MAIZE SEEDLING BLIGHT CAUSED BY *FUSARIUM VERTICILLIOIDES* INVOLVES FUMONISIN B1 MOBILITY AND MODULATION OF NITRIC OXIDE BY THE DENITRIFICATION PATHWAY

by

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(Under the Direction of Anthony E. Glenn)

ABSTRACT

Maize pathogen *Fusarium verticillioides* is the causal agent of seedling blight disease, with production of the fumonisin B_1 (FB_1) mycotoxin being necessary for disease development. FB_1 was previously observed to accumulate in the roots and leaves during systemic infection of the plant, but not in the leaves when watered into the soil. Here we detected FB_1 in the roots and leaves during seedling blight infection by an aconidial *F. verticillioides* strain able to infect the mesocotyl but unable to colonize leaves, while wild type demonstrated colonization and FB_1 accumulation in the same locations. Therefore, root colonization by *F. verticillioides* is necessary for accumulation of FB_1 and leaf colonization is dispensable for FB_1 mobility *in planta*. Since phytotoxic effects of fumonisin are known to include nitric oxide (NO) accumulation *in planta*, the biological and virulence effects of NO on *F. verticillioides* was evaluated. Specifically, we addressed the roles of flavohemoglobins FHB1 and FHB2 on NO detoxification by *F. verticillioides*. Microarray analysis revealed a significant induction of *FHB2* (17-fold) transcription with exposure to NO (1.5 mM DETA NONOate, a NO donor) and a 2-fold increase in *FHB1* transcription. In comparison, the highest induction at 246-fold was a dissimilatory

nitrite reductase (*NIR1*), along with high induction of other denitrification genes, including a P450 nitric oxide reductase (*NOR1*). Deletion mutants (*fhb1*, *fhb2*, *nor1*, *nir1*, and *fhb1/fhb2* double deletion) were generated and challenged on NO, nitrate, and nitrite media. *fhb1*, *fhb2*, and *nor1* were restricted in growth with NO exposure, while *fhb1/fhb2* showed no growth. Only 7% of *fhb1/fhb2* conidia survived the NO exposure. All mutants grew on nitrate and nitrite media under normal atmospheric conditions. In contrast, *fhb2*, *nir1*, *nor1*, and *fhb1/fhb2* were unable to grow on nitrite media under hypoxic conditions, thus linking these genes to denitrification. Also, *fhb1/fhb2* had restricted growth on nitrite media under oxygenic conditions, demonstrating the importance of flavohemoglobins in nitrite metabolism. Maize seedling blight assays revealed *fhb1/fhb2* to be more virulent than wild type, with double the production of FB_1. Here we show denitrification is important in NO detoxification and link such detoxification to virulence and FB_1 production.

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INDEX WORDS: Transpiration, Stomatal closure, *Fusarium verticillioides*, Nitric Oxide, Denitrification, Maize seedling blight, Fumonisin, Shootward movement, Bioscreen, Flavohemoglobin, P450 nitric oxide reductase, Nitrite reductase

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DEDICATION

I would like to dedicate this work to my mother, father, and brother. Your support for me is unwavering and has brought me farther than I ever thought possible. To my Aunts and Uncles who supported me when I need them the most.

I dedicate this work to my future wife Suzette, who always brings me immeasurable joy with her cheerful wit and loving embrace.

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"We hurt people that love us. Love people that hurt us. Hurt people that love us. Love people. I'm inspired. So get inspired"

- Kendrick Lamar

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The ascomycetous fungus *Fusarium verticillioides* (tel. *Gibberella moniliformis*) of the order Hypocreales is a non-obligate plant pathogen of maize causing a number of economically significant diseases including root rot, kernel rot, seed rot, stalk rot, and seedling blight (Kommedahl and Windels, 1981; Kuldau and Yates, 2000). *F. verticillioides* can infect maize at all growth phases, and after initial infection will routinely colonize outward, infecting neighboring tissues and often resulting in systemic infection of the host (Bacon and Hinton, 1996; Bush et al., 2004; Duncan and Howard, 2010; Munkvold et al., 1997; Oren et al., 2003). *F. verticillioides* can decrease maize yield and diminishes grain quality by contamination with the fumonisin mycotoxins and possibly other secondary metabolites including fusaric acid, fusarin C, and moniliformin (Bacon et al., 1996; Marasas et al., 1986; Wiebe and Bjeldanes, 1981).

Fumonisin B_1 (FB_1) poses a threat to food safety, causing a number of species-specific diseases. FB_1 makes up a large portion of the total environmentally-available fumonisins and is the most toxicologically active compared to the next most common analogs FB_2 and FB_3. Twenty-five other fumonisin analogs have been identified that occur naturally in low concentrations (Bartok et al., 2010).

FB_1 contamination of field-grown maize has been determined to be the causal agent in farm animal diseases leukoencephalomalacia (Marasas et al., 1988) and porcine pulmonary

edema (Colvin and Harrison, 1992). Long-term feeding studies have shown that FB_1 and naturally contaminated maize-based diets containing fumonisins (FB_1, FB_2, and FB_3) are carcinogenic in liver and kidney of rodents (IARC, 1993, 2002). In addition, fumonisin toxicity has also been described in cattle, monkeys, rabbits, sheep, ducks, and chickens (IARC, 2002). Human consumption of fumonisin-contaminated maize in the Transkei region of South Africa has been associated with increased risk of esophageal cancer (IARC, 1993). A possible link between fumonisin exposure has also been suggested in parts of the world were maize, potentially contaminated with fumonisins, is consumed in large amounts and diets are possibly deficient in folate and B-vitamins (Harris and Shaw, 1995; Marasas et al., 2004). Consequently, the U.S. Food & Drug Administration has issued industry guidance levels for total fumonisin contamination allowable in human food and animal feeds.

