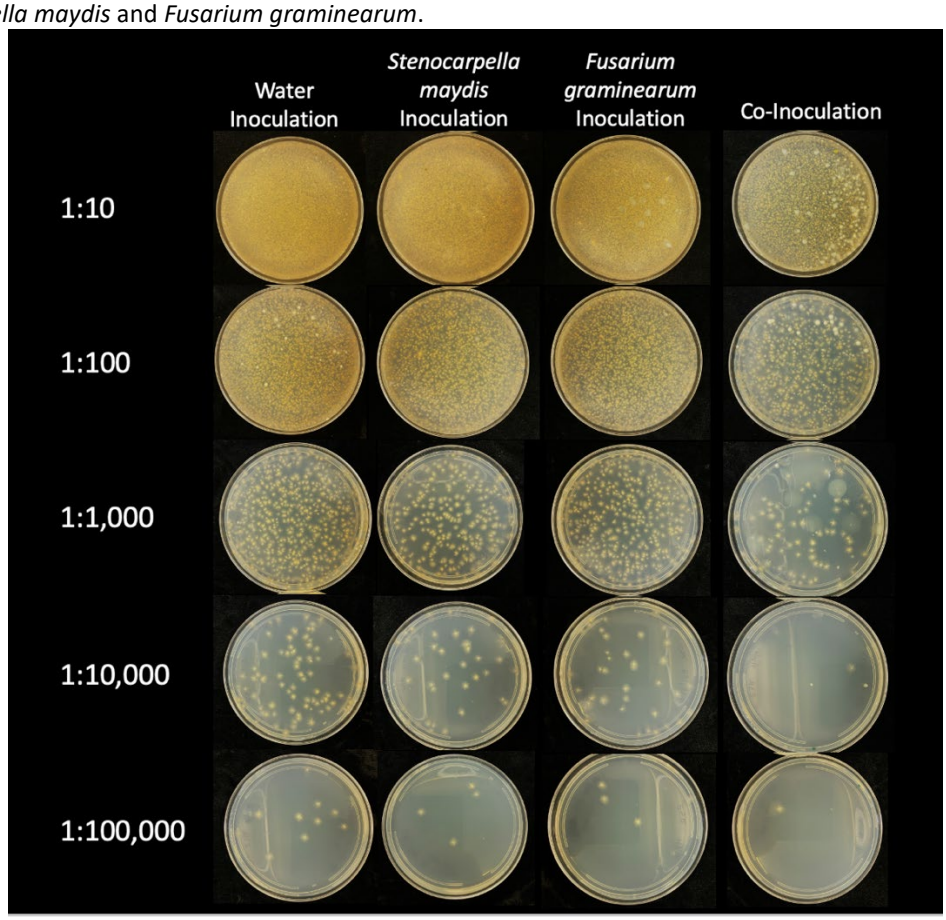

Figure 9. Colony dilution plates from examples of each treatment in experiment. For the water inoculation there were 900,000 colonies/mL of sample. For the *Stenocarpella maydis* inoculation there were 500,000 colonies/mL of sample. For the *Fusarium graminearum* inoculation there were 300,000 colonies/mL of sample. For the co-inoculation there were 100,000 colonies/mL of sample. Sub-cultures of select colonies on 1:100,000 plates were taken, and further analysis will be conducted.

