Investigating mechanisms of root infection and sudden death syndrome development in *Glycine max* caused by *Fusarium virguliforme*

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Abstract

One of the top emerging threats to soybean production in the U.S. is sudden death syndrome (SDS) caused by the fungal pathogen, *Fusarium virguliforme*. The soil-borne pathogen infects roots causing initial root rot symptoms, later followed by foliar symptoms marked by interveinal chlorosis and necrosis. These foliar symptoms are thought to be caused by host-selective toxins and effector proteins that enter xylem during the root infection, since *F. virguliforme* remains in the roots throughout infection. These foliar symptoms can cause premature defoliation, pod drop, and up to 100% yield loss. Two proteins, FvTox1 and FvNIS1, have been identified as important factors in foliar SDS development, but their modes of action remain unknown. To date, genetic resistance to SDS is quantitative and controlled by many quantitative trait loci. Initially, *F. virguliforme* was thought to have a narrow host range, but it has recently shown the ability to colonize other common field crops and weeds, though development of classic SDS symptoms are inconsistent. This proposal aims to elucidate the mechanisms by which *F. virguliforme* enters soybean roots and identify the targets of FvTox1 and FvNIS1. In addition, I will investigate alternate plant hosts for potential resistance to root infection and to foliar symptom development caused by these proteins.

Primary Objective: Characterize how *F. virguliforme* enters soybean roots, causes effector-induced foliar symptoms, and identify mechanisms of resistance to SDS in other crop hosts.

Central Hypotheses: *Fusarium virguliforme* infects soybean roots independently and via direct interactions with the soybean cyst nematode. Non-soybean hosts employ unique resistance mechanisms that prevent root rot and foliar symptom development caused by *F. virguliforme*.

Specific Aims

Aim 1 – Identify modes of *F. virguliforme* entry into soybean root tissues

- Generate a fluorescent strain of *F. virguliforme*
- Search for appressorium development on soybean roots using fluorescent microscopy
- Determine if *F. virguliforme* interacts with and colonizes nematodes using fluorescent microscopy

Aim 2 – Identify FvTox1 and FvNIS1-interacting proteins that induce foliar SDS symptoms

- Confirm less virulent phenotypes of *fvtox1* and *fvnis1* mutants
- Tag FvTox1 and FvNIS1 with a fluorescent marker
- Vacuum infiltrate fluorescently tagged FvTox1 and FvNIS1 into soybean leaf disks to identify subcellular localization using fluorescent microscopy
- Identify potential interacting proteins using co-immunoprecipitation and liquid chromatography – mass spectrometry (LC-MS)
- Confirm potential interacting proteins with yeast two-hybrid experiments

Aim 3 – Investigate alternate-host resistance mechanisms to SDS

- Identify toxin-interacting homologs in other crops through gene homology studies
- Vacuum infiltrate fluorescently tagged FvTox1 and FvNIS1 into alternate host leaf disks to identify subcellular localization using fluorescent microscopy
- Identify potential interacting proteins using co-immunoprecipitation and LC-MS
- Confirm potential interacting proteins with yeast two-hybrid experiments

Background and Significance

History and current state of soybean SDS in the U.S.

The causal agent of soybean sudden death syndrome (SDS) in the U.S. was identified in 1971 in Arkansas as *Fusarium solani* f. sp. *glycines*[1–3]. Since then, genetic and morphological studies have shown that *Fusarium solani* f. sp. *glycines* consists of multiple distinct species, with *Fusarium virguliforme* (Fv) being the only SDS-causing species present in the U.S.[4–8] However, recent surveys have identified other SDS-causing *Fusarium* species in Michigan, although Fv remains the most prominent species (M. Chilvers and J. Jacobs, unpublished). Initial symptoms of SDS appear in the roots as discoloration and rotting, with foliar symptoms typically developing much later. Severe foliar symptoms include interveinal chlorosis and necrosis that can lead to premature leaf drop and pod abortion, causing a significant decrease in yield. These above-ground symptoms occur while Fv remains in the roots secreting proteins and other compounds that induce the foliar symptoms[9] (Fig. 1). Soybean SDS is now present in nearly all soybean producing states in the U.S., having caused an average 27.7 million bushel yield loss between 2004 and 2009[10,11].

Soybean SDS is a persistent, recurring, and spreading problem. Fv is persistent in fields because effective management strategies are not consistently effective across regions and Fv has been shown to colonize many common crop and weed species, even though classical SDS symptoms do not typically develop[12]. Management strategies such as crop rotation, soil tillage practices, and seed treatments with fungicides have all shown mixed results, making it difficult to give appropriate recommendations to growers about how to manage SDS[9]. Genetic resistance to SDS would be an effective management strategy, but studies in this area have determined that resistance is complex and controlled by quantitative trait loci (QTLs), which can contribute to root rot resistance or foliar resistance separately[13–16]. Some of the QTLs associated with SDS resistance overlap with known QTLs for other pests, making it difficult to decipher the role and impact of each resistance QTL[17].

Fusarium virguliforme entry into root tissues

Fusarium virguliforme is a soil-borne pathogen capable of infecting soybean roots and can only be re-isolated from roots or lower stems[1,2]. To enter plant roots, many fungal pathogens generate specialized hyphae called an appressorium to build up turgor pressure, forcing a hyphal penetration peg between the cells of a plant host[18,19]. Once inside the host, fungal pathogens typically do one of three things; develop hyphae that remain in the extracellular apoplast, develop intracellular invasive hyphae, or develop specialized feeding structures called haustoria that also invade plant cells[20]. Scanning electron microscopy of Fv appears to show appressoria formation on soybean root surfaces and root cross sections show evidence that Fv colonizes the vascular tissues[21]. Appressorium development by Fv is surprising, since better-characterized *Fusarium* relatives (*F. oxysporum*, *F. graminearum*, and *F. verticillioides*) either develop intermittent appressorium-like structures[22] or no appressorium at all[23,24]. Instead, *Fusarium* species typically rely on cell wall degrading enzymes[25–27], and the Fv genome contains many genes for plant cell wall degrading enzymes[28]. It is possible that Fv may use both appressorium development and cell wall degrading enzymes to enter soybean roots. In this study, a fluorescent strain of Fv will be used to examine fungal development on soybean root tissues and search for additional evidence of appressorium development.