The concentrations of fumonisin in corn products intended for human consumption are recommended not to exceed 2- 4 ppm depending on how the corn is processed, and the concentrations deemed acceptable in animal feed vary between species (Voss et al., 2007). Furthermore, the European Union places a maximum legal limit for cumulative amounts of FB_1 and FB_2 in certain food products, including unprocessed maize (2,000 µg per kg), processed maize products (1,000 µg per kg), processed maize products for direct consumption (400 µg per kg), and foodstuffs intended for consumption by infants and children (200 µg per kg) (EU, 2006).

Despite these regulatory measures, incidences of high fumonisin exposure occur, often in developing countries where corn is a staple crop. Additionally, culling diminished-quality ears or providing proper storage are generally economically unfeasible in these countries (IARC, 1993). In a survey program monitoring 2004 – 2011 mycotoxin presence in feed and feed raw materials

worldwide showed South American samples were the most frequently contaminated with fumonisin with 77% of samples testing positive and an average concentration of 2,691 µg/kg (Schatzmayr and Streit, 2013). Also, alarmingly high rates were measured in China in 2011 at a maximum of 77,502 µg/kg (Streit et al., 2013). However, fumonisin contamination does not occur exclusively in developing countries.. Streit et al. (2012) compared the occurrence of mycotoxins in European feed and feed raw materials since 2004. Maize samples in 3 EU countries tested 44% positive for fumonisin with a max of 5,114 µg/kg in 2008 (Monbaliu et al., 2010) and feed material tested 66% positive for fumonisins in Southern Europe, with a range of 25-36,390 µg/kg (Griessler et al., 2010). Furthermore, Poland and Switzerland also tested positive for fumonisin contamination in greater than 50% of feed samples with maximum concentrations of 14,470, and 265,000, respectively (Dorn et al., 2011; Grajewski et al., 2012). These recent reports in developed countries show contamination of corn-based animal feed at maximum concentrations far exceeding those known to be toxic to animals

THE SYMPTOMLESS INFECTION

The term “Symptomless Infection” was utilized by Bacon and Hinton in 1996 to describe the undetected *F. verticillioides* associated with maize in most crop fields. This symptomless endophytic association between the fungus and maize is a notable phenomenon, as without overt symptoms there is an increased possibility of undetected infection leading to fumonisin accumulation in the crop and maize products.

F. verticillioides mode of infection and accumulation of fumonisin in maize is in part determined by the genetics of the host plant and the fungus. Infection is also dependent on environmental conditions (Desjardins et al., 2007), particularly moisture (Bush et al., 2004) and

temperature (Murillo-Williams and Munkvold, 2008). Another complicating issue determining symptomatic versus symptomless infection is the origin of infection. Infection via insect or herbivore damage often results in clear symptoms and signs of infection in any maize part and the resulting colonization is more opportunistic and pathogenic than endophytic (Bacon et al., 2006). Seedborne diseases often present symptomless infections (Bacon et al., 2006). In contrast, infections originating from the soil have been described as having a more virulent association with maize (Oren et al., 2003).

The mode of kernel infection was recently discovered to utilize the stylar canal in susceptible lines of maize. The lines that were not susceptible to this type of infection contained a closed stylar canal and had a characteristically tighter wrap with more husk layers and a longer silk channel (Duncan and Howard, 2010). The silk, stalk, and seeds are the major infection points that lead to systematic infection of the plant. The progression of the disease to the kernels has been demonstrated in laboratory experiments and is described in four possible parts: (1) seed to seedling transmission, (2) colonization of the stalk, (3) movement into the ear, and (4) spread within the ear (Munkvold et al., 1997). Though the described processes for kernel infection are well understood, the mechanism for colonization by the fungus to uninfected plant tissue is not (Maiorano et al., 2009). The associations between the presences of the fungus, the fumonisin mycotoxin, and the development of plant disease symptoms are similarly not well understood (Maiorano et al., 2009).

The ecological purpose of maize's association with the symptomless endophytic *F. verticillioides* is unclear; however, there is some evidence that when maize seedlings are infected with endophytic strains more prolific growth is observed in those seedlings compared to non-infected seedlings (Yates et al., 1997). This endophytic type of association may or may not align

with the goals of limiting consumption of fumonisins. For instance two fumonisin tolerant Tama Flint maize lines were reported containing high concentrations of 170 nmol g^{-1} (dry weight) FB_1, without development of pathogenic symptoms when watered with 10 µM of FB_1 (Desjardins et al., 2007). It is possible that efforts to breed resistance against *F. verticillioides* could have inadvertently led to selection of a tolerance to fumonisin along with the endophytic nature of the fungus (Bacon and Hinton, 1996).

SEEDLING BLIGHT DISEASE

Plant disease type and severity depends on several factors including the environment, genetics of both the fungus and the host, and point of infection. Crop residues left in the field can contain *F. verticillioides* mycelia able to produce both macroconidia and microconidia prolifically and are capable of contaminating soils (Leslie and Summerell, 2006). The maize seedling infection results from either planting kernels in these infested fields or, in the previous season, by kernel infection originating from colonization of the silks by microconidia, spread by wind, water splash, or insect vectors (Munkvold et al., 1997). The seeding blight disease is an important and unique aspect of the *F. verticillioides* infection.