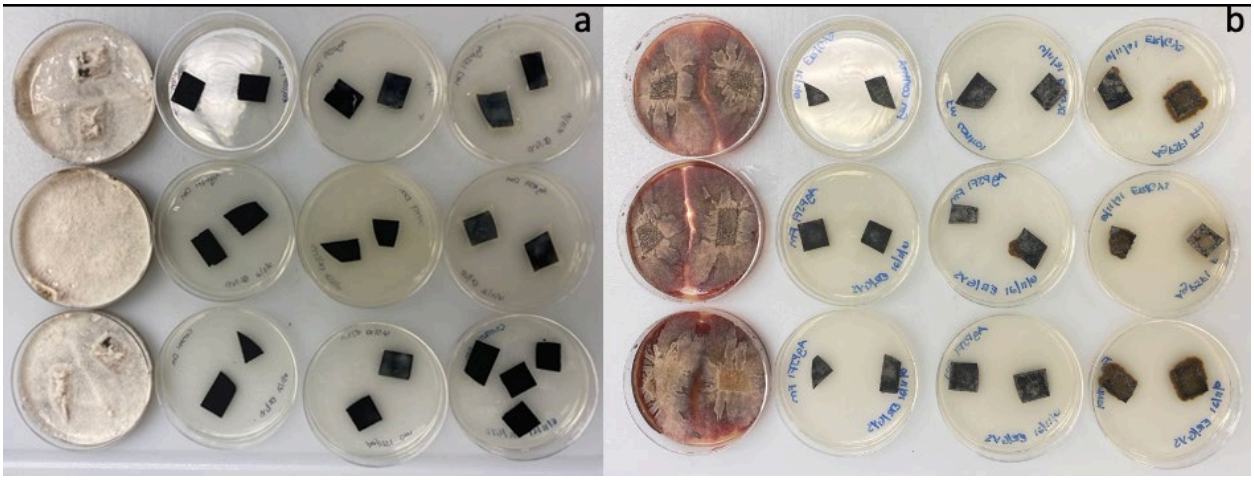

Figure 10. (a) pJF1 Agrobacterium-transformed strains of *Stenocarpella maydis* were plated on PDA plates mediated with 0, 50, 100, and 250 µg/mL Hygromycin (from left to right). Photographs were taken 17 days after cultures were plated. (b) pJF1 Agrobacterium-transformed strains of *Fusarium meridionale* were plated exactly like (a) from left to right. Photographs were taken 17 days after cultures were plated.

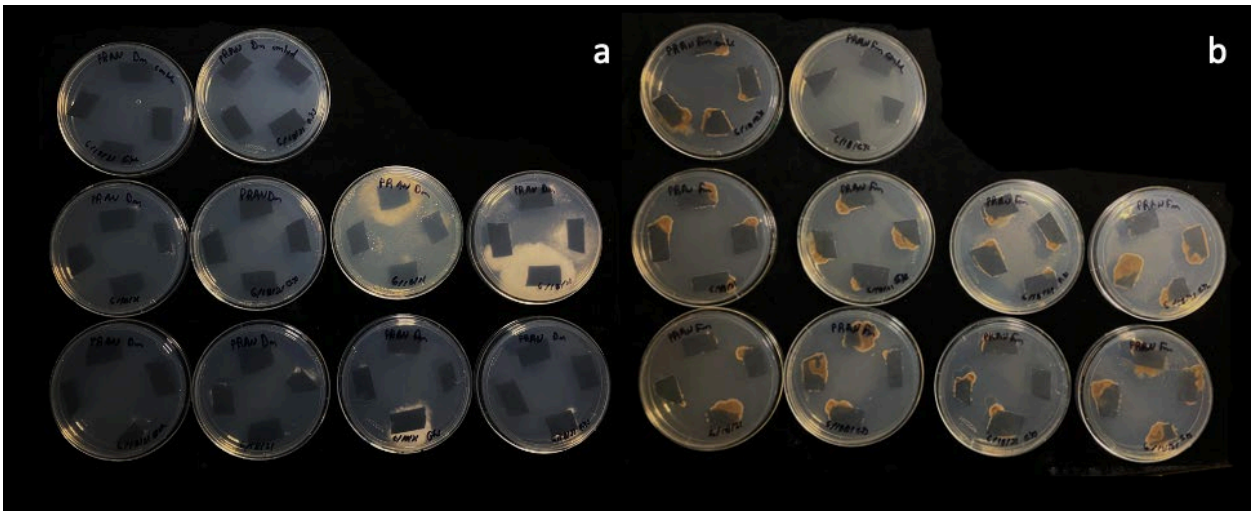

Figure 11. pRAN Agrobacterium-transformed strains of both *Stenocarpella maydis* and *Fusarium meridionale* were grown on PDA plates mediated with 50 µg/mL of Hygromycin. Photographs of growth were taken after 11 days.

