DETERMINATION OF GENETIC AND METABOLIC FEATURES WITHIN *SAROCLADIUM ZEAE* RESPONSIBLE FOR ITS TOLERANCE TO ANTIMICROBIAL PHYTOCHEMICALS PRODUCED BY MAIZE AND ITS PHYSICAL AND XENOBIOTIC INTERACTIONS WITH *FUSARIUM VERTICILLIOIDES* IN THE PLANT

by

GARRETT THOMPSON HIBBS

(Under the Direction of Scott Gold & Anthony Glenn)

ABSTRACT

Fusarium verticillioides is a fungal phytopathogen of maize. *F. verticillioides* produces the fumonisin mycotoxins, which pose a threat to human and animal health. *Sarocladium zeae* is a nonpathogenic fungal endophyte of maize suggested as a potential biocontrol for *F. verticillioides*. *S. zeae* produces the secondary metabolites, pyrrocidine A & B, that are antagonistic towards *F. verticillioides* and inhibit its production of fumonisin. It has been shown that the *FvMBL1* gene is required for *F. verticillioides* to resist the maize antimicrobial phytochemical 2-benzoxazolinone (BOA). We found *S. zeae* possesses an ortholog of *FvMBL1* that is also required for its ability to resist BOA. Our other findings show that *S. zeae* reduced the amount of fumonisin B1 recovered from maize ears when co-inoculated with *F. verticillioides*, the presence of *S. zeae* and *F. verticillioides* was surveyed in starburst kernels collected in Georgia, and the biosynthetic gene cluster that produces pyrrocidine was identified.

INDEX WORDS: *Fusarium verticillioides, Sarocladium zeae,* maize, biocontrol, MBL1, pyrrocidines

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GARRETT THOMPSON HIBBS

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by

GARRETT THOMPSON HIBBS

Major Professor:	Scott Gold
Committee:	Anthony Glenn
	Marin Brewer
	Robert Kemerait

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
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DEDICATION

This work is dedicated to my supportive family who I love more than anything I have ever known. To my beautiful mother, Judy Hibbs, whose love and support has made me into the person I am today, to my father and forever my hero, Henry Hibbs, and to my brother and best friend always, Robert Hibbs.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Fusarium verticillioides is a ubiquitous mycotoxigenic fungus that can exist as both a phytopathogen, or as an asymptomatic endophyte of maize. *F. verticillioides* is the most frequently reported plant pathogen infecting maize, and can cause several symptoms.

Sarocladium zeae (formerly known as *Acremonium zeae*) is another endophyte commonly found in maize. *S. zeae* produces the secondary metabolite, pyrrocidine A & B, found to be antagonistic toward *F. verticillioides*. Our recent findings have also shown that pyrrocidine is able to suppress fumonisin production in *F. verticillioides*. Because of this, *S. zeae* is a potential biological control agent for *F. verticillioides*. To resist invasive microbial colonization, maize produces the antimicrobial phytochemical, 2-benzoxazolinone (BOA). For *F. verticillioides* to successful colonize maize, it must possess the ability to resist BOA. A previous microarray analysis of *F. verticillioides* exposed to BOA identified two unlinked loci, each containing multiple genes in the *F. verticillioides* genome referred to as FBD1 and FBD2 (Glenn, et al., 2016). *FvMBL1* was among some of the most highly induced genes within these clusters. Deletion of *FvMBL1* rendered *F. verticillioides* unable to grow on media containing BOA (Glenn, et al., 2016). *S. zeae* is also a successful colonizer of maize and therefore must also posses the ability to resist BOA. After conducting a BLAST search of DNA sequence coding for *Fv*MBL1 against the genome of *S. zeae*, we identified a gene with high identity (e value = 0.0). In one objective of this study we generated a Δ*Szmbl1* deletion strains in *S. zeae* to study whether deletion of *SzMBL1* would also render *S. zeae* unable to grow within the presence of BOA. Verified *Szmbl1* deletion strains were severely inhibited on BOA containing media indicating that, like *FvMBL1* in *F. verticillioides, SzMBL1* is required for resistance to BOA in *S. zeae*. The next two objectives of this study were to build evidence for the use of *S. zeae* as a biological control agent for *F. verticillioides*. One of these objectives involved co-inoculating maize ears with both *F. verticillioides* and *S. zeae* to build a greater understanding of the ability of *S. zeae* to reduce fumonisin B1 levels. The results of this experiment showed that *S. zeae* was able to reduce the amount of fumonisin B1 recovered from samples 62% compared to maize ears that were only inoculated with *F. verticillioides*. Another objective of this study was focused on observing the ability of *S. zeae* as a biological control agent for *F. verticillioides* in a field setting. To study this, we conducted a field survey in Georgia to investigate the presence or absence of *S. zeae* and *F. verticillioides* in asymptomatic kernels and kernels expressing the *F. verticillioides* starburst symptom. The final objective of this research was to identify the biosynthetic gene cluster responsible for producing pyrrocidine in *S. zeae*. We found that the deletion of the, here named, *SzPYC1* gene resulted in the inability of *S. zeae* to produce pyrrocidine. This provided strong evidence that the biosynthetic gene cluster that *Sz*PYC1 was located in is required for pyrrocidine production.

LITERATURE REVIEW

Fusarium verticillioides as a pathogen of maize

In general, Fusarium species have been recognized as plant pathogens causing a variety of plant symptoms and are commonly found in tropical and temperate regions (Nelson et al., 1994). *F. verticillioides* can exist as a pathogen of maize causing seedling blight, stalk rot, and ear rot, and can be pathogenic towards maize at any stage in its development (Munkvold et al., 1997; Foley, 1962). Stalk rot caused by *F. verticillioides* can cause significant economic losses to maize producers in the United States. In the northern United States *Fusarium* stalk rot was ranked fifth or sixth in greatest losses caused by plant disease in maize, and in the southern United States *Fusarium* stalk rot was ranked first or second for maize disease losses between 2012 and 2015 (Mueller et al., 2016).

Fumonisin

Fumonisin is a polyketide-derived secondary metabolite produced by a biosynthetic gene cluster found in *F. verticillioides* (Brown et al., 2007). Fumonisins are mycotoxins that pose a threat to human and animal health. In animals, fumonisins can cause diseases such as leukoencephalomalacia in equine (Marasas et al., 1988), pulmonary edema in swine (Colvin & Harrison, 1992), and liver toxicity in rats (Kriek, Kellerman, & Marasas, 1981). Negative health effects that fumonisins present to humans are still not well understood, but there is epidemiological evidence to support that fumonisins may be a risk factor for the development of esophageal cancers and neural tube birth defects in areas where maize consumed as a dietary staple possesses high incidence of *F. verticillioides* infection (Bulder et al., 2012; Gelineau-van Waes et al., 2009; Gelderblom et al., 1988; Marasas, 2001). Fumonisins have also been associated with human stunting (Riley & Merrill, 2019). With these health threats in mind, industrial countries such as the United States can suffer steep economic losses due to rejection of food and feed that exceed maximum tolerated levels of fumonisins (Wu, 2006).

Sarocladium zeae as a biological control agent for *F. verticillioides*

S. zeae shows potential as a biological control agent for *F. verticillioides* because of its production of the secondary metabolites, pyrrocidine A & B, shown to be antagonistic toward *F.*

verticillioides (Wicklow & Poling, 2009; Wicklow, et al., 2005). Our recent findings have also shown that pyrrocidines are able to suppress fumonisin production in *F. verticillioides* at levels well below those required for visible growth inhibition (2.5 μg/ml vs >20 μg/ml pyroccidine B; Gold et al., 2019).

Histopathological studies have shown that when both *S. zeae* and *F. verticillioides* are together within maize kernels, *S. zeae* inhabits the larger embryo and endosperm region while *F. verticillioides* is typically confined to the pedicel and abscission layers (Sumner, 1968). These observations may indicate that *S. zeae* can effectively suppress *F. verticillioides* from colonizing the entire kernel keeping *F. verticillioides* subdued to the much smaller area of the pedicel.

For a microorganism to be an effective biocontrol in maize it must posses the ability to colonize the same area of the plant infected by the pathogenic organism, and organisms already adapted to vegetative parts of the plant may hold an advantage over others (Alberts et al., 2016). *S. zeae* is a fungal endophyte of maize that fits these criteria, being one of the most common fungi found colonizing maize and typically found together in asymptomatic maize kernels with *F. verticillioides* (King, 1981; Wicklow et al., 2005).

2-benzoxazolinone

Benzoxazinoid secondary metabolites are generated by maize and are used as one of the mechanisms by which the plant resists invasive microbial colonization (Saunders & Kohn, 2008). Due to benzoxazinoids' unstable nature in aqueous solutions, when plant vacuoles containing these compounds are disrupted they rapidly degrade to the benzoxazolinones, 6-methoxy-2- benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA) (Saunders & Kohn, 2008). BOA has been shown to possess antifungal activity due to lipophilic and electronic properties enforced by its aromatic ring substituents (Bravo, Copaja, & Lazo, 1997).

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CHAPTER 2

THE *SAROCLADIUM ZEAE* ORTHOLOG OF *FVMBL1* CONFERS RESISTANCE TO 2-BENZOXAZOLINONE

INTRODUCTION

Fusarium verticillioides is a ubiquitous mycotoxigenic fungus that can exist as both a phytopathogen, or as an asymptomatic endophyte of maize (Bacon & Hinton, 1996). *F. verticillioides* is the most frequently reported plant pathogen infecting maize, and can cause seedling blight, stalk rot, and ear rot (Munkvold, et al. 1997).

F. verticillioides' greatest impact comes from its threat to human and animal health due to its production of the fumonisin mycotoxins. Fumonisins can cause animal diseases such as leukoencephalomalacia in equine (Marasas et al., 1988), pulmonary edema in swine (Colvin & Harrison, 1992), and liver toxicity in rats, sheep and primates (Kriek, Kellerman, & Marasas, 1981). While the negative health effects of fumonisins on humans are still not well understood, there is epidemiological evidence to support that fumonisin may be a risk factor for the development of esophageal cancers and neural tube birth defects in areas where maize consumed as a dietary staple has a high incidence of *F. verticillioides* infection (Bulder et al., 2012; Gelineau-van Waes et al., 2009; Gelderblom et al., 1988; Marasas, 2001).

In addition to *F. verticillioides*, another endophyte commonly found in maize is *Sarocladium zeae* (syn. *Acremonium zeae*). *S. zeae* and *F. verticillioides* are the most common fungi found colonizing preharvest maize (Wicklow et al., 2005). *S. zeae* has been suggested as a potential biological control agent for *F. verticillioides* because of its production of the secondary metabolites, pyrrocidine A & B, shown to be antagonistic towards *F. verticillioides* (Wicklow & Poling, 2009; Wicklow et al., 2005). Additionally, our recent findings showed that pyrrocidines are able to suppress fumonisin production in *F. verticillioides* (Gold et al., 2019). Within maize, both *S. zeae* and *F. verticillioides* are commonly found in asymptomatic kernels (King, 1981). Histopathological studies showed that when both *S. zeae* and *F. verticillioides* are together within a kernel, *S. zeae* inhabits the larger embryo and endosperm region while *F. verticillioides* is typically confined to the pedicel and abscission layers (Sumner, 1968).

One of the mechanisms that maize uses to resist invasive microbial colonization is through its production of benzoxazinoid secondary metabolites (Saunders & Kohn, 2008). Due to the unstable nature of benzoxazinoids, when plant vacuoles containing these inactive glycosylated compounds are disrupted they are released as active aglycones and rapidly degrade to the benzoxazolinones, 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA) (Saunders & Kohn, 2008). BOA has been shown to possess antifungal activity due to lipophilic and electronic properties enforced by its aromatic ring substituents (Bravo et al., 1997).

For *F. verticillioides* to successfully be a primary colonizer of maize, it must possess a mechanism to resist BOA. Using a previous microarray analysis of *F. verticillioides* exposed to BOA, Glenn et al. (2016) identified two unlinked loci in the *F. verticillioides* genome each containing multiple genes referred to as the *FDB1* and *FDB2* clusters. Multiple genes within these loci were highly induced suggesting that *FDB1* and *FDB2* were functional gene clusters (Glenn et al., 2016). Mutagenesis of open reading frames within *FDB1* and *FDB2* gene clusters and screening for sensitivity to BOA identified the gene FVEG_08291 encoding *Fv*MBL1 having strong amino acid similarity to the metallo-β-lactamase (MBL) superfamily. Deletion of *FvMBL1* rendered *F. verticillioides* unable to grow on media containing BOA (Glenn et al., 2016).

Similar to *F. verticillioides*, *S. zeae* is a successful colonizer of maize and is therefore hypothesized to also possess the ability to resist BOA. A BLAST search of DNA sequence coding for *Fv*MBL1 against the genome of *S. zeae*, identified a gene with high similarity (E value = 0.0; 76.4% identical with 305 of 399 residues; 85.5% similar). In this study we generated a Δ*Szmbl1* deletion strains in *S. zeae*. Verified Δ*Szmbl1* deletion strains were severely inhibited on BOA containing media indicating that, like *FvMBL1* in *F. verticillioides, SzMBL1* is required for resistance to BOA in *S. zeae*.

MATERIALS AND METHODS

Fungal strains and growth conditions

Strains of *S. zeae* used in this study were grown from frozen stocks of conidia maintained for long-term storage at -80°C in 15% glycerol. Wild-type strain NRRL 6415 was used for generating Δ*Szmbl1* deletion strain. The fungus was grown routinely using potato dextrose agar (PDA; Neogen Food Safety, Lansing, MI, USA) and potato dextrose broth (PDB; Neogen Food Safety, Lansing, MI, USA.) at 27°C in the dark.