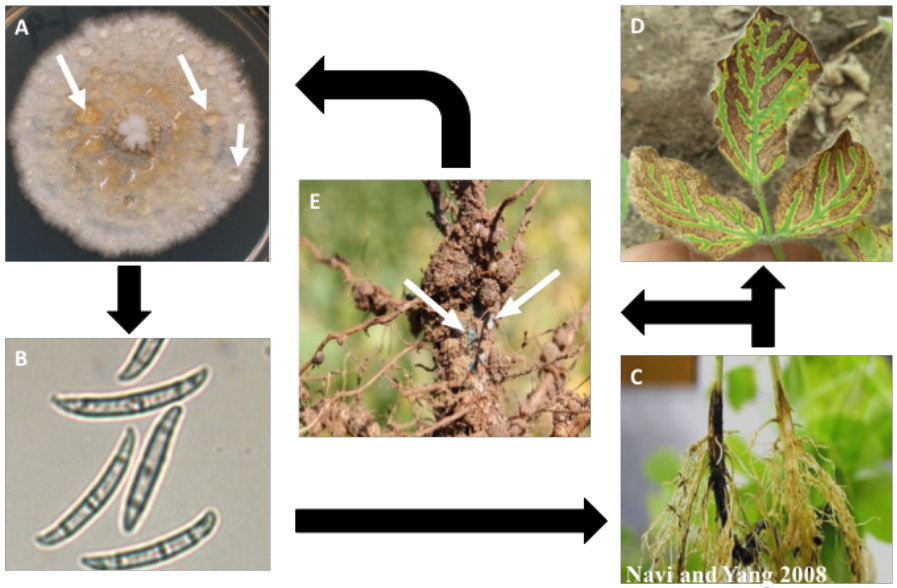


Figure 1. Infection cycle of *Fusarium virguliforme* in soybean. (A) Sporodochia develop (white arrows), (B) containing many 3-5 septate macroconidia. (C) Conidia germinate and infect soybean roots, causing root rot symptoms. (D) Toxin production, secretion, and translocation causes foliar SDS symptoms. (E) *F. virguliforme* remains in roots, developing pigmented (sometimes blue) masses of chlamydospores for overwintering (white arrows).

A more efficient mode of entry into soybean roots may be provided by the soybean cyst nematode (SCN). Independently, SCN is a major threat to soybean production throughout the U.S. However, Fv and SCN are commonly found in the same fields, leading some to believe that they may be able to directly interact with one another. Currently, there is conflicting evidence about the impact of SCN on SDS. A synergistic relationship between SCN and Fv could make soybean SDS symptoms worse, and an antagonistic interaction could result in a competition for nutrients, thus allowing only one pathogen to become a prominent problem. Initially, the two pathogens were thought to compete with one another because evidence suggested that Fv could infect and parasitize SCN cysts and juveniles[29,30]. This idea has been somewhat dismissed because the studies used *F. solani* f. sp. *glycines* isolates, which may not have specifically been Fv. Subsequent field studies have measured SDS severity in the presence of both pathogens and found that foliar SDS symptoms occur sooner and are more severe when both pathogens are present, compared to single inoculations[31–35]. In contrast, other greenhouse[36] and field studies[37–39] have shown little to no correlations between SDS severity and SCN quantity, with Fv being the only significant factor contributing to SDS. Interestingly, one greenhouse study occasionally found mild foliar SDS development in the presence of SCN and the absence of Fv inoculation[36]. This curious observation might be explained by a phosphate deficiency, which can be mistaken as SDS. Alternatively, the researchers may have unknowingly vectored Fv with SCN, since SCN is an obligate biotroph and can only be reared on soybeans. To date, all studies examining SDS in the presence of SCN have had very similar experimental designs, yet they have yielded inconsistent results and provided little advancement in our knowledge to the relationship between Fv, SCN, and SDS. These discrepancies could be due to the different Fv isolates, SCN races, and soybean cultivars used in each study. A new approach is needed to significantly

advance knowledge about these biological relationships. I propose to use a fluorescent strain of Fv in co-culture experiments with SCN to examine their relationship using fluorescent microscopy. Some *Fusarium* species have been shown to colonize *Caenorhabditis elegans*[40], hence Fv may be able to colonize SCN. This type of interaction could allow SCN to transmit Fv into soybean roots through mechanical wounds.

SDS symptom development

The mechanisms employed by Fv to cause root rot symptoms are unknown and have not been studied at a molecular level. However, many studies have investigated the cause of the striking foliar SDS symptoms (Fig. 1D). The development of foliar symptoms while Fv remains in the roots triggered the hypothesis that Fv can secrete host selective toxins (HSTs), phytotoxins, and effector proteins into the soybean vascular tissue that translocate to the foliar tissues[41–43]. Subsequent studies identified that cell free culture filtrates alone are capable of inducing foliar SDS symptoms, supporting this hypothesis[42–44]. Major evidence that fungal pathogens can secrete proteins inside plant xylem came with the identification of secreted in xylem (SIX) proteins in tomatoes infected by *Fusarium oxysporum* f. sp. *lycopersici*[45,46]. It was later shown that Fv could secrete proteins into soybean xylem[47], although the study did not identify the three well-characterized phytotoxic proteins produced by Fv. Interestingly, Ji et al. showed that light is required for cell-free culture filtrates to induce foliar SDS and that these symptoms are associated with the degradation of the large subunit of Rubisco[48]. The ToxA protein produced by *Pyrenophora tritici-repentis* also requires light to cause disease symptoms in wheat and functions by direct interaction with a chloroplast protein, which disrupts chloroplast function[49–51]. Some have speculated that FvTox1 also localizes to chloroplasts[52,53], though subcellular localization of FvTox1 in soybean leaves needs to be confirmed.

Fungal proteins that induce plant disease symptoms are often called host selective toxins (HSTs) or effectors in the literature. To date, no studies have investigated whether specific Fv proteins can cause disease symptoms in other plants. Therefore, I will refer to all Fv proteins involved in symptom development as effectors instead of HSTs. Fv contains a single gene that encodes a 13.5kDa protein named Toxin1 (FvTox1)[54]. This gene has been well characterized and is known to play a role in foliar chlorosis in soybeans, as *fvtox1* mutants cause a 2-fold lower severity of foliar SDS[55]. Like many small, secreted proteins, FvTox1 is processed from a pro-peptide for secretion and is cysteine rich with 5% cysteine residues[54]. Another effector produced by Fv was originally identified in 1996 as a 17 kDa protein and was re-discovered in 2016 and named FvTox2, but still has not been well characterized[43,56]. The most recent protein was identified as FvNIS1 and is plays a role in foliar necrosis development of soybeans[56]. FvNIS1 is an ortholog to the Necrosis Inducing Secreted peptide 1 (NIS1) from *Colletotrichum orbiculare*, which is a small secreted protein but only contains one cysteine residue[57]. In addition to these proteins, Fv is also capable of producing toxic secondary metabolites like radicicol (monorden), fusaric acid, and citrinin that can accumulate and cause cell death in soybean leaves[41,56].