Seedling blight results in a decrease in seedling root mass, stunted aerial growth, and manifestation of various leaf abnormalities (Glenn et al., 2008; Williams et al., 2006; Williams et al., 2007). Also noted are lesions that appear on the roots similar to signs of root rot in mature maize plants (Bacon and Hinton, 1996). As with all *F. verticillioides* diseases, the severity of seedling blight is highly dependent upon the genetics of the host and the fungus. However, another important factor in the severity of the seedling blight is the production of fumonisins. Several studies have focused on the effect of mycotoxin production on the virulence of *Fusarium*

species. In the case of *F. verticillioides* and fumonisin, only the maize seedling blight infection has clear evidence positively correlating virulence to fumonisin production. Research has concluded that for other diseases, such as ear rot and stem rot in mature maize, fumonisin is unnecessary (Jardine and Leslie, 1999; Proctor et al., 2002). However, strains that produce the fumonisins are associated with hyper-aggressiveness toward mature maize plants, resulting in significantly larger stem lesions (Jardine and Leslie, 1999).

Genetic analysis of fumonisin-producing strains in correlation with seedling blight severity first linked fumonisin production to increased virulence, but was later determined to not be necessary or sufficient for disease development (Desjardins et al., 1995). A positive correlation exists, however, between leaf lesion manifestation, decreased root weight, increased seedling stunting, and presence of soil fumonisins during seedling blight infection (Williams et al., 2006). Also determined was that the fumonisin non-producing strains of *F. verticillioides* did not exhibit leaf lesions or as severe root and stem stunting (Williams et al., 2006). A conclusive study by Glenn et al. (2008) established that fumonisin production is a virulence factor impacting maize seedling disease. When comparing progeny crossed from a strain producing high levels of fumonisin and a strain producing no fumonisin there was a strong correlation between production of fumonisin and virulence. In the same study, a banana-infecting strain formally *F. verticillioides* and now considered *Fusarium musae* (Van Hove et al., 2011), produces no fumonisins and is incapable of causing maize seedling pathogenicity. Complementing this strain with the fumonisin biosynthetic gene (FUM) cluster responsible for biosynthesis of FB_1, FB_2, and FB_3 by transformation of the FUM cluster resulted in the production of fumonisins and ability to cause maize seedling blight.

Additionally, watering non-inoculated maize seedlings with FB_1 caused a significant dose-dependent reduction in root and shoot mass and increased incidence of leaf lesions (Williams et al., 2007). Similarly, inhibition of maize callus growth (Vanasch et al., 1992) and radical elongation has been noted (Doehlert et al., 1994). Spraying FB_1 on maize seedling leaves, however, did not result in leaf lesions, root, or aerial tissue stunting (Abbas and Boyette, 1992).

Considering that FB_1 uptake in roots can act solely to cause disease symptoms on seedlings suggest its phytotoxicity. Also, fumonisins are structurally similar to the *Alternaria alternata* f. sp. *lycopersici* (AAL) toxin, and both FB_1 and AAL toxin mimic sphingoid bases and induce similar toxicity symptoms in tomato, including decreased root and shoot mass and an accumulation of the free sphingoid bases, sphinganine and phytosphingosine (Abbas et al., 1994). Fumonisin B1 watering experiments in maize demonstrated similar toxic symptoms and an examination of the roots found an accumulation of sphinganine, phytosphingosine, and their respective 1-phosphates caused by the inhibition of ceramide synthase (Williams et al., 2007). Accumulation of these sphingoid bases and sphingoid base-1-phosphates are suggested to contribute to the disease symptoms of plant leaves and roots due to their cellular toxicity at high concentrations (Abbas et al., 1994; Williams et al., 2007).

Similarly, FB_1 is responsible for the disruption of ceramide synthase in mammalian tissues and is responsible for observed species-specific diseases (Merrill et al., 2001a). One downstream effect studied in mammalian systems is the disruption of lipid rafts responsible for cell-to-cell communication (Landry and Xavier, 2006) and regulation of apoptosis (Ayllon et al., 2002; Gajate and Mollinedo, 2005; Scheel-Toellner et al., 2004). FB_1 has been shown to induce apoptosis through disruption of lipid rafts in rat oligodendrocytes (Decker, 2004). In plants there

is sufficient evidence of lipid rafts being produced, however, their role in the plasma membrane and potential for fumonisin disruption is unknown.

The underlying mechanisms of how ceramide synthase disruption leads to pathogenic symptoms is also unknown, however, most likely involve the accumulation sphingoid bases and sphinganine in particular (Abbas et al., 1994). At the genetics level fumonisin tolerance is linked to a single heritable trait in maize. Backcrosses of maize FB_1-tolerant line Tama Flint and FB_1 susceptible line B73 showed resistance to fumonisin was predictable (Desjardins et al., 2007). However, the mechanism behind fumonisin tolerance is still unknown. Fumonisin involvement in the *F. verticillioides* seedling blight infection is likely multi-factorial and highly dependent upon the genetic context of the host-fungal interaction and environmental complexities (Desjardins et al., 2007).