OSCAR-ATMT

A red fluorescent protein expressing strain of *S. zeae (Sz*-RFP) and other transformants containing ectopic integrations of the *SzMBL1* deletion construct were generated using the

OSCAR protocol (Gold et al., 2017; Paz et al., 2011). Deletion constructs generated using the OSCAR protocol were transformed into *Escherichia coli* (One Shot® MAX Efficiency® DH5αTM-T1R, Invitrogen, Carlsbad, CA, USA) and transformants were cultured overnight for 12 hr at 37 °C and 250 rpm in 5 mL of Luria-Bertani (LB) medium with the addition of 100 μg/mL spectinomycin (Thermo Fisher Scientific, Waltham, MA, USA). Construct structure was confirmed by sequencing. An *E. coli* transformant containing the verified OSCAR deletion plasmid was stored in 15% glycerol at -80 °C. *E. coli* plasmid was extracted (Qiagen Miniprep Kit, Qiagen, Inc., Valencia, CA, USA) and transformed into *Agrobacterium tumefaciens* AGL-1 and selected on low sodium (0.5 g/L) LB medium amended with 100 μg/mL spectinomycin at 27 °C for 48 hours. *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *S. zeae* was carried out by standard protocol (Gold et al., 2017; Paz et al., 2011).

CRISPR-cas9

Because of the failure of ATMT to generate deletion strains we reasoned that creation of targeted double stranded breaks could facilitate homologous recombination (HR). Δ*Szmbl1* deletion strains were generated by the "In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates" method, as shown in Figure 2.1 (Abdallah et al., 2017). Primers used in this study are listed in Table 2.1. Two Cas9 ribonucleoproteins (RNPs) were generated that were designed to create double-strand breaks (DSBs) directly upstream and downstream of the *SzMBL1* ORF. A HygR cassette repair template was generated with a single PCR reaction using TaKaRa high fidelity Taq (TaKaRa Bio, Kyoto, Japan) following the manufacture's protocol using primers MBL1 hyg cassette Fwd and MBL1 hyg cassette Rev (Table 2.1). The HygR cassette contained two 38 bp microhomology arms to direct the cassette to insert at the site of the DSBs. All of these components were delivered to the cell at one time using a PEG mediated transformation of protoplasts generated from wild type *S. zeae* strain NRRL 6415. Transformants were selected on 1% Bacteriological Agar (Neogen Food Safety, Lansing, MI, USA) amended with hygromycin B (150 µg/ml; Roche Diagnostics, Indianapolis, IN, USA).

To determine if *SzMBL1* was successfully deleted from the *S. zeae* genome, transformants were screened by gene-specific PCR. PCR was performed using One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer (OneTaq; New England Biolabs, Ipswich, Massachusetts, USA) with primers *SzMBL1* ORF Fwd and *SzMBL1* ORF Rev (Table 2.1). Cycling parameters for the 792 bp amplicon were 94°C for 30 s; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min; and final extension of 68°C for 5 min. Single spore isolates were obtained from putative *SzMBL1* deletion transformants based on this PCR reaction. These candidates were then screened again using an "anti-Southern" confirmation method, which consisted of four PCR reactions for each transformant. The first of these reactions used a forward primer located just upstream of the RNP cut site that was itself located upstream of the 5′ end of the *SzMBL1* gene and a reverse primer located within the open reading frame (ORF) of the HygR cassette near the 5′ end (*SzMBL1* 5′-out & 1/4). The second reaction used primers located within the ORF of the *SzMBL1* gene to confirm its presence or absence in the genome (*SzMBL1* ORF Fwd and *SzMBL1* ORF Rev). The third reaction consisted of primers located within the ORF of the HygR cassette to confirm its presence within the genome (1/46 & 1/47). The final reaction used a forward primer located within the HygR ORF near the 3′ end and a reverse primer downstream of the RNP cut site that was located downstream of the 3′ end of the *Sz*MBL1 gene (1/3 & *SzMBL1* 3′-out). Each of these PCR reactions was generated using OneTaq with the thermocycler parameters used previously in the first screening of transformants. All primers used in this anti-Southern reaction are listed in Table 2.1.

Plate assay

Δ*Szmbl* and wild-type *S. zeae strains* were assessed for tolerance on BOA medium (Glenn et al., 2001). Stock solutions of BOA were prepared at 100 mg/mL (Sigma-Aldrich Chemical Co.) in 100% ethanol and were stored at 4°C (Glenn et al., 2016). BOA media were prepared at two concentrations: 1.0 mg/mL, 0.75 mg/mL, and a control. 100% Ethanol was added as needed so that all media contained 1% ethanol. Ten mL of each BOA medium was pipetted into the three separate sections of 100 mm Y-Petri plates. Δ*Szmbl* and wild-type *S. zeae* strain NRRL 6415 were initially grown on PDA and at 27°C in the dark for 5 days. After 5 days, three 4 mm agar plugs were taken from the margin of the growing colony on each of these plates and transferred to Y-plates containing BOA media. These plates were then incubated at 27°C in the dark. Noticeable differences in BOA sensitivity between Δ*Szmbl1* and wild-type *S. zeae* were observed after 14 days of growth at these conditions. This experiment was conducted twice.

RESULTS

The OSCAR-ATMT gene deletion method provided evidence that S. zeae had a low rate of homologous recombination

The One Step Construction of Agrobacterium-Recombination-ready-plasmids (OSCAR) method was initially used to attempt to generate a Δ*Szmbl1* deletion strain. A gene deletion construct was designed to target *SzMBL1* and its structure confirmed by sequencing (data not shown). The construct was delivered to *S. zeae* via *Agrobacterium tumefaciens*-mediated transformation (ATMT). Fungal transformants were generated and individually screened by PCR with primers that amplified a region within the open reading frame (ORF) of *SzMBL1*. PCR amplification of a portion of the *Escherichia coli* hygromycin phosphotransferase (HygR) ORF conferring resistance to hygromycin B was used as a control. After screening over 100 fungal transformants generated using the OSCAR method, none were found to be deletion strains, but rather were found to all be the products of ectopic integration (Table 2.2).

These data suggest that *S. zeae* has a low rate of HR at least via the OSCAR-ATMT based approach. To further test this, three other genes of interest were selected that were located in other regions of the *S. zeae* genome (PKS-NRPS c383, PKS-NRPS c700, and PKS-NRPS c822). OSCAR-ATMT was used to generate and screen transformants for the three other genes of interest. The results were similar to those in the attempt to delete *SzMBL1*. Transformants produced by the deletion constructs designed to target the three other genes of interest were all found to contain ectopic insertions (Table 2.2).

The OSCAR method was unable to produce a *S. zeae* deletion strain in these experiments, but we found it to be a useful tool for inserting foreign DNA into the *S. zeae* genome. In each transformant containing an ectopic insertion, HygR was incorporated into the *S. zeae* genome. Additionally, using ATMT, we delivered the plasmid pCK1287 (a gift from Dr. Chang-Hyun Khang, University of Georgia; Jones, et al., 2016), containing a hH1-tdTomato construct to generate a *S. zeae* strain (*Sz*-RFP) with strongly red fluorescent nuclei for use in later experiments, shown in Figure 2.2.

CRISPR-Cas9 method successfully generated ΔSzmbl1 deletion mutant

Given our results using the OSCAR method we determined that fungal gene deletion methods strictly using HR appear insufficient to generate deletion strains in *S. zeae*. We then used a CRISPR-Cas9 based gene deletion method (Abdallah et al., 2017).

Transformants generated using this method were screened in the same manner as transformants produced by the OSCAR method using PCR to detect for the presence or absence of the *SzMBL1* ORF with the HygR ORF serving as a control. Four deletion candidates and nineteen ectopic transformants were identified among the twenty-three transformants screened (Figure 2.3). Single spore isolates were obtained from putative *ΔSzmbl1* deletion transformants based on this PCR reaction. These candidates were then screened again using an "anti-Southern" as a final confirmation method (Figure 2.4). We found that the CRISPR-Cas9 method was more efficient in generating deletion strain as compared to the OSCAR-ATMT method that relies only on HR (Table 2.2).

Plate assay demonstrates that MBL1 is critical for tolerance to BOA in S. zeae

A Δ*Szmbl1* deletion strain generated using CRISPR-cas9 was used to test the role of *Sz*MBL1 in tolerance to BOA. Δ*Szmbl1* was compared to wild-type *S. zeae* by plating both strains on media amended with three different concentrations of BOA; 1.0 mg/mL, 0.75 mg/mL, and 0.0 mg/mL. Albeit with slowed growth, wild-type *S. zeae* tolerated BOA at 0.75 mg/mL, while the growth of the Δ*Szmbl1* mutants was strongly inhibited at that concentration (Figure 2.5). Both wild-type *S. zeae* and Δ*Szmbl1* strains grew similarly on media containing no BOA. The phenotype observed in the Δ*Szmbl1* deletion strain when challenged with BOA indicates that *Sz*MBL1 is required for resistance to BOA (Figure 2.5).

DISCUSSION

S. zeae is a frequent endophyte inhabiting maize seed (Wicklow et al., 2005). To be such a successful colonizer of maize, the fungus must have a way to cope with the benzoxazinoid antimicrobial defensive compounds including BOA produced by maize. *F. verticillioides*, another frequent colonizer of maize, is often observed co-inhabiting maize seed simultaneously with *S. zeae* (Sumner, 1968). Previous studies have indicated that *F. verticillioides* copes with BOA by detoxifying it with the metallo-β-lactamase encoded by (FVEG_08291), the *FvMBL1* gene (Glenn et al., 2016). Here we show that the *S. zeae* ortholog of this gene (*SzMBL1*) is also required to degrade the maize antimicrobial defense compound BOA in this fungus.

While both *S. zeae* and *F. verticillioides* are among the most commonly found endophytes inhabiting maize seed, *S. zeae* is relatively understudied compared to its mycotoxigenic endophytic neighbor. Because of its pathogenic nature toward maize, and its production of the fumonisin mycotoxins posing a threat to human and animal health, *F. verticillioides* has been the subject of many studies. Several of these have involved gene manipulation to better understand *F. verticillioides* on a molecular level. Here, for the first time, we report the molecular transformation and genome editing in *S. zeae*. The potential of *S. zeae* to inhibit growth and especially the recent discovery that its secondary metabolites, the pyrrocidines, suppress fumonisin production has focused our attention toward more fully understanding its seed-borne nature as a biological control agent for *F. verticillioides*. To carry out incisive and informative genetic analysis of the potential to use of *S. zeae* as an effective biological control agent we developed transformation protocols and successfully employed CRISPR-Cas9 gene editing to delete the gene suspected of conferring resistance to BOA.

Our first attempt to develop a Δ*Szmbl1* deletion strain began with use of the OSCAR-ATMT method. After screening over 100 fungal transformants none were found to be deletion strains, but rather all possessed ectopic integrations. While OSCAR did not serve the purpose of generating a deletion strain, it did give us insight into the low rate of HR in *S. zeae* and its apparent high rate of non-homologous end joining (NHEJ) as a DNA repair pathway. OSCAR-ATMT did prove to be an efficient way of incorporating foreign DNA into its genome. Using OSCAR, we generated a red fluorescent protein strain of *S. zeae* (*Sz*-RFP) by delivery of the pCK1287 plasmid on the first attempt (Figure 2.2). This marks the first time a new strain of *S. zeae* was generated through intentionally delivering foreign DNA to incorporate into its genome. *Sz*-RFP contains resistance to hygromycin B making it a useful tool in future experiments to study *S. zeae* colonization of maize and its seed-to-seed transmission. The pCK1287 plasmid delivered to *S. zeae* has no homology to the *S. zeae* genome and therefore inserted via NHEJ. The ease at which the T-DNA of pCK1287 incorporated into the genome may be an indication that *S. zeae* possesses a high rate of NHEJ.

With our previous results indicating that *S. zeae* may have a low rate of HR, we next used a CRISPR-Cas9 based gene deletion method (Abdallah et al., 2017, see Figure 2.1). With the Cas9 protein creating double strand breaks up- and down-stream of the *SzMBL1* ORF we anticipated a higher rate of HR. This method successfully generated the desired Δ*Szmbl1* deletion strains.

To determine the role of *Sz*MBL1 in the ability of *S. zeae* to resist BOA, Δ*Szmbl1* and wild-type *S. zeae* were grown on media amended with two different concentrations of BOA; 1.0 mg/mL, 0.75 mg/mL, and a control. This experiment showed that, wild-type *S. zeae* tolerated BOA at 0.75 mg/mL, while the growth of the Δ*Szmbl1* mutant was strongly inhibited at that concentration. Both wild-type *S. zeae* and Δ*Szmbl1* strains grew similarly on media containing no BOA. The phenotype observed in the Δ*Szmbl1* deletion strain when challenged with BOA indicates that *Sz*MBL1 is required for resistance to BOA. These results are identical to those seen in *F. verticillioides* when the deletion of *FvMBL1* resulted in the loss of ability to grow on media containing BOA (Glenn et al., 2016). These results suggest that MBL1 orthologs play important roles in multiple genera of fungi that are colonizers of maize and other BOA producing plant species.

We have successfully identified methods useful for creating deletion strains of *S. zeae* as well as methods to introduce new genetic material to its genome. The use of the CRISPR-Cas9 based gene deletion method resulted in the generation of Δ*Szmbl1* that was used to demonstrate that *Sz*MBL1 confers the ability of *S. zeae* to resist BOA. We next plan to test if Δ*Szmbl1* mutants lose the ability to colonize maize through growth room plant inoculation experiments, further assessing the importance of BOA tolerance as a mechanism to allow select fungi the ability to be primary colonizers of maize.

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Table 2.1. List of primers used in this study.