Identifying new phytotoxic compounds and effector proteins is an important step towards understanding the complexity of the interactions between any pathogen and host. However, previous studies have shown significant roles for both FvTox1 and FvNIS1 in foliar symptom development[55,56]. This warrants further investigation into their modes of action, which is the central focus of this proposal. Identifying targets of FvTox1 and FvNIS1 which induce foliar symptoms may allow new targets for improving soybean resistance to SDS (Fig 2). Instead of a

targeted approach, recent studies have focused on developing synthetic peptides or mammalian-derived antibodies that generally bind to FvTox1[52,58]. However, binding FvTox1 does not necessarily inhibit its function and synthetic constructs need to be validated *in planta* before they can be deemed safe and effective. In addition, mammalian-derived transgenes in plant tissues make them unfit for human consumption under the current USDA regulations. Instead, I will identify the host proteins that FvTox1 and FvNIS1 target to cause foliar SDS symptoms, revealing specific points of intervention that could enhance SDS resistance. These mechanisms are important because they may function in very different ways. For instance, *Pyrenophora tritici-repentis*, which causes tan leaf spot of wheat, produces two major effectors that can have two different modes of action to cause disease symptoms[51]. As mentioned, ToxA functions inside plant cells inducing rapid cell necrosis after localizing to the chloroplast[49–51]. In contrast, ToxB functions outside plant cells in the apoplast inducing gradual chlorosis[51,59]. However, both contain a single host susceptibility gene in wheat[49], indicating the intricate nature of plant-pathogen interactions. By investigating the modes of action of individual effectors produced by Fv, in soybean and in alternate hosts, unique modes of resistance for FvTox1 and FvNIS1-induced symptoms may be identified (Fig. 2).

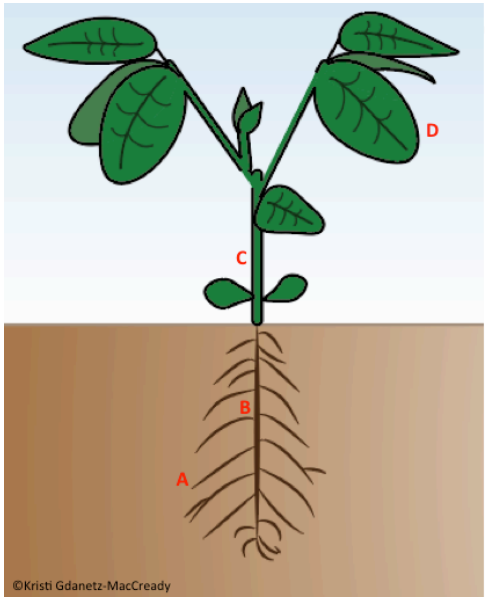


Figure 2. Points of interaction for *Fusarium virguliforme* toxins and plant hosts. (A) *F. virguliforme* infects soybean root tissues and begins expressing toxins. (B) Toxins enter xylem tissues and begin translocating to the foliar tissues. (C) Toxins travel through xylem into foliar tissues. (D) Toxins reach leaf tissues and interact with their target, inducing cell death. In alternate hosts, a lack of symptom development may result from (A) no expression of toxins, (B) the inability for toxins to enter xylem, (C) degradation of toxins within the xylem, or (D) a lack of an appropriate binding partner to induce symptom development.

Significance

Soybeans are one of the top grossing crops in the United States each year, netting an average production value of $39.9 billion over the past five years. Its oils are valuable in many industries including cooking oil production and biofuels. Its protein content also makes valuable feed supplements for livestock. However, Fv continues to spread world-wide, now found in Malaysia and South Africa[60,61] threatening global agriculture. Identifying a role for SCN in Fv infection in this study may provide more useful management advice for growers to prevent SDS. Specific soybean proteins that interact with Fv effectors can become targets for novel genome editing tools to edit genes to specifically inhibit the action of FvTox1 and/or FvNIS1.

Preliminary Studies

Assessment of Risk Factors for Predicting SDS

To date, management strategies for reducing SDS have yielded inconsistent results. The best management strategy is growing soybean cultivars with enhanced genetic resistance, but no cultivar has shown 100% resistance. Therefore, we sought to determine our ability to predict the risk of a field developing SDS by collecting samples before and throughout the growing season in a field naturally infested with *F. virguliforme*. Accurately predicting SDS development may provide soybean growers with useful information for making better management decisions, like using seed with higher resistance, using a seed treatment, or planting a different crop altogether.

This two-year field study took place in Decatur, MI during 2014 and 2015. This field site was found to be naturally infested with Fv in 2009, and SDS continues to be a problem. Often during SDS outbreaks, foliar symptoms develop in "patches" within a field, but it is unclear if this is due to a heterogeneous distribution of Fv or other environmental factors. Soil samples were collected prior to planting in a grid sampling design to prevent biased sampling, and consisted of 96 samples over a 446m^2 area. These samples were processed for DNA extraction, and the DNA was used for quantification of Fv using a qPCR assay shown to have high sensitivity and specificity across many labs[62,63]. The abundance of nematodes, including soybean cyst nematodes (SCN), were also determined from these soil samples by MSU Diagnostic Services. The same cultivar was planted throughout the plot to control for cultivar effects on SDS development (Asgrow AG2534 in 2014, Asgrow AG2535 in 2015, Monsanto Company, St. Louis, MO, USA). At young vegetative (V3) and mature reproductive (R5) soybean growth stages[64], three plants were collected from each of the 96 sample points to assess SDS severity. Roots of the three individual plants were visually rated as a percentage of rotten root tissue, and leaves of the three individual plants were visually rated for foliar SDS on a scale from 0-9 in 0.5 increments (Jason Bond, Southern Illinois University, unpublished). Lastly, in-field SDS incidence (percentage of plants expressing any SDS symptoms, from 0-100%) and severity (average severity of plants expressing SDS symptoms, from 0-9) ratings were taken at soybean reproductive stages R4, R5, and R6 and converted into a disease index score by taking $\frac{\text{Incidence} * \text{Severity}}{9}$. This provided disease index values on a continuous scale from 0-100, instead of a discrete 0-9 scale[65]. In 2014, disease index ratings were obtained on a plot-wide basis, providing 24 data points. In 2015, disease index ratings were obtained on a three-foot swath of plants at each sampling location, providing 96 data points.