APPLICATION OF MYCOTOXIN BIOMARKERS IN PLANT SYSTEMS

Fumonisins from *F. verticillioides* along with aflatoxin, deoxynivalenol, ochratoxin and zearalenone produced by various *Aspergillus* or *Fusarium* species are considered the most toxigenic contaminates in agricultural systems worldwide (CAST, 2003). Food contamination results in disease outbreaks in farm animals and in the case of aflatoxins in humans; however, identifying mycotoxins as the causal agent can be problematic. Moreover, the effects of mycotoxin toxicity may be hidden or even synergistic with other disease agents, such as hepatitis B virus infection (Groopman et al., 2005). This has stimulated research into the mechanisms of mycotoxin toxicity and led to development of mechanism-based “biomarkers”, which in the case of aflatoxin have linked exposure to human diseases. For the other mycotoxins mechanism-based

and /or exposure-based biomarkers have been used successfully (Van der Westhuizen et al., 2013).

Currently, biomarkers are at different stages of development depending on the mycotoxin and the system. The aflatoxin biomarkers are considered the most advanced and well supported for linking aflatoxin exposure to disease progression in farm and laboratory animals, as well as humans (Groopman et al., 2005). In Chapter 2 we review these biomarkers and suggest aflatoxin to be the gold standard for biomarker development. Additionally, we present our research as an example of how the accumulated knowledge of biomarkers in plant systems are an effective phytopathological tool to track disease development.

NITRIC OXIDE IN DEVELOPMENT AND PATHOGENICITY

Pivotal signaling molecules for life include the Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) e.g. hydrogen peroxide (H_2O_2), superoxide (O_2^-), and nitric oxide (NO). These molecules are highly reactive, short lived, and are noted for reacting with a multitude of other cell components. This biochemistry makes ROS and RNS important and potent signaling molecules across kingdoms of life. However, this same biochemistry makes accurate measurements and mechanisms of action difficult to delineate. Therefore, the exact mode of action of these species is not well understood since effects can be dependent of the dose and the system involved. Also, high concentrations of these reactive species by overproduction in the cell leads to oxidative or nitrosative stress and cytotoxic effects (Apel and Hirt, 2004; Thomas et al., 2008).

ROS and RNS have been investigated in animal and plant systems. In plant systems, the oxidative burst of ROS has been established as a main aspect of pathogen response in plants

since Dr. Noriyuki Doke's ground breaking work in 1983. Potato tuber protoplasts were inoculated with *Phytophthora infestans* or exposed to the cell wall preparation from *P. infestans* and were found to readily produce O_2^- (Doke, 1983). This induction, termed the oxidative burst, is one of the quickest and first detectable events during the incompatible host-pathogen interactions and is crucial for induction of defense genes, manifestation of the hypersensitive response, cross-linking of cell wall components, callose deposition, and phytoalexin production (Apostol et al., 1989; Daudi et al., 2012; Desikan et al., 2000; Devlin and Gustine, 1992; Lamb and Dixon, 1997; O'Brien et al., 2012).

Some similarities between ROS and NO are evident in plant systems, yet the role of NO has received less attention by researchers. NO is a RNS free radical and a form of nitrosative stress. As with ROS, NO acts as a signaling molecule. Highly reactive with an unpaired electron, NO can exist in a variety of reduced states including the NO^- (nitroxyl ion) and NO^+ (nitrosonium ion) each having their own unique reaction capability giving NO exceptionally diverse biochemistry (Gow and Ischiropoulos, 2001). Moreover, some important physical aspects of NO include its near instantaneous mobility through membranes (Ignarro et al., 1987; Palmer et al., 1987). The complexity of NO's effects is also demonstrated in its dual nature as a cytoprotectant and cytostimulant at low concentrations, whereas at high concentrations NO is cytotoxic (Kroncke et al., 1997). NO was first elucidated as a messenger in the immune, nervous and cardiovascular systems in mammals and is the most powerful endogenous signal for vasodilatation yet discovered (Anbar, 1995; Grisham et al., 1999; Lundberg et al., 2008; Pfeiffer et al., 1999).

Phytopathological studies recently demonstrated NO as an important aspect of plant defense against a variety of pathogens. Similar to the ROS oxidative burst that marks the

beginning of pathogen recognition, there is an early NO burst. This has been seen in barley epidermal cells infected with powdery mildew *Blumeria graminis* f. sp. *hordei* (Prats et al., 2005) and in pelargonium infected with necrotrophic pathogen *Botrytis cinerea* (Floryszak-Wieczorek et al., 2007). Also, NO was noted in *Arabidopsis* challenged with the bacterium *Pseudomonas syringae* pv. *tomato* (Bennett et al., 2005), the tobacco mosaic virus in tobacco (Durner et al., 1998), and the oomycete pathogen *Bremia lactucae* on *Lactuca* (Sedlarova et al., 2011). Additionally, cryptogein, a fungal elicitor from *Phytophthora cryptogea,* induced the NO burst in tobacco epidermal cells (Foissner et al., 2000). However, there is some conflicting evidence about the function of NO in pathogenicity. Exposure of the susceptible tomato cultivar *Lycopersicon esculentum* Mill. cv. "Perkoz" to the pathogenic *Botrytis cinerea* demonstrated an increase in NO levels paired with a decrease in H_2O_2 levels and an increase in virulence (Malolepsza and Rozalska, 2005). Also, *B. cinerea* endogenous production of NO diffuses to the host plant, triggering the hypersensitive response (HR), a form of localized programmed cell death, which leads to rapid tissue collapse and is highly effective against biotrophic pathogens that depend on the plant for nutrition (Richael and Gilchrist, 1999). However, in this case the HR may benefit the infecting necrotrophic pathogen, being activated by the fungus as a pathogenic strategy (Turrion-Gomez and Benito, 2011). Interestingly, recent studies have shown the sphingoid bases, precursors to sphingolipids, are potent activators of the NO burst in *Arabidopsis* (Guillas et al., 2013). This could have interesting implication on the effect of fumonisin on nitric oxide production in maize.