Primer name	Description	Primer sequence
Sz MBL1 ORF Fwd	Forward primer located within the ORF of the *SzMBL1* gene to confirm its presence or absence in the genome	TACGACTGGTCCTTCATCAT
Sz MBL1 ORF Rev	Reverse primer located within the ORF of the *SzMBL1* gene to confirm its presence or absence in the genome	CTCGATGAACTTGTCGATGG
SzMBL1 5′-out	Forward primer located just upstream of the RNP cut site located upstream of the 5′end of the *SzMBL1* gene used in *SzMBL1* Anti-Southern.	GGGAGTTTATCTGTTCTTCTTCT
SzMBL1 3′-out	Reverse primer located just downstream of the RNP cut site located downstream of the 3′ end of the *SzMBL1* gene used in *SzMBL1* Anti-Southern.	CTTCTACGCCAACAAGAAGA
1/3	Forward primer located within the HygR ORF near the 3′ end used in Anti-Southern	AGAGCTTGGTTGACGGCAATTTCG
1/4	Reverse primer located within the HygR ORF near the 5′ end used in Anti-Southern	GCCGATGCAAAGTGCCGATAAACA
1/46	Forward primer located within the ORF of the HygR cassette to confirm its presence within the genome	GACAGGAACGAGGACATTATTA
1/47	Reverse primer located within the ORF of the HygR cassette to confirm its presence within the genome	GCTCTGATAGAGTTGGTCAAG
SzMBL1 hyg cassette Fwd	Forward primer used to generate HygR cassette repair template	TTGGGGGAAATAAGCTACAGCCTGGGC GCGCTACGAGGTAGAATTCCTGCAGCC CAACTG
SzMBL1 hyg cassette Rev	Reverse primer used to generate HygR cassette repair template	ACGGAGGAGGAGCTCAAGGAGATGCGT GCGACCATCAAGCTCTAGAACTAGTGG ATCCAA
SzMBL1 O1	OSCAR primer one designed based on the OSCAR protocol for the purpose of generating Δ*Szmbl1*	GGGGACAGCTTTCTTGTACAAAGTGGA ATGGCGGATGTGATTTC

SzMBL1 O2	OSCAR primer two designed based on the OSCAR protocol for the purpose of generating Δ*Szmbl1*	GGGGACTGCTTTTTTGTACAAACTTGTT ATGTGGTATAGCCCGTGTT
SzMBL1 O3	OSCAR primer three designed based on the OSCAR protocol for the purpose of generating Δ*Szmbl1*	GGGGACAACTTTGTATAGAAAAGTTGT TGCGGTTGGATTCTAAG
SzMBL1 O4	OSCAR primer four designed based on the OSCAR protocol for the purpose of generating Δ*Szmbl1*	GGGGACAACTTTGTATAATAAAGTTGT ATCCTCGGTGACTTCAT
CRISPR MBL1 #1	20 bp sequence used for generating Alt-R™ CRISPR-Cas9 crRNA used to generate RNP that generated DSB upstream of the 5′end of *SzMBL1*	GCGTTCATTCCAATGCGACG
CRISPR MBL1 #2	20 bp sequence used for generating Alt-R™ CRISPR-Cas9 crRNA used to generate RNP that generated DSB downstream of the 3′end of *SzMBL1*	GATCGCCCTGGGGCTTCAGC

Table 2.2. Results of transformations using OSCAR-ATMT and CRISPR-Cas9 method. Genes of interest targeted for deletion are listed along with the number of transformants screened that were either ectopic or deletion strains. Two of the four Δ*Szmbl1* strains were confirmed with Anti-Southerns.

Gene of Interest	Transformation Method	# of Transformants	# of Ectopic Strains	# of Deletion Strains
SzMBL1	OSCAR-ATMT	109	109	0
PKS-NRPS c383	OSCAR-ATMT	24	24	0
PKS-NRPS c700	OSCAR-ATMT	21	21	0
PKS-NRPS c822	OSCAR-ATMT	20	20	0
SzMBL1	CRISPR-Cas9	23	19	4

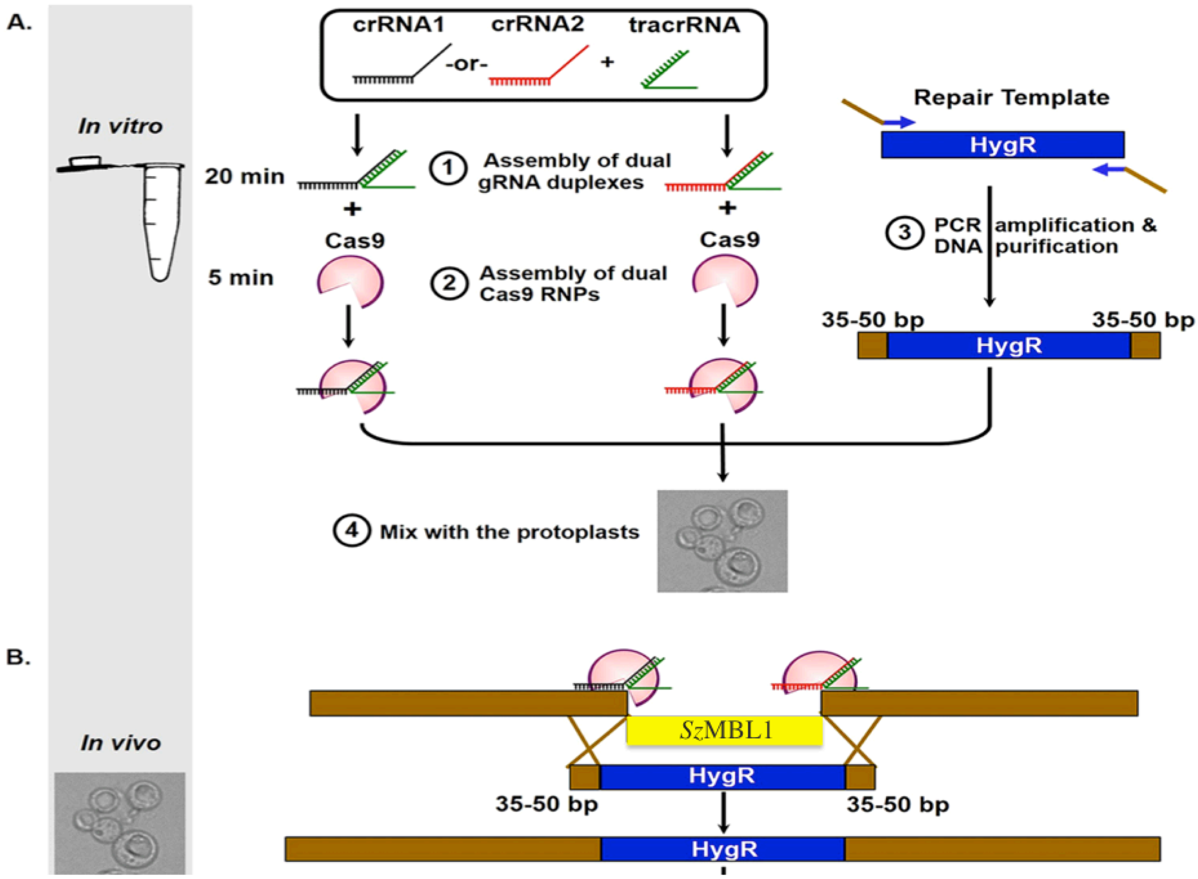

Figure 2.1. Deletion approach for *SzMBL1* by the In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates method. Image modified with permission (Abdallah et al., 2017). License can be viewed at https://creativecommons.org/licenses/by/4.0/legalcode.

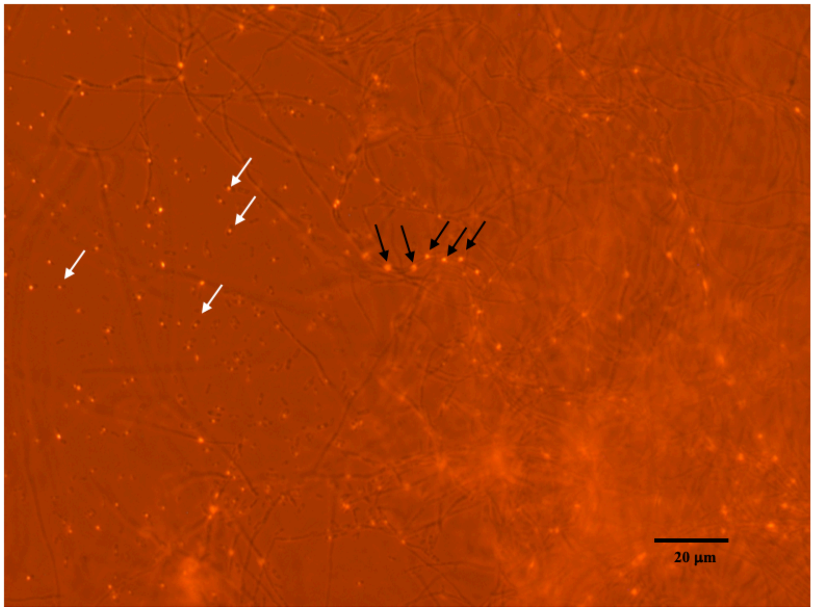

Figure 2.2. *Sarocladium zeae* transformed by ATMT with plasmid pCK1287 expressing td-Tomato fused to the C-terminus of the *Magnaporthe oryzae* Histone H1 (black arrows nuclei along a hypha, white arrows conidial nuclei; size bar 20 μm). This strain was generated using ATMT to deliver the hH1-tdTomato construct. Fluorescence is localized in the nucleus.

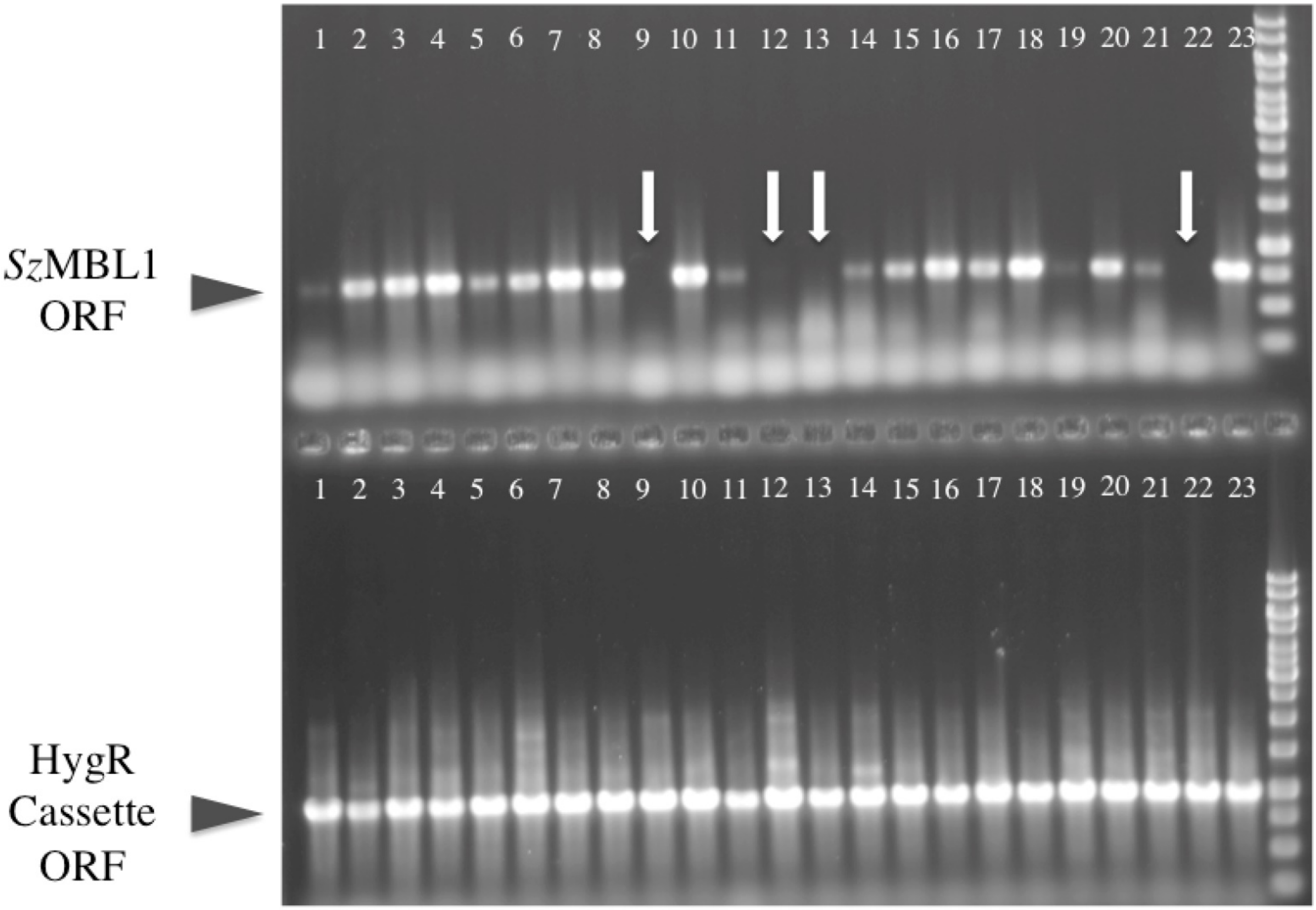

Figure 2.3. Initial PCR screen for Δ*Szmbl1* deletion mutants. White arrows point to Δ*Szmbl1* deletion candidates. Amplicons were analyzed through 0.8% agarose by gel electrophoresis.