Another advantage to the grid sampling design is that each sample point has unique coordinates. Therefore, we plotted each risk factor with coordinates and interpolated values to fill in non-sampled areas, creating contour plots that shows the distribution of each risk factor throughout the field using the 'fields' package in R version 3.2.0 (Fig. 3). The data were interpolated through simple kriging using a stationary covariate model. The quantities of Fv and SCN in the soil before planting were found to be heterogeneous in both 2014 (Fig. 3A) and 2015 (Fig. 3B). In 2014, the abundance of Fv in the soil prior to planting had a significant positive correlation to the SDS disease index at growth stage R5 ($R = 0.41$, $p = 0.047$), while SCN in the soil prior to planting had no significant correlation to SDS disease index at growth stage R5 ($R = 0.22$, $p = 0.296$). In 2015, the abundance of Fv in the soil prior to planting had a significant positive correlation with SDS disease index scores at growth stage R5 ($R = 0.51$, $p = 1.13 \times 10^{-7}$), while SCN also had a significant positive correlation to SDS disease index at growth stage R5 (R

= 0.53, p = 2.99x10^{-8}). In addition, 2014 showed no significant correlation between Fv and SCN quantities in the soil prior to planting (R= 0.3, p = 0.155), but 2015 did (R = 0.40, p = 5.98x10^{-5}). Together, these results indicate that the distribution of Fv in the soil can help aid in the prediction of SDS development even with samples taken before planting. In addition, the distribution of SCN may also help in SDS prediction, yet the relationships between Fv and SCN, and between SCN and SDS remains unclear and will be investigated in this proposal. This risk assessment project has concluded and these findings are in preparation for publication (Roth et al, *in prep*).

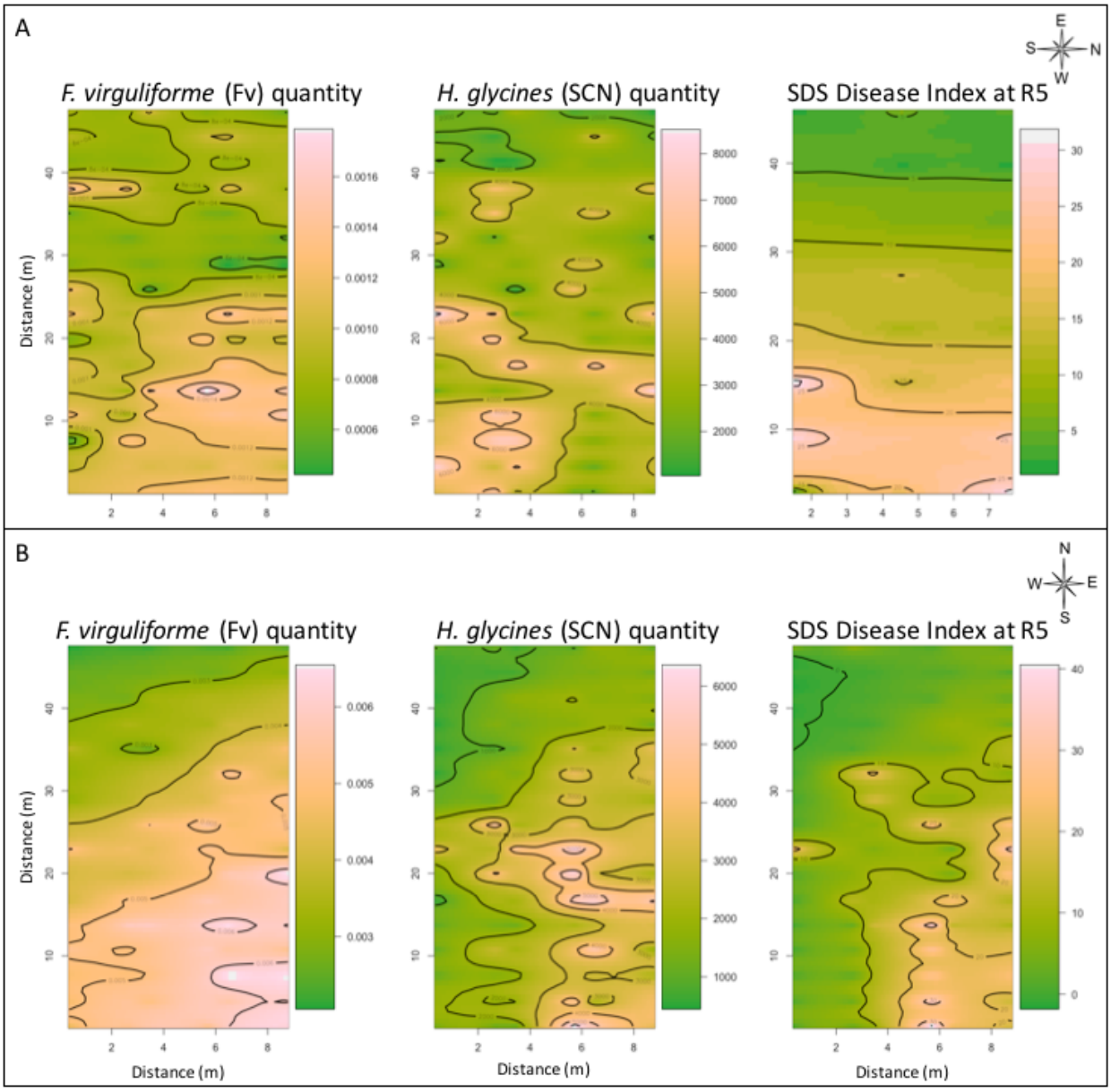


Figure 3. Contour plots of *Fusarium virguliforme*, soybean cyst nematode, and SDS distributions in a field plot during (A) 2014 and (B) 2015. Right, quantity of *F. virguliforme* DNA in the soil as a ratio of total DNA extracted. Middle, counts of SCN present in the soil. Right, SDS disease index ratings at soybean growth stage R5. Tan areas represent high quantities and green areas represent low quantities.

Development of transgenic *Fusarium virguliforme* strains

Previous studies have developed successful transformation methods for knocking out genes or inserting GFP in Fv[55,56,66]. Unfortunately, no other studies have been published since the development of the GFP strain. Fluorescent proteins are commonly used in fungi, and many fluorophores have been optimized to perform better than GFP[67–69]. We have developed an optimized protocol for protoplast generation and genetic transformation of Fv using existing protocols for Fv and for the close relative *F. graminearum*[66,70], and intend to develop a GFP strain for studying the infection process of soybean using fluorescent microscopy.

Our preliminary work has resulted in an *fvtox1* mutant in order to replicate the results of a previous study by Brar et al. Their study knocked out Fvtox1 in the Fv strain Clinton1B, and found that foliar symptoms in the Williams 82 soybean cultivar were reduced 2-fold when using a stem-cut assay containing culture filtrates obtained from the mutant[55]. Our *fvtox1* mutant strain was obtained using the Fv isolate Mont-1 (NRRL 22292). It successfully colonizes potato dextrose agar amended with 150µg/mL hygromycin (Fig. 4A), because the Fvtox1 gene has been successfully replaced by the hygromycin phosphotransferase (hph) gene (Fig. 4B). Our preliminary results show that root rot symptoms in the Sloan soybean cultivar are significantly reduced when inoculated with the *fvtox1* mutant, but foliar symptoms are not different than the wild type (Figure 5). These results need to be replicated and performed with multiple independent *fvtox1* knockouts prior to making final conclusions.