Induction of NO response results in many downstream effects in plants. For instance, the formation of structural barriers by cell wall modifications in the initial phases of the *Colletotrichum coccodes* – tomato interaction (Wang and Higgins, 2005b). Similarly, Prats et al.

(2005) observed significant papilla deposition co-occurring with the NO burst in the barley- *B. graminis* f. sp. *hordei* interaction. Another well studied effect of the induction of NO is production of secondary metabolites known for defense by the plant. This induction is usually mediated by fungal elicitors and includes metabolites of interest because of their pharmaceutical importance, including ginseng saponin, hypericin, puerarin, catharanthine, and taxol (Hu et al., 2003; Wang et al., 2006; Xu et al., 2005; Xu et al., 2006; Xu and Dong, 2005; Zheng et al., 2008).

Most notably, NO plays a crucial role in eliciting the hypersensitive response in the incompatible plant-pathogen interactions. In recent years NO has been incorporated into the HR signaling cascade, and has been demonstrated to work synergistically with ROS. The cell death in HR response requires a precise and careful balance between H_2O_2, O_2^-, and NO. The involvement of the NO and ROS product peroxynitrite $ONOO^-$ has also been implicated to trigger global changes in ion flux, extracellular pH, membrane potential, and protein phosphorylation. These changes activate the plant genome encoding program cell death (Clarke et al., 2000; Delledonne et al., 2003; Delledonne et al., 1998; Delledonne et al., 2001; Romero-Puertas et al., 2004). Apart from pathogenic interactions, NO is a signaling molecule regulating plant germination, growth, development, stomatal closure, and abiotic stress (Besson-Bard et al., 2008; Delledonne et al., 1998; Moreau et al., 2010; Neill et al., 2002; Wilson et al., 2008; Zhang et al., 2008).

The direct mechanism of NO signaling stems from its redox-based post-translational modification of proteins, including S-nitrosylation, which is a covalent bonding of an NO molecule to a sulfhydryl group on cysteine. This modification affects the protein's activity, localization, interactions, and folding conformation (Yun et al., 2012). This form of protein

modification can also occur on carrier molecules such as glutathione forming S-nitrosoglutathione and is the reason why a 5 to 15 s half-life signaling molecule can ramify a signal throughout the plant lasting for days. This carrier molecule can perform protein trans-nitrosylation and acts as a bio-reserve for NO (Kettenhofen et al., 2004).

NO can be generated enzymatically or non-enzymatically. For instance, NO can be generated in the plant via nitrite reduction as described under the acidic conditions of the barley aleurone apoplast (Bethkeet al., 2004). There are a number of both oxidative and reductive pathways for NO production. However, much controversy underlies the enzymatic production of NO in plants. There is no clear cut model for NO production by a single enzyme despite evidence for a simple enzymatic mechanism. The most extensively described NO-producing enzymes have been nitric oxide synthase (NOS) in mammals. NOS catalyzes a two-step oxidation of L-arginine to NO and citrulline (Moncada and Higgs, 1991). Evidence of "NOS like" activity in plants has been demonstrated in soybean, tobacco, maize and *Arabidopsis* (Delledonne et al., 1998; Durner et al., 1998; Ribeiro et al., 1999). However, there has been no direct homolog to the NOS gene found thus far in plants. The only close homolog in *Arabidopsis* (AtNOA1) was shown to have an indirect role in NO production, but not direct synthesis (Guo et al., 2003). A number of other potential NO producing enzymes in plants are cytochrome P450 monooxygenases (Boucher et al., 1992) and the nitrate and nitrite reductases (Rockel et al., 2002). When *Arabidopsis* was challenged with the bacterial pathogen *Pseudomonas syringae* pv. *maculicola*, nitrate reductase was found to be the major producer of NO as double *nia1*/*nia2* mutants failed to produce a NO burst. However, some NO production was still determined to originate from NOS-like activity (Modolo et al., 2005). How NO is produced in the plant is unclear, but remains as an important aspect of plant defense.

Another level of complexity is that fact that NO is also a powerful exogenous and endogenous signaling factor in fungi as well. NO both promotes germination and decreases viability of the blastoconidia of *Candida albicans* (Abaitua et al., 1999). In *Neurospora crassa*, NO has been shown to inhibit photoconidiation (Ninnemann and Maier, 1996). NO influences germination in *Colletrotrichum coccodes* (Wang and Higgins, 2005a), and in *Coniothyrium minitans* (Gong et al., 2007) and sporangiophore development in *Phycomyces blakesleeanus* (Maier et al., 2001). Appressorium formation in the obligate biotrophic powdery mildew fungus *Blumeria graminis* also requires the endogenous production of NO and scavenging by cPTIO can disrupt this process (Prats et al., 2008). The same was shown for *Magnaporthe oryzae* during germination, early development, and appressorium formation (Samalova et al., 2013). In both cases endogenous NO seems to drive the infection and is crucial for pathogenicity. Similar to plants, the agent responsible for production of endogenous NO in fungi is yet to be elucidated and may in fact be more ambiguous. “NOS-like” enzymes have been suggested in *Aspergillus oryzae* and *Glomerella graminicola*, but these are the exceptions since most fungi do not contain NOS sequences in their genomes (Gorren and Mayer, 2007; Turrion-Gomez and Benito, 2011). Nitrite-induced NO production from cultures of *Pythium*, *Botrytis*, and *Fusarium* have been confirmed, but the exact mechanism still remains elusive (Conrath et al., 2004).