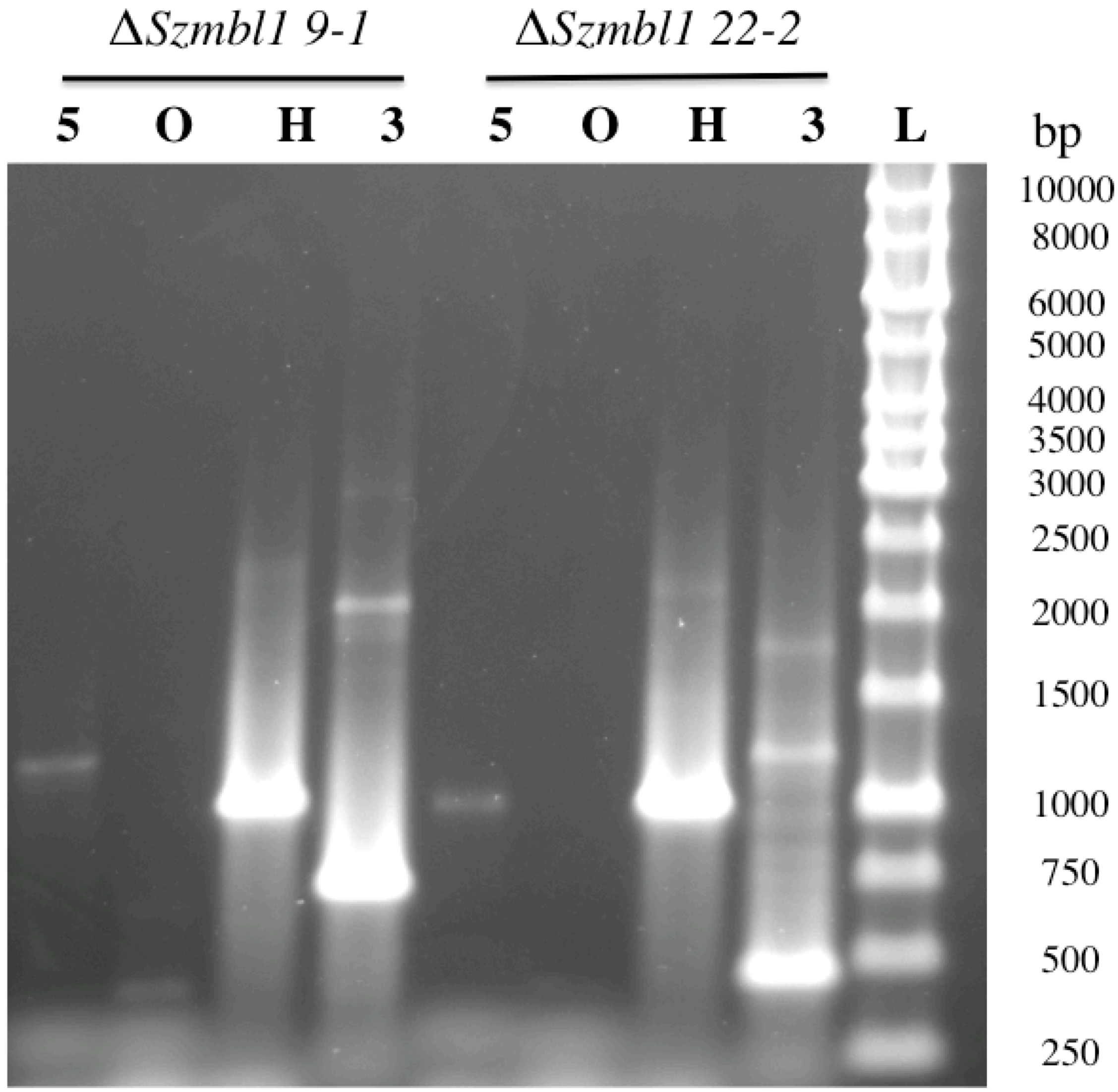

Figure 2.4. Anti-Southern confirmation of two Δ*Szmbl1* deletion mutants. Amplicons in lane 5 of each mutant depict that the 5′ end of the HygR cassette was inserted in the correct location within the *S. zeae* genome and were generated with *SzMBL1* 5′-out & 1/4 primers. PCR products in lane O of each mutant were generated with primers located within the open reading frame of the *SzMBL1* gene (*SzMBL1* ORF Fwd and *SzMBL1* ORF Rev). Lane H of each mutant were generated with primers located within the open reading frame of the HygR cassette (1/46 & 1/47). PCR products in lane 3 of each mutant depict that the 3′ end of the HygR cassette was inserted in the correct location within the *S. zeae* genome and were generated with primers 1/3 & *SzMBL1* 3′-out.

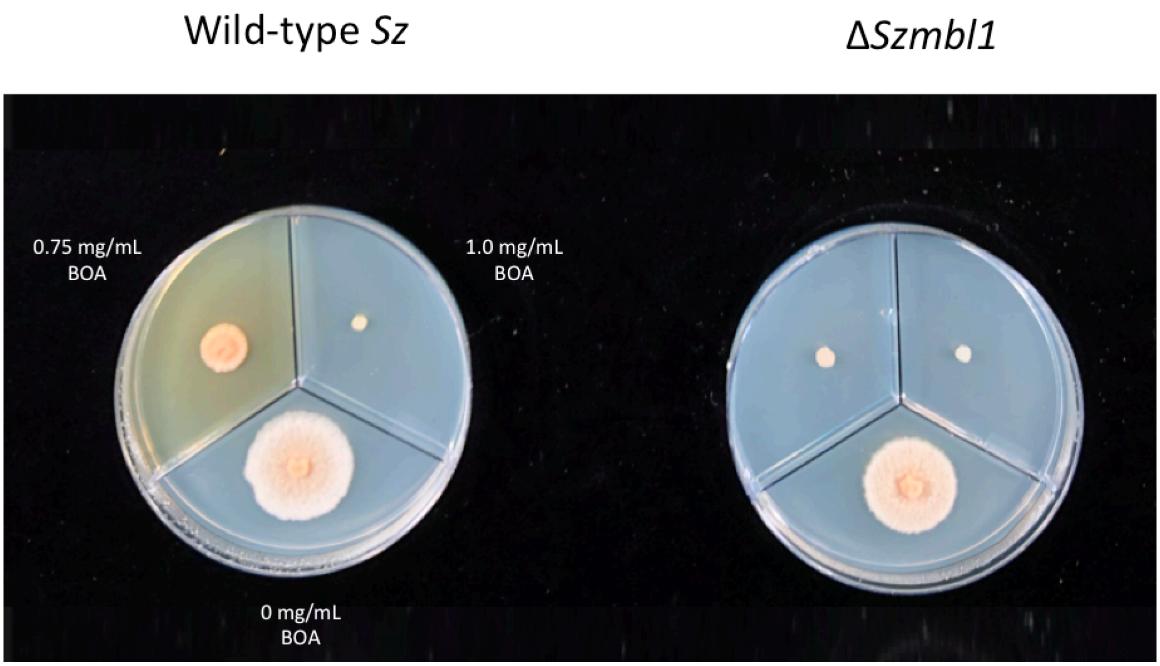

Figure 2.5. Comparison of growth between wild-type *Sarocladium zeae* strain NRRL 6415 and a Δ*Szmbl1* mutant. BOA medium amended with two different concentrations of BOA; 1.0 mg/mL, 0.75 mg/mL, and a control.

CHAPTER 3

SAROCLADIUM ZEAE, A POTENTIAL BIOLOGICAL CONTROL AGENT FOR *FUSARIUM VERTICILLIOIDES* AND ASSOCIATION BETWEEN CO-INFECTION AND KERNEL SYMPTOM DEVELOPMENT

INTRODUCTION

Maize is a major staple food and source of feed and fuel worldwide. The United States produces more maize than any other country in the world, where there are more acres of maize planted than any other major crop (Capehart & Proper, 2019; USDA-NASS, 2019). In 2017 the United States produced more than 14.5 billion bushels of maize on over 83 million acres, with a value of over $48 billion (USDA-NASS, 2019; USDA-NASS 2017). However, plant disease poses a threat to maize production every year. It has been previously estimated that loss of maize yield caused by plant pathogens in the United States can range from 2% to 15% (Munkvold & White, 2016). Within this range, plant disease in maize can cause the loss of billions of dollars annually in the United States.

The most frequently reported plant pathogen infecting maize is *Fusarium verticillioides*, which can cause ear rot, seedling blight, and stalk rot (Munkvold, et al., 1997). In the northern United States stalk rot caused by *F. verticillioides* was ranked fifth or sixth in greatest losses caused by plant disease in maize, and in the southern United States *Fusarium* stalk rot was ranked first or second for maize disease losses between 2012 and 2015 (Mueller et al., 2016).

Another threat that *F. verticillioides* poses to the maize industry is its production of the fumonisin mycotoxins. Fumonisins are a concern for both human and animal health. In humans, consumption of fumonisin is associated with esophageal cancer, stunting of growth, and birth defects (Riley et al & Merrill., 2019). Additionally, fumonisins cause a variety of animal toxicoses such as pulmonary edema in swine (Colvin & Harrison, 1992), leukoencephalomalacia in horses (Marasas et al., 1988), and liver toxicity in rats (Kriek, Kellerman, & Marasas, 1981). Because of theses health threats, industrial countries such as the United States can suffer steep economic losses due to rejection of food and feed that exceed maximum tolerated levels of this mycotoxin (Wu, 2006).

The health threats and economic losses caused by *F. verticillioides* bring importance to the ability to control the pathogen. Chemical control of *Fusarium* diseases has generally been described as unsuccessful and control has relied more on other methods such as mitigating insect damage through planting transgenic varieties, cultivars with tighter and more complete husks, or through planting at earlier dates (Blacutt et al., 2018). Other control methods for *F. verticillioides* have involved the use of biological controls (Alberts et al., 2016).

For a microorganism to provide effective biocontrol in maize it must posses the ability to colonize the same area of the plant infected by the pathogenic organism, and organisms already adapted to vegetative parts of the plant may hold an advantage over others (Alberts et al., 2016). *Sarocladium zeae* is a fungal endophyte of maize that fits these criteria, often being found together in asymptomatic maize kernels with *F. verticillioides* (King, 1981).

S. zeae is one of the most common fungi found colonizing preharvest maize and shows potential as a biological control agent for *F. verticillioides* because of its production of the secondary metabolites, pyrrocidine A & B, shown to be antagonistic towards *F. verticillioides*

(Wicklow & Poling, 2009; Wicklow, et al., 2005). Our recent findings have also shown that pyrrocidines are able to suppress fumonisin production in *F. verticillioides* at levels well below those required for visible growth inhibition (2.5 μg/ml vs >20 μg/ml pyroccidine B; Gold et al., 2019).

Histopathological studies have shown that when both *S. zeae* and *F. verticillioides* are together within maize kernels, *S. zeae* inhabits the larger embryo and endosperm region while *F. verticillioides* is typically confined to the pedicel and abscission layers (Sumner, 1968). These observations lead to the idea that *S. zeae* may be effectively suppressing *F. verticillioides* from colonizing the entire kernel and is keeping *F. verticillioides* subdued to a much smaller area of the kernel. In particular, it would be interesting to know how *S. zeae* may be effectively reducing symptoms caused by *F. verticillioides*.

One symptom produced by *F. verticillioides* that is frequently found in *F. verticillioides* infected kernels is the starburst symptom that consists of a streaking pattern on the pericarp that radiates out from the silk scar. These streaks appear white because of a loss in transparency of the clear outer coat of the pericarp cells due to their disintegration by the fungus (Duncan & Howard, 2010). While kernel rot symptoms are often associated with mechanical damage to the ear, Duncan and Howard showed evidence that *F. verticillioides* may enter non-damaged ears by traveling through the silk channel, perhaps aided by capillarity, and enter the kernel via the stylar canal (Duncan & Howard, 2010). With the hypothesis that *S. zeae* may be suppressing *F. verticillioides* from colonizing the entire kernel, then perhaps *S. zeae* could inhibit the formation of the starburst symptom if already present in the kernel prior to infection by *F. verticillioides*. In this study we conducted a field survey in Georgia to investigate the presence or absence of *S. zeae* and *F. verticillioides* in asymptomatic kernels and kernels expressing the starburst symptom. In addition, we tested the ability of *S. zeae* as a biological control agent for *F. verticilliodes* through growth chamber co-inoculation trials

MATERIALS AND METHODS

Fungal strains and growth conditions

Strains of *S. zeae* and *F. verticillioides* used in this study were grown from frozen stocks of conidia maintained for long-term storage at -80°C in 15% glycerol. The red fluorescent protein strain of *S. zeae* used in the growth chamber experiment, that contains resistance to hygromycin B, was generated by delivering the plasmid pCK1287 (a gift from Dr. Chang-Hyun Khang, University of Georgia; Jones, et al., 2016) to wild-type *S. zeae* strain NRRL 6415 (*Sz*-RFP). pCK1287 contains hH1-tdTomato, the *Magnaporthe oryzae* histone H1 fused at the carboxy terminus to the tdTomato version of the DsRed fluorescent protein. *Fv*-GFP, a hygromycin B-resistant, green fluorescent protein-expressing strain of *F. verticillioides* earlier generated by delivering the plasmid pCT74 to wild-type *F. verticillioides* FRC M3125 was used in the growth chamber experiments. These fungi were grown routinely using potato dextrose agar and potato dextrose broth (Neogen Food Safety, Lansing, MI, USA) at 27°C in the dark.

Plant growth room experiments

Growth room inoculation experiments were conducted on the dwarf popcorn maize variety, Tom Thumb (High Mowing Organic Seeds, Wolcott, VT, USA) because it fit well in our growth room due to its short height. The experiment consisted of six 6-gallon pots, 43.81 cm in diameter, and filled with moist Fafard 3 potting mix soil (Agawam, MA, USA). Prior to planting, seeds were treated to eliminate pre-existent endophytes and surface microbes through a surface and internal sterilization process (Glenn et al., 2008). After this, the soil of each pot was soaked with water and 10 seeds were planted per pot at a planting depth of 2.5 cm.

The growth chamber was set to 16 hours of light and 8 hours of darkness. The temperature plan was 20°C at midnight, 23°C at 6:00am, 26°C at 7:00 am, 30°C at 8:00 am, 26°C at 8:00 pm, then 23°C at 9:00 pm. Over hanging lights were lowered until they were 30.5 cm above the top of the plant and were adjusted to maintain this distance throughout their growth. All bulbs in light fixtures were monitored throughout the experiment and replaced as needed so that all bulbs were operational at all times. The positions of the pots were rotated in a consistent manner once a week throughout the experiment to avoid height differences in plants later in growth. Plants were watered as needed with distilled water. Once plants reached a height of around 61 cm they were fertilized once every 2 weeks with 22.18 mL of Miracle-Gro® Water Soluble All Purpose Plant Food (ScottsMiracle-Gro, Marysville, OH, USA) and 22.18 tablespoons of epsom salt mixed in 5.68 L of distilled water. To ensure a higher rate of pollination, once both tassels and silks formed on the plants, one branch of each tassel from every plant was plucked and dusted across the corresponding plant's silk. Additionally, when tassels reached the point of shedding pollen, plants were shaken once a day to release more pollen.