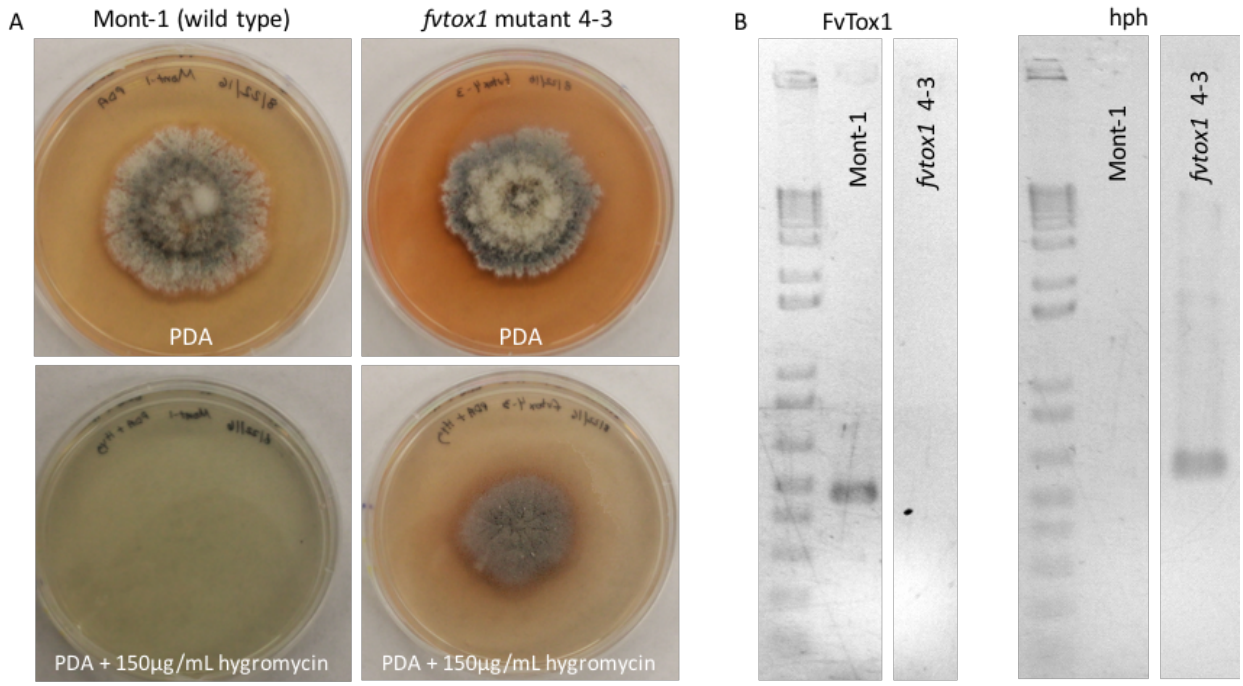


Figure 4. Genetic knockout of FvTox1. (A) The wild type strain (Mont-1) is able to grow on potato dextrose agar, but not when amended with 150 µg/mL hygromycin. The *fvtox1* 4-3 mutant is capable of growing on potato dextrose agar, even when amended with 150 µg/mL hygromycin. Images were taken 21 days post inoculation. (B) Mont-1 is PCR positive for the FvTox1 gene, while the *fvtox1* 4-3 mutant is negative. Mont-1 is PCR negative for the hygromycin resistance gene (*hph*) while the *fvtox1* 4-3 mutant is positive.

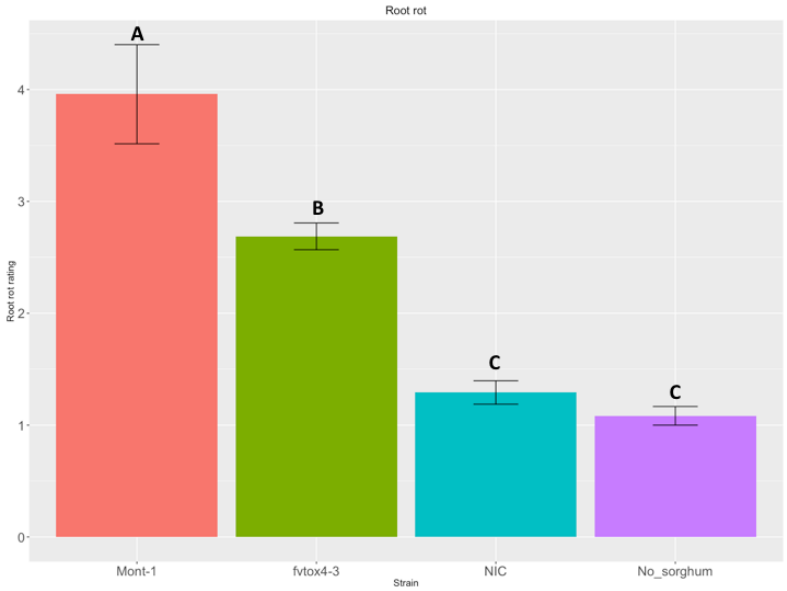


Figure 5. Virulence screen of the *fvtox4-3* mutant. Root rot severity was significantly decreased compared to inoculation with the wild type strain (Mont-1), but still caused significantly more damage the either negative control (NIC = non-inoculated sorghum control).

These preliminary studies have laid the foundation for my future studies. Our risk assessment project determined that Fv can be heterogeneously distributed throughout a field, and that sampling soil before planting may provide useful information to growers while making management decisions. One of these decisions may be the use of genetically resistant soybean cultivars to reduce yield losses across a field, although full genetic resistance is currently unavailable. Our risk assessment project also indicated a potential interaction between SCN and Fv in a field setting, particularly in 2015, that needs further validation. The molecular protocols developed so far will be used to generate useful tools to study SDS development and the interaction between Fv and SCN.