One final aspect of NO yet to be discussed is the need for pathogens to detoxify NO during the NO burst. The high concentration of NO during this process is cytotoxic and a compatible pathogen is required to overcome this obstacle. Detoxification for successful pathogenicity has been studied in *Candida albicans* virulence on mice (Ullmann et al., 2004) and in the bacterial pathogen *Erwinia chrysanthemi* on *Saintpaulia ionantha* (Favey et al., 1995). Both systems utilize the highly conserved flavohemoglobins, which are comprised of a globin

domain fused to a ferredoxin reductase like FAD- and NAD-binding modules (Gardner et al., 1998). Flavohemoglobins are sometimes referred to as Nitric Oxide Dioxygenases (NOD) in bacterial systems because of their ability to detoxify NO by oxidation to nitrate (NO_3^-). Flavohemoglobins are prolific and conserved throughout all kingdoms of life (Gardner, 2005).

Mutating the *Erwinia chrysanthemi HmpX* flavohemoglobin resulted in a significant increase in NO level accumulation, disease resistance, and an HR response in the *S. ionantha* host (Favey et al., 1995). Interestingly, insertion of *hmpx* in to the avirulent *Pseudomonas syringae* pv. tomato avrB suppressed the HR response in *Arabidopsis*, reverting the *Pseudomonas syringae* pv. tomato avrB back to a competent pathogen (Boccara et al., 2005). Similarly, transformation of *Arabidopsis* with the flavohemoglobin from *Escherichia coli* (NOD) also suppressed the NO burst and HR response when challenged with *Pseudomonas syringae* .spv. tomato avrB (Zeier et al., 2004). Finally, *Cryptococcus neoformans* was also shown to require a flavohemoglobin, gene *FHB1*, for both virulence on mice and NO detoxification as found by insertional mutagenesis (Idnurm et al., 2004). The function of flavohemoglobins in detoxification of NO is a virulence factor for a number of pathogens. NO represents a complex and potent signaling compound and antimicrobial compound in pathogenic interactions and the role of NO in the *F. verticillioides* – maize interactions is yet to be explored.

HYPOTHESES

The hypotheses put forth in this dissertation were designed to give insight into the roles of fumonisin and nitric oxide in the complex relationship between *F. verticillioides* and maize. The insights given here will further our understanding of the complex interplay of these metabolites and will aid in our ability to limit disease and mycotoxin accumulation in maize.

Chapter 3: The objective of this study was to determine the *in planta* transport of fumonisins and potential mechanisms of uptake into the roots.

Hypothesis 1 - Transport of fumonisin is maize genotype specific.

Hypothesis 2 – Fumonisin is mobile *in planta*, accumulates in the leaves, and requires the presence of the fungus in the roots but doesn't require direct fungal colonization of the leaves.

Hypothesis 3 – Fumonisin accumulation in the roots is genotype specific.

Hypothesis 4 – Fumonisin accumulation in the roots is mediated by transpiration and therefore inhibited by the addition of abscisic acid, a plant hormone.

Chapter 4: The objective of this study was to determine the effects of fumonisin on maize transpiration and stomatal aperture.

Hypothesis 1 – Fumonisin B1 causes an increase in transpiration rates.

Chapter 5: The objective of this study was to determine the role of nitric oxide in *Fusarium verticillioides* pathogenicity on maize.

Hypothesis 1 – *Fusarium verticillioides* utilizes flavohemoglobins for detoxification of nitric oxide.

Hypothesis 2 – Exposure of exogenous nitric oxide to *Fusarium verticillioides* triggers induction of detoxification genes.

Hypothesis 3 –*Fusarium verticillioides* has endogenous production of nitric oxide.

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CHAPTER 2

THE CURRENT STATE OF MYCOTOXIN BIOMARKER DEVELOPMENT IN HUMANS AND ANIMALS AND THE POTENTIAL FOR TRANSLATION TO PLANT SYSTEMS