Plant inoculations in growth chamber experiment

Plants were inoculated 20 days after 70% of plants in the growth chamber had begun silking. There were two different inoculation types and a control in this experiment. Two pots in each trial were assigned one of these inoculation types with every plant within these pots receiving the same inoculation type. The first inoculation type was 2 mL of sterile distilled water containing a *Fv*-GFP conidial suspension adjusted to 10^4 conidia/mL (*Fv* only). The second inoculation type was 1 mL of a *Fv*-GFP conidial suspension adjusted to $2X10^4$ conidia/mL and 1 mL of a *Sz*-RFP conidial suspension adjusted to $2X10^5$ conidia/mL (*Fv & Sz*). Uninoculated ears served as controls. Conidia were made fresh by inoculating PDB flasks with *Sz*-RFP and *Fv*-GFP strains that were stored at -80°C in 15% glycerol. Flasks were incubated for 5 days at 27°C in the dark before conidia were harvested. Suspensions of each inoculum type were delivered to ears via a hypodermic needle and syringe through the husk near the center of the ear. The needle was used to wound one developing kernel by pressing the needle through one of the kernels and then was pulled back slightly to inject inoculum into the area between the husk and the developing kernels. Figure 3.1 is a depiction of the experimental design.

Ninety days after planting, ears were harvested. Each ear was stripped of its husk. Once the husk was removed, the number of starburst kernels, kernels with ear rot, and total number of kernels were recorded for each ear. Kernels were counted as starburst kernels if they clearly showed white streaking that is typical of the symptom. Ear rot kernels were characterized as any kernel that made contact with *F. verticillioides* hyphal growth that colonized the areas between kernels, or on their surface. The total numbers of kernels on each cob was estimated as the product of kernels in one seed column by kernels in one central row around the circumference of the ear. All kernels were then stripped off each ear and placed into individual pre-labeled sterile 50 mL conical tubes.

Fumonisin content of inoculated ears

To prepare the collected kernels for fumonisin extraction, kernels from each ear were ground into a fine material using a Kleco 8200 8 canister ball mill pulverizer (Visalia, CA, USA). All kernels from one ear were placed together at once into a metal canister with a large metal ball bearing. Material was pulverized to a fine powder for 30 s (repeated once if material not fully a fine powder). Pulverized kernel tissue was returned to original 50mL tubes. Metal canisters and large metal ball bearings were washed with a Liquinox and water solution, then rinsed with distilled water, then sprayed with 100% methanol (Fisher Scientific, Hampton, NH, USA) and were left to dry between each sample to avoid contamination between samples.

We measured the amount of fumonisin detected in each sample using liquid chromatography mass spectrometry as previously described (Williams et al., 2007). For each 50 mL tube of ground kernels, we homogenized the material by rapid shaking and 1g of this material was collected from each sample. The 1 g of ground material was then placed into a Falcon® 15 mL polystyrene conical tube (Corning, Tewksbury, MA, USA) with 10 mL of 1:1 acetonitrile:ddH_2O + 5% formic acid. The tube was then capped and vigoursly shaken. A 1 mL aliquot was taken from this sample and was diluted 1,000-fold with 3:7 acetonitrile:ddH_2O + 0.1% formic acid, then subjected to mass spectrometry analysis. Parameters were optimized using a fumonisin standard (Sigma-Aldrich, St. Louis, MO, USA) and was eluted with a linear solvent gradient increasing from 30% acetonitrile to 100% acetonitrile (+0.1% formic acid) over 9 minutes.The column providing separations was an Imtakt Cadenza CW –C18, 150 mm x 2 mm, 3μm. Peaks were monitored by summing the total ion current from the MS2 of the molecular ions.

Collection of ears used in field survey

For this objective, samples were collected from 10 unirrigated fields of field corn in 10 different counties of Georgia. These counties included: Whitfield, Ben Hill, Turner, Jefferson, Worth, Colquit, Ware, Jeff Davis, Washington, and Burke. At each site, more than 20 ears were collected in 2018 near harvest in an "X" shaped pattern across the field.

Kernel collection method from selected ears

Dried mature ears were collected in the field and stored at 4°C. Ten ears were selected from each site that expressed both asymptomatic and symptomatic starburst kernels. Starburst kernels were those that showed white streaking across their surface in a starburst pattern. On each of the selected ears, 5 asymptomatic kernels and 5 symptomatic starburst kernels were collected and placed in separate, sterile, Falcon® 14 mL Round Bottom Polystyrene Test Tubes, with Snap Cap (Corning, Tewksbury, MA, USA).

DNA extraction of selected field survey kernels

Three of the five kernels from both asymptomatic and symptomatic kernels collected from each of the ears were used for DNA extraction, leaving two extra asymptomatic and symptomatic kernels from each sample to be used later if needed. Each of these kernels was ground individually using a mortar and pestle. Each kernel ground in this manner was wrapped such that material was isolated in its own individual glassine weighing paper to avoid contamination from sample to sample. Each of these ground kernels was then transferred to a lysing matrix A tube and DNA was extracted using a FastDNA™ Spin Kit at 6 m/s with 3 pulses of 30 s and a 1 min intervening pause at room temperature between each pulse (MP Biomedicals, LLC, Santa Ana, CA, USA) following the manufacturer's protocol. DNA extracted using this method was then stored at -20°C until use.

Screening kernel DNA for the presence of *S. zeae* and *F. verticillioides*

To determine the presence or absence of *S. zeae* and *F. verticillioides* DNA, samples were screened by gene-specific PCR. Each DNA sample collected was assayed using three separate primer sets. The first primer set amplified a region within the *FUM1* gene that is specific to *F. verticillioides* (FUM1 Fwd & FUM1 Rev; 278 bp product), the second primer set amplified a region within the β-tubulin gene that was specific to *S. zeae* (Sz β-tubulin Fwd & Sz β-tubulin Rev; 438 bp product), and the third primer set was used as a positive control amplifying a maize R gene specific to maize (maize Fwd and maize Rev; around a 600 bp product). Sequence for all primers used in this experiment can be viewed in Table 3.1. PCR was performed using One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer (OneTaq; New England Biolabs, Ipswich, Massachusetts, USA). Cycling parameters for these PCR reactions were 94°C for 30 s; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min; and final extension of 68°C for 5 min.

RESULTS

Sarocladium zeae suppresses fumonisin levels in corn ears

Our growth chamber experiment consisted of injecting maize ears with two different inoculum types and then measuring the amount of fumonisin B1 (FB1) recovered from samples of each. The two inoculation types were: "Fv only" where ears were inoculated with only *Fv*-GFP conidia, "Fv & Sz" where ears were inoculated with both *Fv*-GFP and *Sz*-RFP conidia.

“control” where ears were un-inoculated. Visual analysis of ears from both the *Fv* only and *Fv* & *Sz* groups showed the *F. verticillioides* symptoms of ear rot and starburst kernels. However, there was much more ear rot seen in ears from the *Fv* only group as compared to the *Fv* & *Sz* group. No *F. verticillioides* symptoms were seen on ears collected from the control group. Data from the fumonisin extraction of each group showed that the mean value of FB1 extracted from the *Fv* & *Sz* group was 62% less than the mean value of FB1 collected from the *Fv* only group (Figure 3.2). FB1 was recovered from ears in the Control group, but only at trace amounts (<μg/g). The data collected from this experiment showed that the presence of *S. zeae,* when co-inoculated with *F. verticillioides,* significantly reduced the mean levels of FB1 recovered from samples ($p = 0.0081$).

Georgia field survey suggests that *S. zeae* and *F. verticillioides* are frequently both present in both symptomatic starburst kernels and in asymptomatic kernels.

Field survey ears were analyzed from three counties in Georgia. One each located in the northern, middle and southern regions of the state. DNA was extracted from three asymptomatic kernels and three symptomatic starburst kernels off of each ear previously selected. Three primer sets were designed that amplify DNA in regions that were specific to *F. verticillioides*, *S. zeae,* and maize as a control. A description on how PCR products were run using gel electrophoresis can be seen in Figure 3.3. Data collected from PCR product initially indicated that both *F. verticillioides* and *S. zeae* were present in both asymptomatic and symptomatic kernels collected from each of the three counties (Figure 3.4). However, after all PCR products from this experiment were screened, a PCR reaction was run with water as the template for each primer set to act as a negative control test. The resulting reaction produced PCR product suggesting that contamination may have been present in the water used for the previous PCR reactions (Figure

3.5). This result leaves questions to whether the results from the initial PCR reaction of kernel DNA were actually false positives due to contamination. Because of this, kernel DNA collected from this experiment will be run once more at a later date after contamination issues have been resolved.

DISCUSSION

F. verticillioides is a plant pathogen of maize that poses a threat to maize production as well as to human and animal health because of its production of the fumonisin mycotoxins. These threats make developing effective control strategies for *F. verticillioides* important. One potential control method for *F. verticillioides* is through the use of biological controls. *S. zeae* has been shown to be a potential biological control agent for *F. verticillioides* because of its production of the secondary metabolites, pyrrocidine A & B, shown to be antagonistic to fungal growth (Wicklow, et al., 2005). In this study we looked for evidence of *S. zeae's* potential to control *F. verticillioides* through growth room co-inoculation experiments and by conducting a field survey to build a greater understanding of the relationship between the starburst kernel symptom produced by *F. verticillioides* and the presence of *S. zeae* within the kernel as well.

To assess the potential of *S. zeae* as a biological control agent for *F. verticillioides,* our first objective was conducting a growth room co-inoculation experiment. In this study maize ears that were injected with *F. verticillioides* inoculum were compared to ears injected with both *F. verticillioides* and *S. zeae* to look at the ability of *S. zeae* to reduce the amount of FB1. The data shown are from a single experiment. Two other trials were attempted, but due to a growth chamber malfunction in the second trial and due to the December 2018 U. S government shut down, a third trial was also lost. Because of this, repeating this experiment will be necessary to build more confidence in the ability of *S. zeae* to reduce FB1. The results obtained from this trial showed that the presence of *S. zeae* did have a significant effect on reducing FB1 levels. Mean quantities of FB1 extracted from the *Fv* & *Sz* group had 62% less fumonisin as compared to the mean quantity collected from the *Fv* only group.

While the results indicate *S. zeae* reduced FB1, the mechanism of this reduction is yet to be fully understood. Before grinding and homogenizing ears in preparation of FB1 analysis, ears were visually assessed for the amount of ear rot present as well as the amount of starburst symptomatic kernels. When assessing the amount of FB1 extracted from ears, there appeared to be a strong correlation with the quantity of FB1 extracted from an ear and the amount of ear rot that was present on that sample before grinding and homogenizing the sample. Ears that had high levels of ear rot produced much higher levels of FB1. The starburst symptom did not appear to have a large effect on FB1 levels that were later collected. This observation matches those made in previous studies showing that there was almost seven times the amount of total fumonisins recovered from kernels expressing ear rot compared to those that were showing the starburst symptom (Lanza et al., 2017). Overall, there was far less ear rot observed on ears of the *Fv* & *Sz* group as compared to the *Fv* only group.

The antagonistic nature of *S. zeae* towards *F. verticillioides* is attributed to its production of the secondary metabolites, pyrrocidine A & B (Wicklow, et al., 2005). Additionally, our recent findings showed that pyrrocidines are able to suppress fumonisin production in *F. verticillioides* (Gold et al., 2019). Based on the results of this study, a key question arises, what is the precise mechanism of this fumonisin reduction caused by *S. zeae* inoculation? Is *S. zeae* limiting FB1 levels because of its ability to reduce *F. verticillioides* ear rot symptoms, is its production of pyrrocidines inhibiting *F. verticillioides's* ability to produce FB1, or is it a combination of both? To get a better understanding of this mechanism, future experiments will look for the levels of pyrrocidine recovered from samples compared to FB1 levels to investigate whether there is a negative correlation between the two. Additionally, more trials of this experiment need to be conducted to see if the results remain consistent.

While our first objective looked at the ability of *S. zeae* as a potential biocontrol for *F. verticillioides* within the controlled settings of a growth room, our next objective was focused on studying the ability of *S. zeae* to reduce symptoms of *F. verticillioides* in a field setting. To do this, we conducted a field survey within Georgia to look for the presence or absence of *S. zeae* in kernels that posses or lack the *F. verticillioides* starburst symptom. Previous studies have shown that *S. zeae* and *F. verticillioides* are often found together within maize kernels with *S. zeae* inhabiting the larger embryo and endosperm region of the kernel and *F. verticillioides* colonizing the pedicel and abscission layers (Sumner, 1968). With this observation in mind, we were interested in studying if the presence of *S. zeae* within the kernel was inhibiting the colonization of the kernel by *F. verticillioides*. Because of the observations made by Sumner, we hypothesized that starburst kernels would not contain *S. zeae* with the thought that if *S. zeae* was present it would inhibit *F. verticillioides* from colonizing the kernel to the point of producing the symptom. Additionally we hypothesized that asymptomatic kernels would either contain both *S. zeae* and *F. verticillioides*, *S. zeae* only, or contain neither of the endophytes. The results of the study initially indicated that *S. zeae* and *F. verticillioides* were present in all kernels collected from all three counties in this study. However, later in the experiment a negative control PCR that used water as the template showed PCR products. This leads to the possibility that the results seen previously were false positives caused by contamination. To complete this study, PCR must be repeated once the issue of contamination is resolved. To achieve this new primers of every set will be ordered and only sterile nuclease free water will be used. A subset of kernel DNA from this study will be used to determine if contaminants are still present.

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Table 3.1. List of primers used in this study.