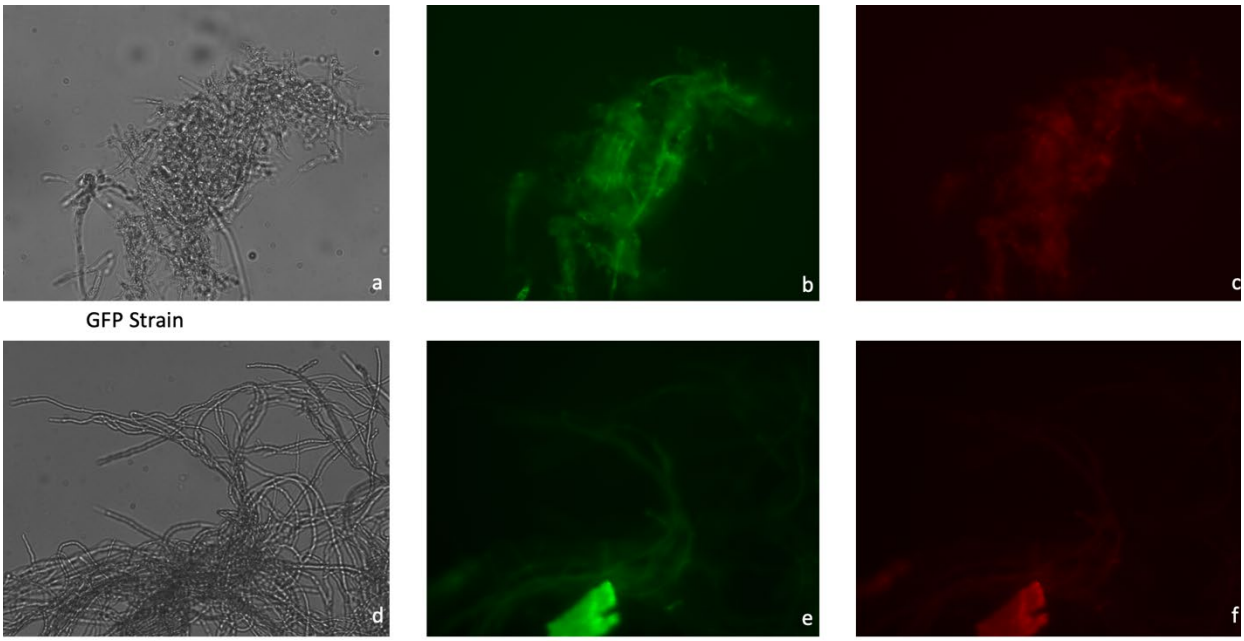

Figure 12. Sub-cultured strains of pRAN Agrobacterium-transformed *Stenocarpella maydis* strains were viewed under a fluorescent microscope along with untransformed strains of *Stenocarpella maydis* for verification of fluorescence. (a) Transformed strain under regular light on microscope. Exhibits expected placement for mycelial fluorescence. (b) Transformed strain under blue light to show green fluorescence. Exhibits fluorescence of mycelia. (c) Transformed strain under green light to show red fluorescence. Exhibits auto-fluorescence that could be from agar. (d) Control strain under regular light on microscope. Exhibits placement of mycelia. (e) Control strain under blue light to show green fluorescence. Exhibits auto-fluorescence of agar under light. (f) Control strain under green light to show red fluorescence. Exhibits auto-fluorescence of agar under light.