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ABSTRACT

Filamentous fungi that contaminate livestock feeds and human food supply often produce toxigenic secondary metabolites known as mycotoxins. Among the hundreds of known mycotoxins, aflatoxins, deoxynivalenol, fumonisins, ochratoxin A and zearalenone are considered the most commercially important. Intense research on these mycotoxins, especially aflatoxin, has resulted in the development of 'biomarkers' used to link exposure to disease risk. In the case of aflatoxin this effort has led to the discovery of both exposure and mechanism-based biomarkers, which have proven essential for understanding aflatoxin's potential for causing disease in humans, including subtle effects on growth and immune response. Fumonisin biomarkers have also been used extensively in farm and laboratory animals to study the fumonisin-induced disruption of cellular and systemic physiology which leads to disease. This review summarizes the status of mycotoxin biomarker development in humans and animals for the commercially important mycotoxins. Since the fungi responsible for the production of these mycotoxins are often endophytes that infect and colonise living plant tissues, accumulation of mycotoxins in the plant tissues may at times be associated with development of plant disease symptoms. The presence of mycotoxins, even in the absence of disease symptoms, may still have subtle biological effects on the physiology of plants. This review examines the question of whether or not the knowledge gained from mechanistic studies and development of biomarkers in animal and human systems is transferable to the study of mycotoxin effects on plant systems. Thus far, fumonisin has proven amenable to development of mechanism-based biomarkers to study maize seedling disease caused by the fumonisin producer, *Fusarium verticillioides*. Expanding our knowledge of mechanisms of toxicity and the overt and subtle effects on animal,

human, and plant systems through the identification and validation of biomarkers will further our ability to monitor and limit the damage and economic impact of mycotoxins.

Key Words: *Fusarium*, *Aspergillus*, aflatoxin, deoxynivalenol, trichothecene, fumonisin, ochratoxin, zearalenone

INTRODUCTION

Mycotoxins are low molecular weight secondary metabolites produced by filamentous fungi that frequently occur on foods and are potentially toxic to humans and animals (CAST, 2003). While several mycotoxins are known or suspected to be involved in human disease, many more are known or suspected to contribute to disease in farm and laboratory animals. Because of their widespread occurrence on foods and their potential for toxicity, several mycotoxins pose a threat to food safety and security (CAST, 2003). The mycotoxins that are both known causes of disease and occur in significant amounts in commercially important commodities consumed by humans are limited to aflatoxins, deoxynivalenol (DON), fumonisins (primarily fumonisin B_1 – FB_1), ochratoxin A (OTA) and zearalenone (ZEA). These commercially important mycotoxins will be the focus of this review. While there are other mycotoxins that are known to cause or contribute to animal disease, such as ergot alkaloids, macrocyclic trichothecenes and T-2 toxin, they will not be covered in this review.

Despite their widespread presence in grains and other commodities, little is known about the selective advantages conferred to the fungi that produce mycotoxins *in planta*. Nonetheless, the ubiquitous production of these energetically costly metabolites by naturally occurring fungal strains, within species and across genera, suggests they confer an advantage over non-producing strains. Some have suggested mycotoxins impart an advantage by inhibiting the growth of competing microorganisms for the same niche environment (Fox and Howlett, 2008) while others have suggested that mycotoxins may reduce herbivory by insects (Tanaka et al., 2005) and animals (Clay, 2009). Yet others have suggested that infection and colonisation of the host plant by mycotoxin-producing fungi is enhanced due to phytotoxic effects or an altered physiological state of the plant to better suit the fungus (reviewed in Reverberi et al., 2010). In animal systems,

mechanism-based approaches using biomarkers have contributed greatly to the understanding of mycotoxins and their mechanisms of action as potential contributing factors to human and animal disease. Similar approaches may prove useful for studying plant pathogenicity and the broader physiological effects of mycotoxins *in planta*. Compared to the knowledge base in animal systems, the biological effects of most mycotoxins in plants are poorly understood and require more in-depth investigation of mycotoxin-based phytotoxicity and related mechanisms of action.

For this review, the mechanism of action of mycotoxins is defined as the initial underlying biochemical or molecular interaction resulting in the principle downstream effects which may include disruption of metabolic and signaling pathways, altered physiological responses, altered cell proliferation and differentiation, and cell death (reviewed in Riley et al., 2011). These initial interactions could be the direct result of the mycotoxin or a metabolite of the mycotoxin. For any mechanism of action it is critical to identify the proximate cause, which is that event that sets everything downstream into motion. An example of an easy to understand mechanism of action is that of a classical agonist binding to an extracellular receptor, which sets into motion a series of downstream effects ending ultimately in some measurable biological response. These responses can sometimes be monitored through the utilisation of detection tools known as ‘biomarkers’.

The Biomarkers Definition Working Group (Atkinson et al., 2001) defined biomarkers as characteristics that objectively measure and indicate the biological state of a pathogenic process or pharmacologic response. In this review we are defining biomarkers as measurable biochemical or molecular indicators of either exposure or biological response to a mycotoxin that can be specifically linked to the proximate cause. Mechanism-based biomarkers often include

changes in the level of specific proteins, cellular metabolites, or gene expression resulting from specific alterations in metabolic or signaling pathways, stress responses, cell proliferation, or cell death. In contrast to mechanism-based biomarkers, exposure-based biomarkers are most often the mycotoxin itself or the metabolized mycotoxin by-product (e.g. glutathione or glucuronide conjugates). These compounds can be detected in easily-accessed biological fluids or tissues. Perhaps the best example is in the case of aflatoxin where the exposure biomarkers are also potential indicators of the proximate cause. Specifically, the covalent binding of metabolically activated reactive metabolites to essential macromolecules, most importantly DNA and proteins (Groopman et al., 2005). Within the context of biomarker utility, many observed down-stream effects may be linked to the proximate cause of toxicity, but their lack of specificity precludes them from being useful for either exposure or effect. An example of this lack of sufficient specificity includes oxidative stress response in both animals and plants. Such broad-based stress responses can be triggered by a number of abiotic and biotic factors.