Primer name	Description	Primer sequence
FUM1 Fwd	Forward primer used to amplify a region within the FUM1 gene that is specific to *F. verticillioides;* 278 bp product	TGCTGCCCTGTATCACAACCA
FUM1 Rev	Reverse primer used to amplify a region within the FUM1 gene that is specific to *F. verticillioides;* 278 bp product	AATGTGCGCTTGATCCAGTT
Sz β-tubulin Fwd	Forward primer used to amplify a region within the β-tubulin gene that is specific to *S. zeae;* 438 bp product	CACGCTACATGCTCTCAGAA
Sz β-tubulin Rev	Reverse primer used to amplify a region within the β-tubulin gene that is specific to *S. zeae;* 438 bp product	GAGCATCTCCGAAGGTATACAA
maize Fwd	Forward primer used to amplify a region within a maize R gene specific to maize; around a 600 bp product	AAGCTTCAGCTTACCTCAGT
maize Rev	Reverse primer used to amplify a region within a maize R gene specific to maize; around a 600 bp product	CCAATCCAACAATGGCCAAAC

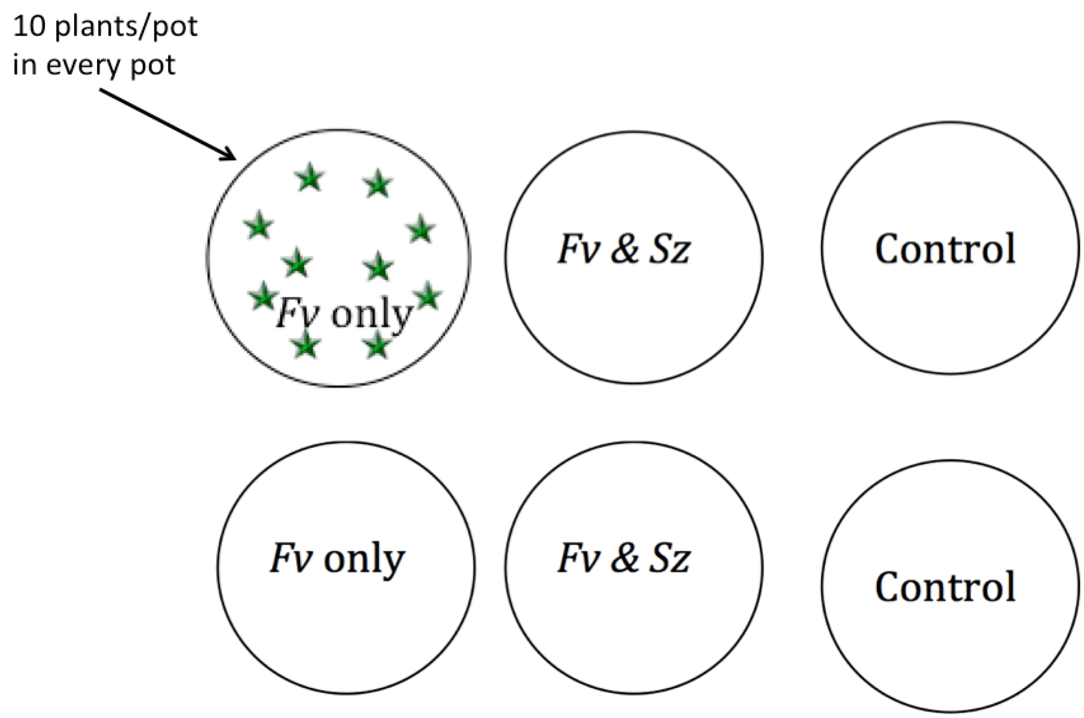


Figure 3.1: Diagram representing an aerial view of growth chamber experiment. Circles seen above represent pots containing maize. Every ear produced within a pot received one inoculation type. *Fv* only: plants were injected with 2 mL of *Fv*-GFP conidia at concentration of 10^4 conidia/mL. *Fv* & *Sz*: plants were injected with 1 mL of *Fv*-GFP conidia adjusted to a concentration of $2.0X10^4$ conidia/mL and with 1 mL of *Sz*-RFP conidia adjusted to a concentration of $2.0X10^5$ conidia/mL. Control: un-inoculated plants.

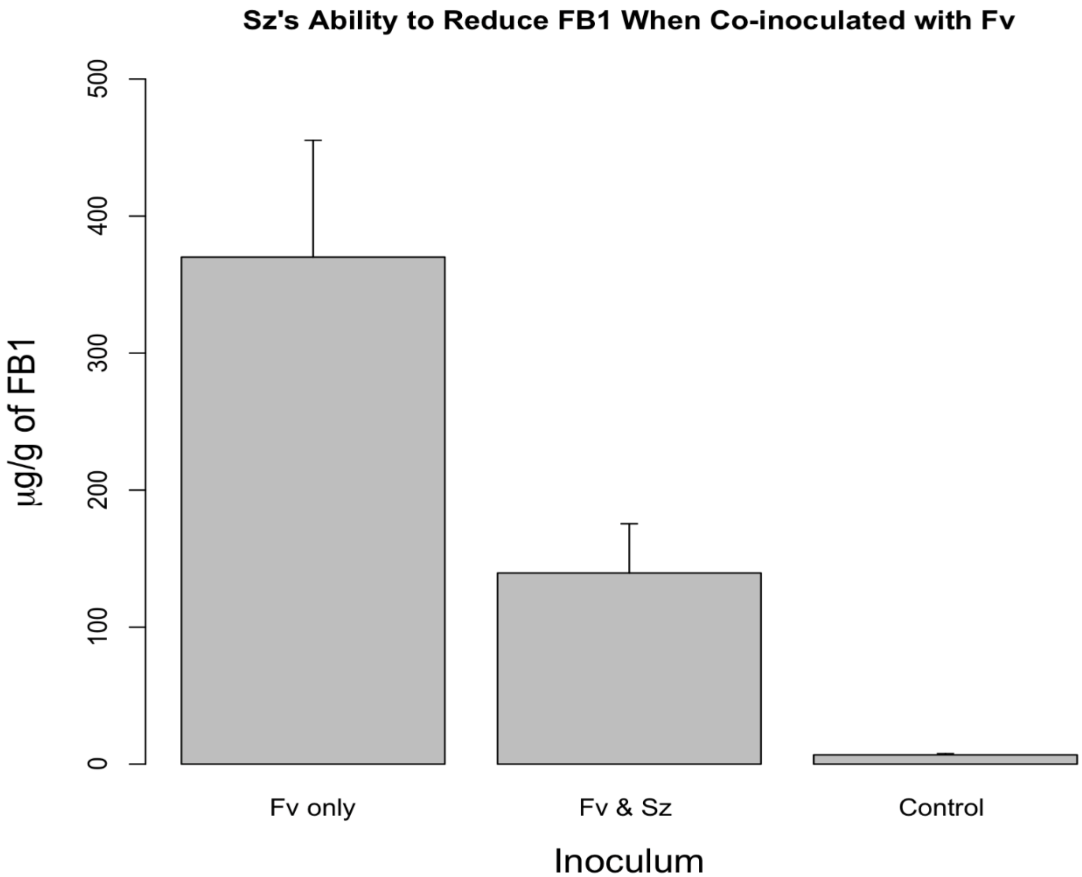


Figure 3.2. Results of one trial in our growth room inoculation experiments. FB1= fumonisin B1. The "Fv only" group represents the mean value in μg/g of fumonisin B1 collected from ears that were inoculated with only *Fv*-GFP conidia. The Fv & Sz group represents the mean value in μg/g of fumonisin B1 collected from ears that were inoculated with both *Fv*-GFP and Sz-RFP conidia. The "Control" group represents the mean value in μg/g of fumonisin B1 collected from un-inoculated ears. Significant difference between the Fv only group and the Fv & Sz group was observed ($p = 0.0081$). Error bars represent standard error.

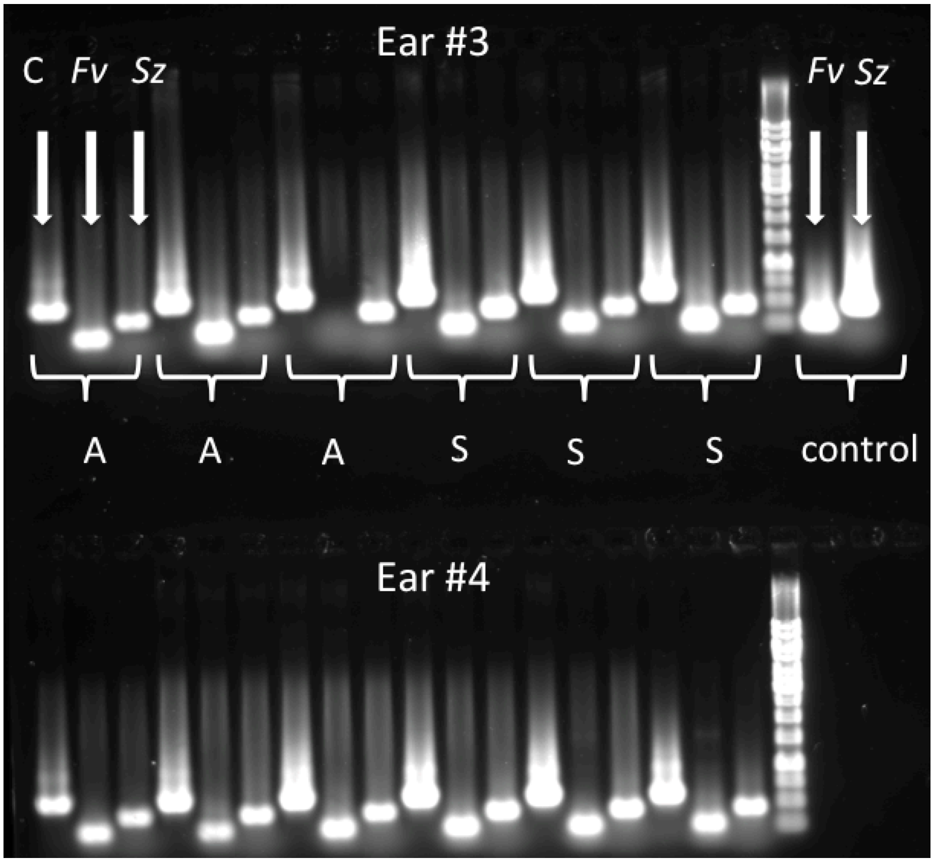
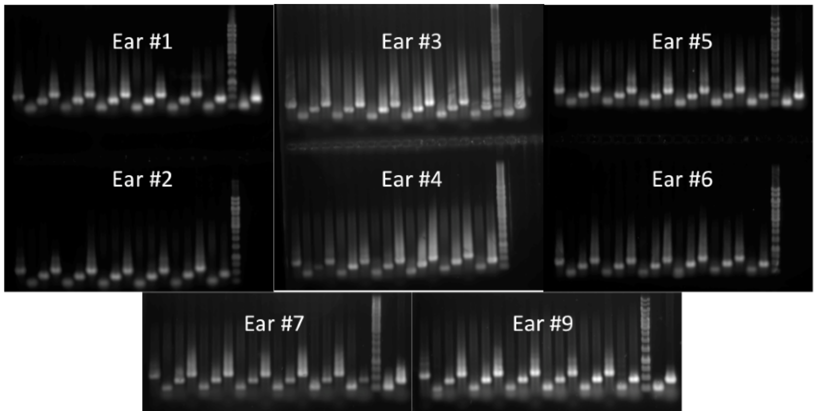
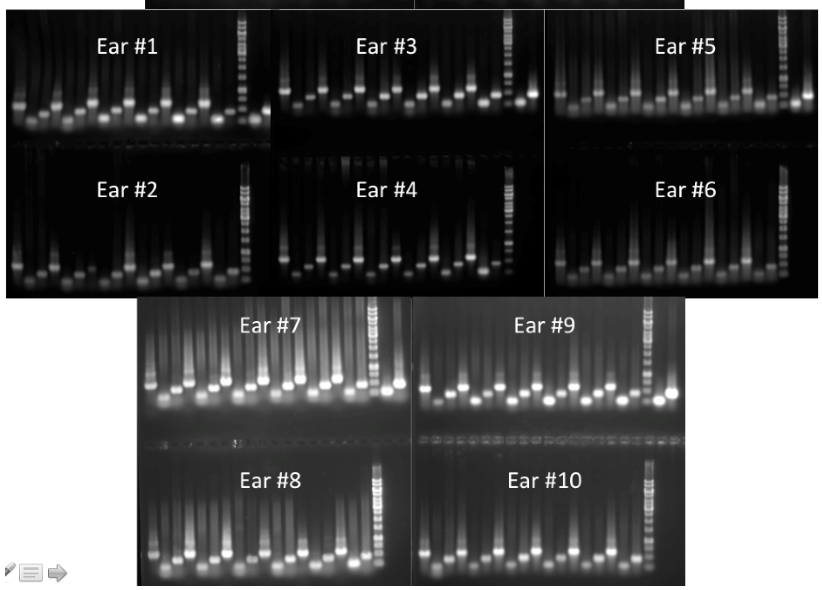


Figure 3.3. Kernel colonization assay strategy. Each row represents samples collected from one of the ten ears selected from each county. DNA from each kernel was used in three PCR reactions each having a different primer set. C= product generated with maize specific primers. *Fv*= product generated with *F. verticillioides* specific primers. *Sz*= product generated with *S. zeae* specific primers. A= DNA template from asymptomatic kernels. S= DNA template from starburst symptomatic kernels. The last two lanes in the first row following the GeneRuler1 kb ladder (FisherSci) represent positive controls where purified *F. verticillioides* DNA was template in the *Fv* lane and purified *S. zeae* DNA template in the *Sz* lane.