Experimental Design

Aim 1 - Identify modes of F. virguliforme entry into soybean root tissues

The first mode of entry I will investigate is the individual capability of *F. virguliforme* (Fv) to penetrate soybean root tissues. Although evidence of appressorium development have been reported for Fv[21], a recent *in silico* analysis suggested that Fv may rely more heavily on plant cell wall degrading enzymes to enter plant tissues[28]. To address these two different findings, I plan to generate a fluorescent strain and observe soybean root infection over time via fluorescent microscopy. The transformation construct needed to produce a fluorescent strain of Fv will be generated on a plasmid using PCR and Gibson Assembly. The plasmid will contain the enhanced GFP (eGFP) gene under the control of the strong GPD promoter and TrpC terminator from *Aspergillus nidulans* (plasmid pDS23[71]), the *hph* gene (plasmid pCB1004[72]), an *E. coli* origin of replication (OriC), and a kanamycin resistance gene (aminoglycoside O-phosphotransferase) (Fig. 6A). Primers used to amplify these four segments contain 5' overhangs that overlap with the fragment next to it, therefore incorporating these overlaps into the PCR product. These PCR products can be assembled into a plasmid using Gibson assembly methods[73]. Briefly, a T5 exonuclease digests a single strand of each PCR fragment from the 5' end, generating 3' sticky ends that interact with the overlapping segments introduced from the neighboring PCR product. Then, a DNA polymerase fills in any missing sequences that may have been digested away by the exonuclease, and gaps in the phosphate backbone are sealed by a T4 DNA ligase. When designed correctly, the result is a plasmid that can be directly transformed into *E. coli* for mass production. I have specifically designed two restriction sites into the plasmid, providing the ability to remove the OriC and kanamycin resistance gene and only use the eGFP and *hph* construct to transform Fv (Fig. 6B). Alternatively, this plasmid could be used as a PCR template to produce a split-construct design to reduce the number of false positive transformants[74,75].

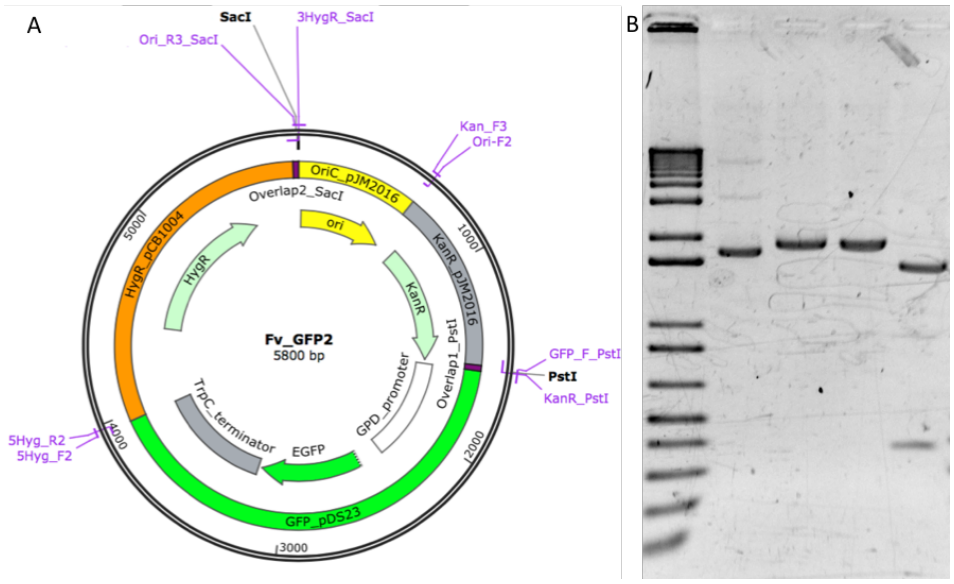


Figure 6. (A) Plasmid "Fv_GFP2", developed via Gibson Assembly. (B) Results of a restriction digest of Fv_GFP2 designed for transforming *F. virguliforme*.
Lane 1 = ladder, Lane 2 = undigested, Lane 3 = SacI single digest, Lane 4 = PstI single digest, Lane 5 = SacI and PstI double digest.

Ascomycete fungi are commonly transformed by using protoplasts, polyethylene glycol, and homologous recombination (HR)[76–80]. This method has also been used successfully in Fv[54,56,66]. Protoplasts are developed by using enzymes like chitinase and β-glucuronidase that break down the fungal cell walls, leaving behind a "naked" cell. The transformation construct is introduced to the protoplasts along with a high concentration of polyethylene glycol that helps make protoplasts competent for DNA uptake. Once the construct is inside, natural DNA repair mechanisms insert the foreign DNA into the fungal genome, often using homologous recombination mechanisms. Protoplasts of the Mont-1 (NRRL 22292) isolate have been obtained using the optimized protocol described in my Preliminary Studies. This isolate was chosen because it consistently produces a high level of root rot and foliar symptoms on inoculated soybeans in greenhouse and growth chamber experiments. Once a fluorescent strain is obtained, we will obtain conidial suspensions and inoculate germinating soybean roots and follow the infection process using fluorescent microscopy, specifically searching for appressorium development.

This fluorescent strain will also be useful in determining the relationship between Fv and SCN, which is the second entry mechanism I will investigate. A study by Muhammed et al. optimized the use of the model nematode *Caenorhabditis elegans* to determine if various *Fusarium* species were animal pathogens, and found numerous *Fusarium* species were able to colonize the internal organs of the nematodes as well as the external cuticles[40]. Therefore, we hypothesize that Fv may be able to colonize SCN cuticles and internal organs, using the SCN as a transmission vector to cause SDS. Using the study by Muhammed et al as a framework, I will determine if Fv has specific interactions with nematodes by using the model nematode *C. elegans*. I will collect Fv macroconidia and *C. elegans* adults and co-culture them together in liquid medium at 25°C for 4+ hours. After incubation, adult *C. elegans* will be picked out of the solution, washed, collected, and placed on microscope slides containing levamisole. This will cause paralysis in the nematodes, render them immobile, and allow observation with a confocal microscope. Conventional and fluorescent microscopy will reveal if Fv is capable of penetrating nematode cuticles or capable of colonizing internal organs, potentially after ingestion by the nematode. Heat-killed Fv conidia will be used as a control in these co-culture experiments. These experiments will then be repeated using SCN to confirm or refute the results obtained with *C. elegans*.

Aim 1 - Potential pitfalls

Developing transformation constructs as plasmids using Gibson Assembly can be expensive, and incorrect nucleotides can be incorporated in the overlapping sequences that are digested by the T5 exonuclease. If prohibitive costs or difficulties with sequencing errors occurs, merge PCR methods will be used as an alternative approach[81]. Merge PCR also uses primers containing 5' overhang sequences that are complimentary to other sequences of interest, but does not result in a plasmid. Instead of an exonuclease creating sticky ends of the two sequences, denaturing the fragments with high temperatures allows some of the sequences to come together and create a merged product containing both initial fragments. Gibson Assembly develops the transformation construct much quicker, and can be propagated as a plasmid through *E. coli*, allowing high reproducibility and the ability to share the construct with other labs.

Rearing SCN in a lab setting is highly complicated because SCN is an obligate biotroph and must be reared *in planta*. In addition, specific soybean cultivars are only susceptible to certain SCN races, but not others[82]. Therefore, the co-culture experiment with fluorescent Fv

macroconidia will benefit from optimization with *C. elegans* first, as this model nematode is also found in soils and is easily cultured in a lab. In addition, we have a *glp4* mutant of *C. elegans* that is temperature sensitive, and cannot reproduce when cultured at 16°C allowing us to use a specific number of nematodes in each experiment. If Fv can colonize *C. elegans* and cause lethality, we will be able to collect data to quantify this relationship.