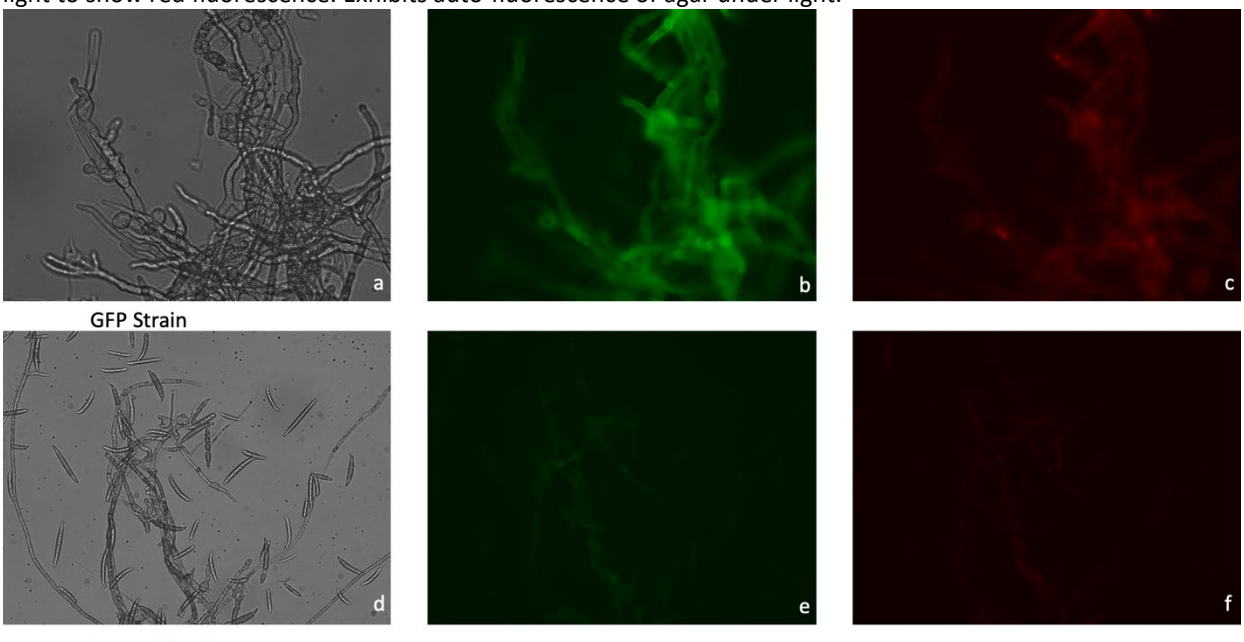

Figure 13. Sub-cultured strains of pRAN Agrobacterium-transformed *Fusarium meridionale* strains were viewed under a fluorescent microscope along with untransformed strains of *Fusarium meridionale* for verification of

fluorescence. (a) Transformed strain under regular light on microscope. Exhibits expected placement for mycelial fluorescence. (b) Transformed strain under blue light to show green fluorescence. Exhibits fluorescence of mycelia. (c) Transformed strain under green light to show red fluorescence. Exhibits auto-fluorescence that could be from agar. (d) Control strain under regular light on microscope. Exhibits placement of mycelia. (e) Control strain under blue light to show green fluorescence. Exhibits auto-fluorescence of agar under light. (f) Control strain under green light to show red fluorescence. Exhibits auto-fluorescence of agar under light.

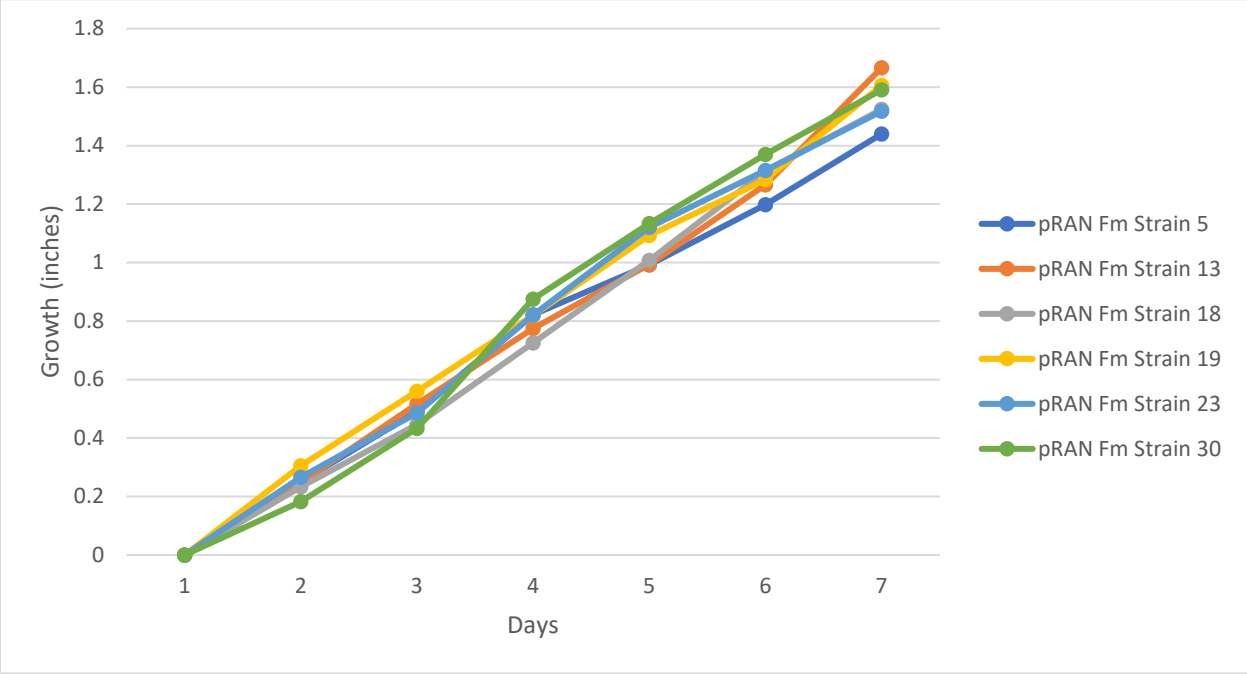

Figure 14. Daily growth of *Fusarium meridionale* transformed strains after single sporing.

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