A)


Ear #1
Ear #3
Ear #5
Ear #2
Ear #4
Ear #6
Ear #7
Ear #9

B)


Ear #1
Ear #3
Ear #5
Ear #2
Ear #4
Ear #6
Ear #7
Ear #9
Ear #8
Ear #10

C)

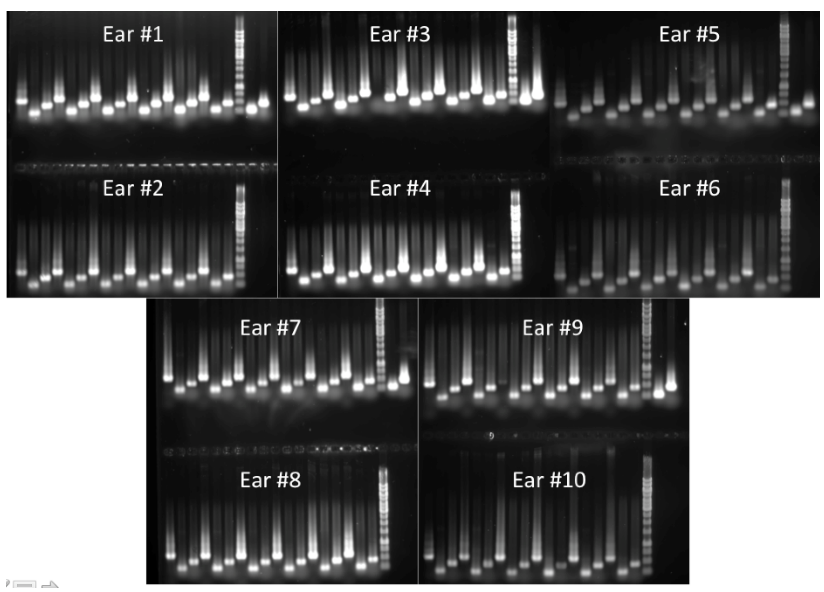

Ear #1
Ear #3
Ear #5
Ear #2
Ear #4
Ear #6
Ear #7
Ear #9
Ear #8
Ear #10

Figure 3.4. All PCR products of samples collected from each of the three counties. (A) Samples collected from Whitfield County located in the Northern region of Georgia. (B) Samples collected from Burke County located in the middle region of Georgia. (C) Samples collected from Ben Hill County located in the southern region of Georgia. Directions on interpreting each gel can be viewed in Figure 3.3.

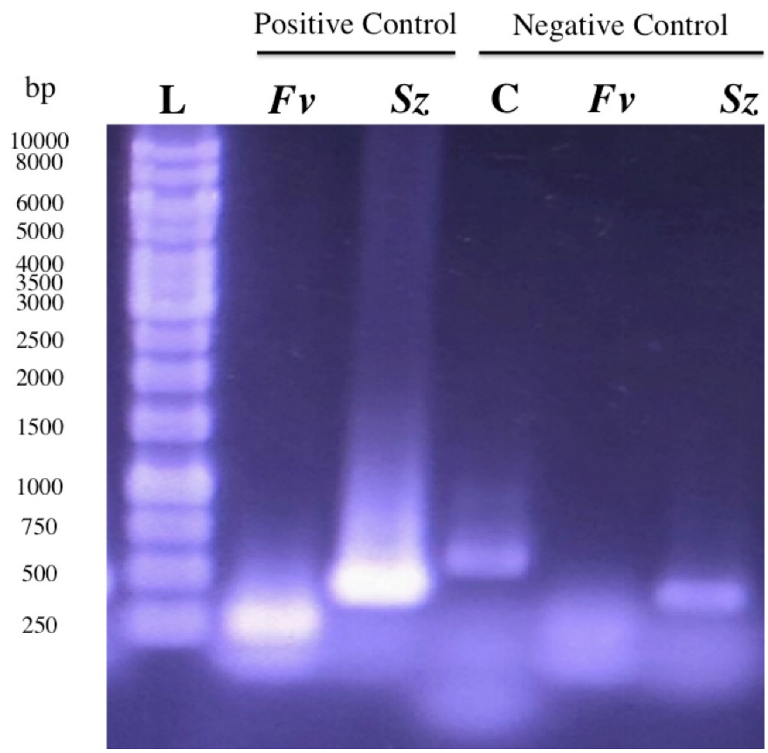


Figure 3.5. PCR product obtained after sample DNA from each county was screened. L= 10 kb ladder. The proceeding two lanes represent product from a positive control test. Primers specific to *F. verticillioides* and pure *F. verticillioides* DNA was used for the template in the *Fv* lane. Primers specific to *S. zeae* and pure *S. zeae* DNA was used for the template in the *Sz* lane. The final three lanes represent product from a negative control test. C= product generated with maize

specific primers with water used for the template. *Fv* = product generated with *F. verticillioides* specific primers with water used for the template. *Sz* = product generated with *S. zeae* specific primers with water used for the template. Water used in the negative control test was the same water used in the PCR reactions from the field survey. The results of the negative control test suggest the presence of a possible contaminant that could have resulted in false positives within the field survey experiment.

CHAPTER 4

IDENTIFICATION OF THE BIOSYNTHETIC GENE CLUSTER RESPONSIBLE FOR PRODUCING PYRROCIDINES IN SAROCLADIUM ZEAE

INTRODUCTION

Fusarium verticillioides is a ubiquitous mycotoxigenic fungus that can exist as both a phytopathogen, or as an asymptomatic endophyte of maize (Bacon & Hinton, 1996). *F. verticillioides* is the most frequently reported plant pathogen infecting maize, and can cause seedling blight, stalk rot, and ear rot (Munkvold, et al. 1997). Additionally, *F. verticillioides* produces the fumonisin mycotoxins, which pose a threat to both human and animal health. In animals, fumonisins can cause diseases such as leukoencephalomalacia in horses (Marasas et al., 1988), pulmonary edema in swine (Colvin & Harrison, 1992), and liver toxicity in rats, sheep and primates (Kriek, Kellerman, & Marasas, 1981). In humans, consumption of fumonisin is associated with esophageal cancer, stunting of growth, and birth defects (Riley & Merrill., 2019).

In addition to *F. verticillioides,* S*arocladium zeae* is one of the most common fungi found colonizing maize (Wicklow et al., 2005). *S. zeae* has been suggested as a potential biological control agent for *F. verticillioides* because of its production of the secondary metabolites, pyrrocidine A & B, shown to be antagonistic towards *F. verticillioides* (Wicklow & Poling, 2009; Wicklow et al., 2005). Additionally, our recent findings have shown that pyrrocidines are able to suppress fumonisin production in *F. verticillioides* (Gold et al., 2019).

Even with the ability of pyrrocidines to suppress fumonisins, a mycotoxin that is associated with major health concerns in humans and animals, the biosynthetic gene cluster that generates the pyrrocidines has yet to be identified within *S. zeae*. Previous experiments have been conducted where a synthetic L-tyrosine probe labeled with oxygen 18 was introduced to a culture of *S. zeae,* and when pyrrocidine was extracted from that same culture it contained the same L-tyrosine probe that was originally introduced to the fungus (Ear et al., 2012). In many cases fungi use iterative hybrid polyketide synthases–non ribosomal peptide synthetases (PKS–NRPS) for the production of secondary metabolites (Fisch, 2013). The PKS modules located in PKS–NRPSs help produce polyketide chains with different reduction and methylation patterns, while the NRPS module fuses an amino acid to the polyketide chain (Fisch, 2013). The experimental results of Ear et al. (2012) show that *S. zeae* was able to incorporate the labeled L-tyrosine into the polyketide chain of pyrrocidine (Ear et al., 2012). This result suggests that a PKS–NRPS may be involved in pyrrocidine biosynthesis. Using antiSMASH software, we identified three PKS-NRPSs that were each present in three different pyrrocidine producing strains of *S. zeae*. In work reported here, we use a CRISPR-cas9 gene editing technique to attempt generating single gene deletion mutants of each of these three PKS-NRPSs. After deleting one of these PKS-NRPSs we found that the newly created mutant strain of *S. zeae* was no longer able to produce pyrrocidines A or B. We have since denoted this gene as *SzPYC1*.

MATERIALS AND METHODS

Fungal strains and growth conditions

Strains of *S. zeae* used in this study were grown from frozen stocks of conidia maintained for long-term storage at -80°C in 15% glycerol. Wild-type strain NRRL 6415 was used for generating Δ*Szpyc1* deletion strains. The fungus was grown routinely using potato dextrose agar (PDA; Neogen Food Safety, Lansing, MI, USA) and potato dextrose broth (PDB; Neogen Food Safety, Lansing, MI, USA.) at 27°C in the dark.

SzPYC1 CRISPR-cas9 gene deletion

Δ*Szpyc1* deletion strain was generated by the "In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates" method (Abdallah et al., 2017). Primers used in this study are listed in Table 4.1 as well as sequence used for generating crRNA. Two Cas9 ribonucleoproteins (RNPs) were generated that were designed to create double-strand breaks (DSBs) directly upstream and downstream of the *SzPYC1* open reading frame (ORF). A HygR cassette repair template was generated with a single PCR reaction using TaKaRa high fidelity Taq (TaKaRa Bio, Kyoto, Japan) following the manufacture's protocol using primers *SzPYC1* hyg cassette Fwd and *SzPYC1* hyg cassette Rev (Table 4.1). The HygR cassette amplicon contained two 38 bp microhomology arms to direct the cassette to insert at the site of the DSBs. All of these components were delivered to the cell at one time using a PEG mediated transformation of protoplasts (Glenn & Bacon, 2009) generated from wild type *S. zeae* strain NRRL 6415. Transformants were selected on 1% Bacteriological Agar (Neogen Food Safety, Lansing, MI, USA) amended with hygromycin B (150 μg/mL; Roche Diagnostics, Indianapolis, IN, USA).

To determine if *SzPYC1* was successfully deleted from the *S. zeae* genome, transformants were screened by gene-specific PCR. PCR was performed using One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer (OneTaq; New England Biolabs, Ipswich, Massachusetts, USA) with primers *SzPYC1* ORF Fwd and *SzPYC1* ORF Rev (Table 4.1). Cycling parameters for the 992 bp amplicon were 94°C for 30 s; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min; and final extension of 68°C for 5 min. Single spore isolates were obtained from putative *Szpyc1* deletion transformants based on this PCR reaction. These candidates were then screened again using an "anti-Southern" confirmation method, which consisted of four PCR reactions for each transformant. The first of these reactions used a forward primer located just upstream of the RNP cut site that was itself located upstream of the 5′ end of the *SzPYC1* gene and a reverse primer located within the open reading frame (ORF) of the HygR cassette near the 5′ end (*SzPYC1* 5′-out & 1/4). The second reaction used primers located within the ORF of the *SzPYC1* gene to confirm its presence or absence in the genome (*SzPYC1* ORF Fwd and *SzPYC1* ORF Rev). The third reaction consisted of primers located within the ORF of the HygR cassette to confirm its presence within the genome (1/46 & 1/47). The final reaction used a forward primer located within the HygR ORF near the 3′ end and a reverse primer downstream of the RNP cut site that was located downstream of the 3′ end of the *SzPYC*1 gene (1/3 & *SzPYC1* 3′-out). Each of these PCR reactions was generated using OneTaq with the thermocycler parameters used previously in the first screening of transformants. All primers used in this anti-Southern reaction are listed in Table 4.1.

Pyrrocidine extraction and analysis

We measured the amount of pyrrocidine A & B detected in each sample using liquid chromatography mass spectrometry (LCMS). *Δpyc1* and wild-type *S. zeae* strain NRRL 6415 were initially grown on PDA at 27°C in the dark for 5 days. One 4 mm agar plug from each strain was taken from these plates and placed onto plates containing 2% Cream of Rice agar (2% COR agar). 2% COR agar consisted of 10g of Uncle Ben's Cream of Rice (Mars Inc., McLean, VA, USA) mixed with 500 mL of diH20 and autoclaved for 15 min at 121° C. After this 7.5 g of Bacteriological Agar (Neogen Food Safety, Lansing, MI, USA) was added, contents were mixed using a stir bar, and were autoclaved once more as they were before. Strains were grown on this medium for 14 days at 27°C in the dark. After this incubation period was completed, three 4 mm agar plugs were taken from near the center of colonies on each COR agar plate, were placed in glass vials, and extracted with 5 mL of ethyl acetate (Fisher Scientific, Hampton, NH, USA) on a rocker for 3 hr. A 1 mL aliquot from each of these vials was then transferred into glass auto injector vials and samples were dried down with nitrogen and then resuspended in 1 mL of 1:1 acetonitrile:ddH_2O containing 0.1% formic acid. Samples were then analyzed by LCMS beginning with 50% acetonitrile increasing on a linear gradient to 100% acetonitrile (+0.1 % formic acid) over 10 minutes. The column providing separations was an Imtakt Cadenza CW – C18, 150 mm x 2 mm, 3 μm. Peaks were monitored using the sum of the intensities of the molecular ions and the formate adducts.

RESULTS

LCMS results provide strong evidence that *SzPYC1* is required for pyrrocidine production

A *ΔSzpyc1* deletion strain of *S. zeae* was generated because it was one of three PKS-NRPSs found within three sequenced pyrrocidine producing strains. The predicted biosynthetic gene cluster that each of these PKS-NRPS are located in are depicted in Figure 4.1. Using LCMS, *ΔSzpyc1* and wild-type *S. zeae* strain NRRL 6415 were screened for their ability to produce pyrrocidine A & B. Both strains were grown under identical conditions before the pyrrocidine extraction method was performed. The results obtained using LCMS indicate that wild-type *S. zeae* strain NRRL 6415 produced both pyrrocidine A & B as expected. However, neither pyrrocidine A or B was detected in samples that were obtained from the *ΔSzpyc1* mutant strain (Figure 4.2). These results provide strong evidence that the NRPS-PKS hybrid gene, *SzPYC1*, is required for pyrrocidine production.

DISCUSSION

The results of our LCMS experiment indicated that the wild-type *S. zeae* strain NRRL 6415 produced both pyrrocidine A & B as expected, but the *ΔSzpyc1* mutant strain was unable to produce either pyrrocidine A or B. These results provide strong evidence that the biosynthetic gene cluster that *PYC1* is located in is required for the synthesis of both pyrrocidine A & B. In a previous experiment conducted in our growth room, maize ears were inoculated with *F. verticillioides*, while other ears were co-inoculated with both *F. verticillioides* and *S. zeae*. The results of this preliminary experiment indicated that co-inoculated ears contained 62% less fumonisin B1 (FB1) as compared to ears that were inoculated with *F. verticillioides* only. Additionally, it was observed in this experiment that co-inoculated ears contained much less ear rot, a symptom produced by *F. verticillioides* that is associated with relatively high levels of FB1 as compared to other symptoms found on the ear (Lanza et al., 2017). In addition, our lab recently showed that pyrrocidine A & B are able to inhibit *F. verticillioides* synthesis of the fumonisins (Gold., 2019). With this in mind, it was unclear if *S. zeae* was reducing the amount of FB1 in co-inoculated ears because of inhibition caused by pyrrocidines, or from other factors of *S. zeae* that reduce the amount of ear rot. the *ΔSzpyc1* mutant strain generated here provides a useful tool to increase our understanding of the mechanism that *S. zeae* uses to reduce the amount FB1. In future growth room experiments, the amount of FB1 recovered from ears co-inoculated with *Δpyc1* and *F. verticillioides* will be compared to ears co-inoculated with wild-type *S. zeae* strain NRRL 6415 and *F. verticillioides* to gain a better understanding of the role pyrrocidine plays in reducing FB1 within the ear. Furthermore, *ΔSzpyc1* has helped to identify the biosynthetic gene cluster that is responsible for producing pyrrocidine. The production of secondary metabolites on an industrial scale can often be achieved through fermentation of genetically engineered bacteria or fungi designed to be overproducers of a desired metabolite (Adrio & Demain, 2010). With the results of this experiment indicating the biosynthetic gene cluster responsible for the production of pyrrocidines, it leads to the possibility of transforming these genes into other microbes that could potentially be used to produce pyrrocidine through industrial fermentation.