Aim 2 - Identify FvTox1 and FvNIS1-interacting proteins that induce foliar SDS symptoms

As previously mentioned, FvTox1 and FvNIS1 are effector proteins produced by Fv that plays a role in foliar chlorosis and necrosis development in soybeans, respectively[55,56]. To our knowledge, FvTox1 has been successfully cloned and C-terminally tagged with useful epitopes for molecular manipulation[52,54], but FvNIS1 has not. Much of the molecular work regarding these effectors have surrounded characterizing the gene and finding ways to inhibit their function. However, no studies have been done to determine their specific mode of action or cellular localization. Without knowing how these proteins function, it is difficult to inhibit their function. I aim to use molecular techniques to characterize the function of these proteins, allowing for a more targeted approach at their inhibition.

First, I will confirm the results achieved by Pudake et al. and Chang et al. to show that FvTox1 and FvNIS1 play a large role in the development of foliar SDS chlorosis and necrosis, respectively[55,56]. I will use our optimized protocol for protoplasting and transforming Fv isolates described above to knock out each gene. I will use PCR to confirm the replacement of each effector gene with the selectable marker gene. Finally, I will prepare wild-type and knockout strains on autoclaved sorghum to act as an inoculation source in growth chamber experiments. Soybean seedlings potted in vermiculite containing inoculum should develop classic SDS symptoms. I will rate the root rot severity, foliar symptom severity, and obtain root dry weight measurements to determine if the knockout strains cause less foliar symptoms as previously reported. I hypothesize that the knockout strains will have lower root rot and foliar SDS severity than that caused by the Mont-1 isolate.

Second, I will clone each gene into an expression vector for the overexpression and purification of the effectors. I will clone each gene into the pPicZ-A plasmid using KpnI and ApaI restriction enzymes, which will add an in-frame c-myc and 6x HIS tag to the 3' end of each gene. When translated, these proteins will have the c-myc and 6x HIS tag directly fused to the c-terminus, allowing purification with affinity chromatography and specific detection using anti-myc antibodies in a Western-blot. This plasmid can be used to transform *Pichia pastoris*, a model yeast for eukaryotic protein expression. Since these proteins are fungal in origin, *P. pastoris* is more likely to fold, process, secrete, and post-translationally modify these proteins correctly, while other model species like *E. coli* may not. I will screen the purified protein for disease-causing activity by introducing it into soybean leaf tissues using a vacuum infiltration assay. Infiltrating soybean leaf disks with purified toxin should induce foliar SDS symptoms, as seen previously.[54] I plan to re-isolate these proteins from infiltrated leaf discs and perform co-immunoprecipitation to identify potential binding partners. Binding partners of these proteins can be identified using liquid chromatography mass spectrometry (LC-MS) at the Michigan State University Proteomics Facility, with purified unbound effector protein submitted as a control. I plan to confirm these putative binding partners by cloning them and performing yeast two-hybrid experiments, where the effector and putative binding partner are fused to "bait" and "prey" proteins that are necessary for transcription of a reporter gene. If the effector and putative binding partner have specific interaction, the two yeast proteins will promote transcription of the

reporter gene, providing significant evidence that there is direct interaction between the effector and putative binding partner.

Additional evidence for the putative binding partners identified in the above experiments will be obtained through fluorescent microscopy. Many fluorescent proteins have been N or C-terminally fused to fungal proteins of interest[49,67,79,83,84]. Both FvTox1 and FvNIS1 contain functionally important N-terminal signal peptides, so I will generate fluorescent protein fusions to the C-terminus of FvTox1 and FvNIS1. These fusion constructs can again be overexpressed in *P. pastoris* and used in vacuum infiltration assays, as described above. However, instead of following up with co-immunoprecipitation and LC-MS, the leaves will be fixed, sectioned, and prepared on slides for viewing with confocal fluorescent microscopy. Localization of the fusion protein should indicate whether the toxin acts internally or in the soybean apoplast. Effectors that co-localize to regions that the putative binding partners are also located will provide additional support for their interaction.

Ultimately, the goal of this project would be to identify soybean proteins that interact specifically with each effector, potentially acting as a receptor. Characterization of these soybean proteins could elucidate their function in the absence of the effectors. If the regular host function is dispensable, soybeans harboring a nonsense mutation in the gene encoding it may provide higher resistance to foliar SDS symptoms and added yield protection.

Aim 2 - Potential pitfalls.

Additions to the C-terminus of FvTox1 have been developed and used in other projects[52], but tagged versions of FvNIS1 have not been reported. The original NIS1 protein discovered in *Colletotrichum orbiculare* was C-terminally tagged with an HA epitope[57], so a similar tag on FvNIS1 is not expected to interfere with its natural function. However, the addition of any tag to a protein may cause improper folding and interference with the normal functionality of the protein. Therefore, if this type of problem arises, an N-terminal tag may be implemented. Since both FvTox1 and FvNIS1 are secreted proteins and contain putative N-terminal secretion peptides that are cleaved upon secretion[54,56], an N-terminal HIS-tag may be naturally cleaved along with the secretion signal, rendering the tag useless. If tagging these proteins becomes too difficult, specific antibodies will be developed to each protein by overexpressing it in *P. pastoris* and sending it to a company for antibody development through injection of a rabbit or goat, and conjugated to a fluorescent dye for fluorescent microscopy. However, the methods described in this aim will be the most efficient use of time and funding to perform the experiments described.

Similarly, fusing a fluorescent protein to a small protein of interest can more than double its size. FvTox1 is 172 amino acids long, FvNIS1 is 152 amino acids long, and eGFP is 238 amino acids long. Therefore, adding the fluorescent tag makes the overall protein much larger. In addition, a direct fusion from the last amino acid in the effector to the starting methionine of the fluorescent protein may inhibit proper folding of one or both domains of the fusion protein. Instead of a direct fusion, various linker sequences can be added that may help each domain fold properly, optimizing effector functionality while retaining fluorescent properties[85,86].

After the development of a functional effector-GFP fusion protein is created, plant-cell auto-fluorescence may become problematic during fluorescent microscopy. Chloroplasts are known to have intense auto-fluorescence, making fluorescent microscopy of leaf tissues more challenging. Alternative fluorescent proteins such as CFP, YFP, mCherry, or mNeonGreen may be fused to the effectors instead of GFP to detect unique fluorescent properties of the toxin and the leaf auto-fluorescence.

Aim 3 - Investigate alternate-host resistance mechanisms to SDS.