Additional experimentation is needed to confirm these preliminary results. Additional independent deletion *Szpyc1* mutants must be generated and complemented. These strains will need to be retested for their ability/inability to produce pyrrocidines.

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Table 4.1. List of primers used in this study

Primer name	Description	Primer sequence
PYC1 ORF Fwd	Forward primer located within the ORF of the *PYC1* gene to confirm its presence or absence in the genome	CTCTCTATGTCGGCTCTATCAA
PYC1 ORF Rev	Reverse primer located within the ORF of the *PYC1*gene to confirm its presence or absence in the genome	CTACTACAGAGGCTACTTCACC
PYC1 5'-out	Forward primer located just upstream of the RNP cut site located upstream of the 5' end of the *PYC1*gene used in *PYC1*Anti-Southern.	GTGTGCAGAGCTTCTTCTT
PYC1 3'-out	Reverse primer located just downstream of the RNP cut site located downstream of the 3' end of the *PYC1*gene used in *PYC1*Anti-Southern.	AATATCAACATGGCCCGG
1/3	Forward primer located within the HygR ORF near the 3' end used in Anti-Southern	AGAGCTTGGTTGACGGCAATTTCG
1/4	Reverse primer located within the HygR ORF near the 5' end used in Anti-Southern	GCCGATGCAAAGTGCCGATAAACA
1/46	Forward primer located within the ORF of the HygR cassette to confirm its presence within the genome	GACAGGAACGAGGACATTATTA
1/47	Reverse primer located within the ORF of the HygR cassette to confirm its presence within the genome	GCTCTGATAGAGTTGGTCAAG
PYC1 hyg cassette Fwd	Forward primer used to generate HygR cassette repair template	CTTGATGTGACCATGTCACACGATGATCTG CCATCGGTTAGAATTCCTGCAGCCCAACTG
PYC1 hyg cassette Rev	Reverse primer used to generate HygR cassette repair template	CGGCCTTGGATCGGATCGGGATGATCGAG CGCGGGGTGGCTCTAGAACTAGTGGATCC AA
CRISPR *PYC1* #1	20 bp sequence used for generating Alt-R™ CRISPR-Cas9 crRNA used to generate	GCTGACAAACGAAAGGGTGG

	RNP that generated DSB upstream of the 5'end of *PYC1*	
CRISPR *PYC1* #2	20 bp sequence used for generating Alt-R™ CRISPR-Cas9 crRNA used to generate RNP that generated DSB downstream of the 3'end of *PYC1*	GAGGACGTGACGTTTCCCCT

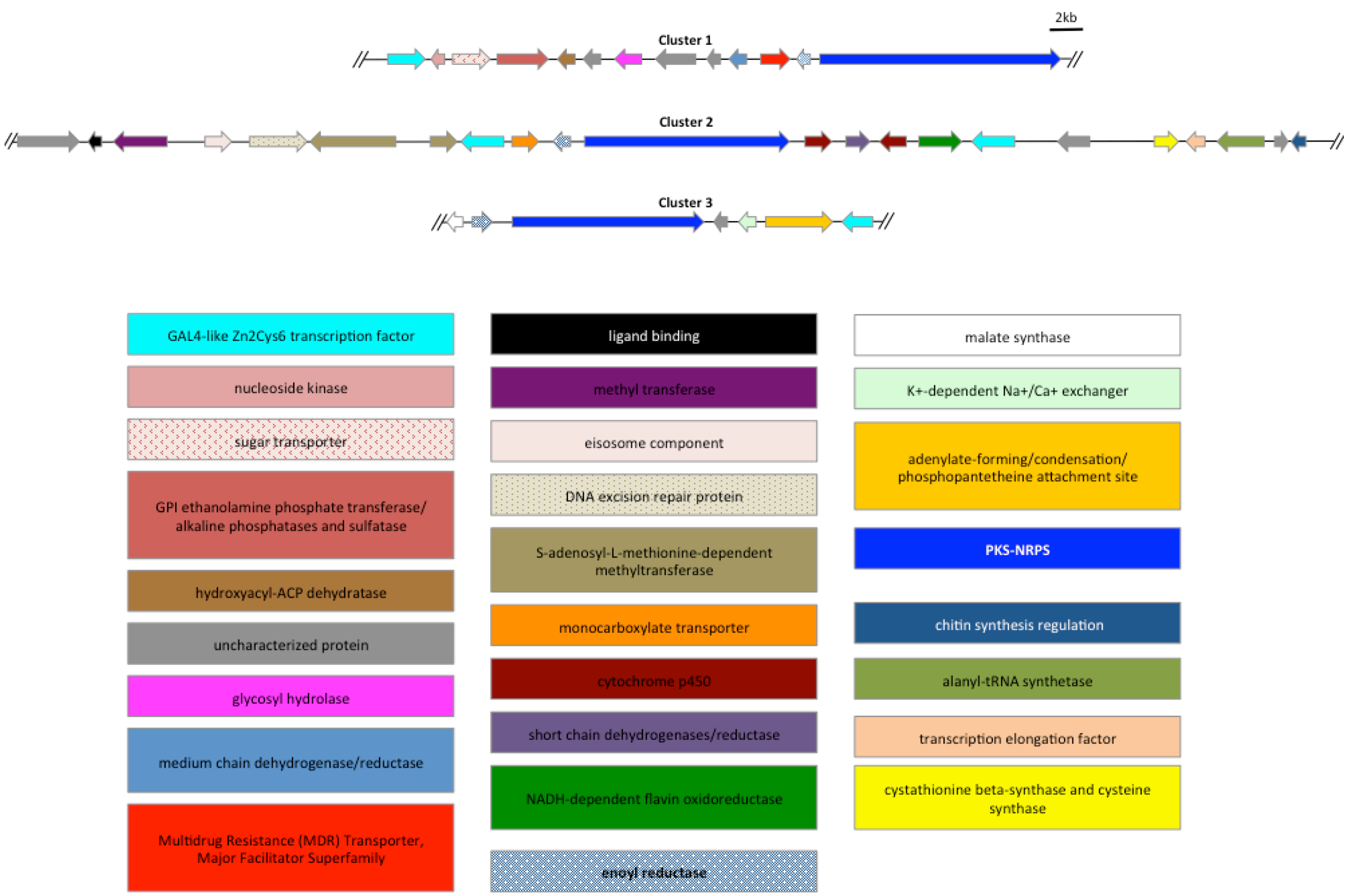

Figure 4.1. *S. zeae* putative gene clusters having a hybrid PKS-NRPS. Each of these clusters was found in three different *S. zeae* strains that are all known producers of pyrrocidine. *PYC1* was the PKS-NRPS located within cluster 1 of this diagram.

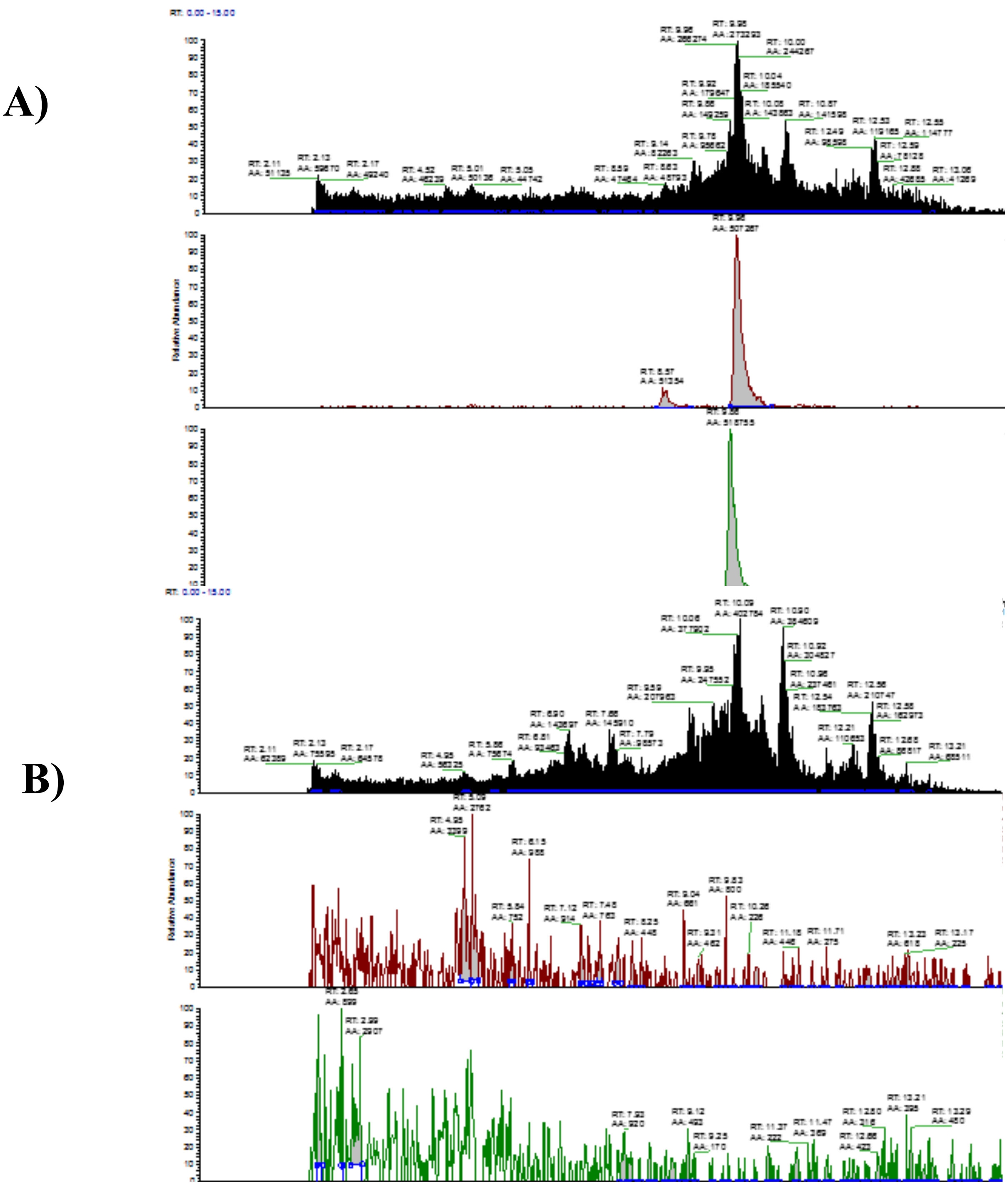

Figure 4.2. LCMS results when screening for the presence or absence of pyrrocidine A & B in wild-type *S. zeae* strain NRRL 6415 and *ΔSzpyc1*. (A) Represents the results of LCMS analysis on wild-type *S. zeae* strain NRRL 6415. The large red peak seen in the second chromatogram depicts the presence of pyrrocidine A (m/z: 486.6 and 532.3) while the large green peak in the third chromatogram depicts the presence of pyrrocidine B (m/z: 488.6 and 534.3). (B) Represents the results of LCMS analysis on *ΔSzpyc1*. In the second chromatogram, no peak was seen at the retention time indicative of the presence of pyrrocidine A. Small peaks seen in this chromatogram represent background noise. In the chromatogram no peak was seen at the retention time indicative of the presence of pyrrocidine B. Small peaks seen in this row represent background noise. These results indicate that *S. zeae* strain NRRL 6415 was able to produce Pyrrocidine A & B as expected, while *ΔSzpyc1* lost the ability to produce pyrrocidine A & B.

CHAPTER 5

CONCLUSIONS

The results from this study have successfully identified methods useful for creating deletion strains of *S. zeae* as well as methods to introduce new genetic material to its genome. The use of the OSCAR-ATMT method was used for generating a red fluorescent strain of *S. zeae* (*Sz*-RFP) that will be a useful tool in later experiments. The use of the CRISPR-Cas9 based gene deletion method resulted in the generation of Δ*Szmbl1* that was used to demonstrate that *Sz*MBL1 confers the ability of *S. zeae* to resist BOA.

In addition, data collected from growth room inoculation experiments shed light on the ability of *S. zeae* as a biological control agent for *F. verticillioides*. When analyzing the amount of fumonisin extracted from different inoculum groups, it was shown that the mean value of FB1 extracted from ears that were inoculated with both *S. zeae* and *F. verticillioides* was 62% less than the mean value of FB1 collected from ears inoculated with *F. verticillioides* only.

Additionally, while conducting a field survey in Georgia to investigate the presence or absence of *S. zeae* and *F. verticillioides* in asymptomatic kernels and kernels expressing the starburst symptom, the results indicated that *S. zeae* and *F. verticillioides* were present in all kernels collected from all three counties in this study. However, later in the experiment a negative control PCR that used water as the template showed PCR products leading to the possibility that the results seen previously were false positives caused by contamination. To complete this study, PCR must be repeated once the issue of contamination is resolved.

Finally, the results obtained using LCMS indicate that wild-type *S. zeae* strain NRRL 6415 produced both pyrrocidine A & B as expected, while neither Pyrrocidine A or B was detected in samples that were obtained from the *S. zeae ΔSzpyc1* mutant strain generated using a CRISPR-Cas9 based gene deletion method. These results provide strong evidence that the NRPS-PKS hybrid gene, *SzPYC1*, is required for pyrrocidine production.