Identifying putative binding partners in Aim 2 will allow me to search for homologs in other crops. This is of particular interest due to the findings of Kolander and colleagues, who found that Fv has the ability to colonize many different crop and weed species[12]. Despite the ability to colonize other plants, foliar symptoms were restricted to soybean and most other legumes (alfalfa, navy bean, and red clover). Root rot symptoms were also found in legumes (soybean, alfalfa, pinto bean, navy bean, white clover, red clover, and pea) and a common weed (Canadian milk vetch). Some crops did not show classic SDS symptoms, but did show a significant reduction in biomass (sugar beet and canola). Yet other crops were colonized, but showed no apparent symptoms of infection (corn, wheat, ryegrass, pigweed, and lambsquarters). Suspicious SDS-like foliar symptoms have also been found in the common weed horsenettle (M.I. Chilvers, A. Byrne, unpublished). If these alternate hosts contain homologs to the putative binding partners identified in soybean, perhaps the toxins can induce foliar symptoms in these alternate hosts under certain conditions.

I aim to use alternate host crops like red kidney bean, sugar beet, and corn that display different levels of SDS symptoms and perform leaf infiltration assays on each using my purified effectors. There are two possible outcomes; the effectors induce disease symptoms (SDS) in alternate hosts, or they do not. If they do, these alternate hosts likely contain a homolog to the binding partner identified in soybean, but may employ a unique resistance mechanism to prevent these effectors from reaching foliar tissues during a natural infection. If they do not cause SDS symptoms, these effectors are truly host-selective and an investigation into specific residues necessary for inducing SDS symptoms is warranted. In addition, if these proteins show SDS-causing capabilities across a broad spectrum of plants, it may be worth considering upscale production and use as a natural herbicide.

Aim 3 - Potential pitfalls.

Working with multiple unrelated species of plants means that leaf tissues will have different compositions, thicknesses, cuticle properties, and tolerances to leaf infiltration assays. The optimized protocol for a leaf infiltration in soybean leaves may not work well for another crop. Therefore, it may be beneficial to practice leaf infiltration assays on model plants, like *Arabidopsis thaliana* and *Nicotiana benthamiana,* even though these plants have not been shown to be alternative hosts of Fv.

A lack of SDS symptoms in non-soybean leaf discs may indicate that the proteins are not being infiltrated, or that they are ineffective at inducing SDS. Therefore, additional immunoprecipitation assays may be necessary after infiltrating alternate-host leaves to confirm that the effectors did enter the leaves, even if it was not capable of causing symptoms in these plants. If exposing cells to the effectors via leaf infiltration proves difficult, a transient expression system like the Effector Detector Vector may be used instead. This vector allows a chimeric fusion of a bacterial and non-bacterial peptide (like fungal proteins) to be injected into *Arabidopsis thaliana* or *Nicotiana benthamiana* by taking advantage of the type III secretion system in *Pseudomonas syringae*[87]. The known bacterial peptide is recognized and cleaved by the plant, allowing the fungal protein to be released. If the plant develops symptoms, the fungal protein is thought to be an effector; if not, then it is not an effector. The system could potentially be used successfully on other types of plants as well, but may require unique pathovars of *Pseudomonas syringae*[88].

Timetable

Aim 1 – Identify modes of *F. virguliforme* entry into soybean root tissues.

In 0-12 months:

- Generate fluorescent strain of *F. virguliforme*
- Fluorescent microscopy of inoculated soybean roots, searching for appressoria
- Fluorescent microscopy of *C. elegans* co-cultured with *F. virguliforme*, searching for fungal colonization

Aim 2 – Identify FvTox1 and FvNIS1-interacting proteins that induce foliar SDS symptoms

Already accomplished:

- *fvtox1* knockout mutants generated and PCR confirmed
- *fvtox1* knockout mutant inoculum prepared

In 6-12 months:

- Generate *fvnis1* knockout strains and inoculum
- Clone FvTox1 and FvNIS1 into *P. pastoris* for overexpression of tagged proteins

In 12-24 months:

- Optimize and perform vacuum infiltration assays on soybean leaf disks

In 18-36 months

- Co-immunoprecipitation and LC-MS of toxin binding partners
- Yeast-2-hybrid confirmation experiments
- Fluorescent microscopy to determine subcellular localization of toxins

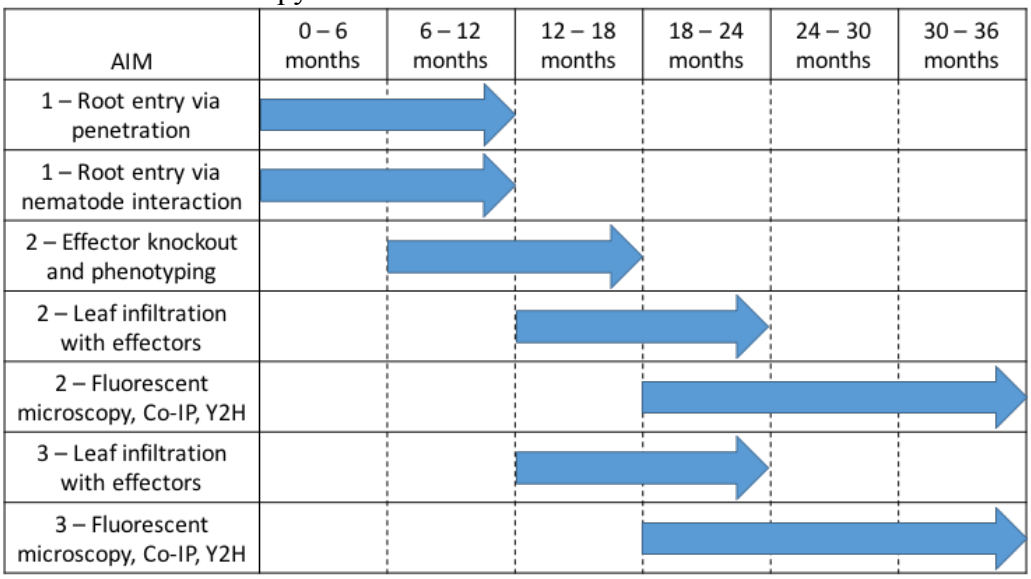
Aim 3 – Investigate alternate-host resistance mechanisms to SDS

In 12-24 months:

- Optimize and perform vacuum infiltration assays on alternate host leaf disks

In 18-36 months:

- Co-immunoprecipitation and LC-MS of toxin binding partners
- Yeast-2-hybrid confirmation experiments
- Fluorescent microscopy to determine subcellular localization of toxins



Potential Funding Sources

The National Institute for Food and Agriculture (NIFA) branch of the USDA provides support for advancements in agriculture through biotechnology while encouraging active education and outreach during the experiments. This project would be suited for a USDA NIFA grant due to the potential development of transgenic crops that could improve soybean production. Given the current state of the public perception of transgenic crops, this project would also benefit from the educational and outreach components of such a grant. Funding from the NSF may also be pursued.

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