In this review we briefly summarise the status of mycotoxin biomarker development in humans and animal research for commercially important mycotoxins. We will also address the question of whether or not the accumulated knowledge of biomarkers in animal and human systems is transferable to studies of mycotoxin effects on plant systems using our work examining the cellular and pathological effects of fumonisin on maize seedlings disease as an example. We have characterised both mechanism-based and exposure-based biomarkers associated with seedling disease development, which provides a better understanding of the underlying mechanism of action and provides clues for other potential physiological effects.

ASSESSMENT OF THE STATE OF BIOMARKER DEVELOPMENT IN ANIMALS AND HUMANS

Table 2.1 summarises the likely mechanism of action for each of the mycotoxins considered in this review and also provides our assessment of the current status of biomarker development for each mycotoxin in animals and humans. The rating assigned to status of biomarker development is based on a generalised scheme (Figure 2.1) derived from the validation scheme proposed by Groopmanet al.(2008) for molecular biomarker research and specifically aflatoxin and hepatitis B virus as risk factors for cancer. Currently, aflatoxin B_1 (AFB_1) is the only mycotoxin for which there are validated biomarkers of both exposure and disease risk in humans and animals (Groopman et al., 2005; Wild and Gong, 2010). The AFB_1-N7 guanine DNA adducts and AFB_1-lysine adducts have been used successfully to study the role of AFB1as a cause or contributing factor for toxicity and diseases in animals and humans (Wild and Gong, 2010). The G-T transversion mutation in codon 249 in the p53tumour suppressor gene has also been used successfully in population based studies in humans (Wild and Gong, 2010). The G-T mutation at the codon homologous to human codon 249 has not been demonstrated in AFB1exposed farm animals (Smela et al., 2001). Nonetheless, studies have shown low levels of p53mutations in AFB_1 treated ground squirrels (Rivkina et al., 1994) and rats (Lee et al., 1998), and studies in transgenic mice suggest p53mutations enhance AFB_1 liver tumourigenicity (Tong et al., 2006). Together, these studies have qualitatively and quantitatively verified exposure, linked exposure to mechanism of action, identified high risk populations, defined dose-response relationships and disease thresholds, tested the efficacy of intervention and decontamination methods, and allowed for the prediction of potential interactions and other factors contributing to susceptibility. The

process of biomarker development with aflatoxin (Kensler et al., 2011) is the 'gold standard' by which the current state of biomarker development for other mycotoxins should be judged.

Exposure and mechanism-based biomarkers are available for use in animal studies with fumonisin, (reviewed in Shephard et al., 2007; Riley et al., 2011; Routledge and Gong, 2011) and while urinary fumonisin has been used successfully in human studies as an exposure biomarker, fumonisin disruption of sphingolipid metabolism has not been demonstrated in humans. Likewise, the blood or urinary levels of DON as exposure biomarkers work well in laboratory animals and humans (reviewed in Pestka, 2010 a,b; Riley et al., 2011; Routledge and Gong, 2011; Turner, 2010). Additionally, some non-specific mechanism based biomarkers have been used in laboratory animal studies, and to a lesser extent in farm animals (reviewed in Pestka, 2010 a,b; Riley et al., 2011), but there have been no studies linking the DON exposure biomarkers with the mechanism-based biomarkers in humans. For OTA, exposure biomarkers have been used widely in human and animal studies (reviewed in Riley et al., 2011; Routledge and Gong, 2011; Scott, 2007), but because the mechanism of action of OTA is unclear and controversial (Mally and Dekant, 2009; Mantle et al., 2010; Marin-Kaun et al., 2008; O'Brien and Dietrich, 2005; Pfohl-Leszkowicz and Manderville, 2007) progress has been slow for development of validated mechanism-based biomarkers for use in either animal models or human studies. The levels of ZEA and its metabolites in excreta have been used successfully as exposure biomarkers in farm and laboratory animal studies (reviewed in Fink-Gremmels and Malekinejad, 2007; Riley et al., 2011) but not in human studies. The easily observable and pathognomonic overt oestrogenic effects of ZEA have been linked to ZEA exposure in animal studies, but this has not been done convincingly in humans.

APPLICATION OF BIOMARKER-BASED APPROACHES TO PLANT STUDIES

We discussed above how the development of biomarkers has aided the understanding of mycotoxin-associated disease development and identification of underlying proximate causes and mechanisms of action in animals and humans. In this section we will explore the potential translation and application of animal-associated mycotoxin effects and biomarker development to plant systems in order to better characterise the phytopathological interactions between mycotoxigenic fungi and their hosts. In essence, can we apply what we know regarding the animal toxicity of these mycotoxins and their mechanisms of action to the host plants in which the mycotoxins are produced and accumulated? Are these secondary metabolites toxic to the host plants? Is there measurable biochemical and molecular disruption or alteration of cellular processes within the plants? Does such disruption or alteration have any negative impact on the plants, whether overt (disease development) or subtle (altered physiology and development)?

In practice, development of plant-based biomarkers would follow similar procedures as outlined for biomarker development in animal and human systems (Figure 2.2). In natural field environments, exposure of a plant to a mycotoxin occurs through either infection of the plant by mycotoxigenic fungi or through environmental exposures such as the presence of the mycotoxin in the soil. Uptake of water and water-soluble mycotoxins from the soil by the roots may result in accumulation in the roots and perhaps transpiration-mediated shootward mobility of the mycotoxins. Whether through this type of environmental exposure and uptake, or from the *in planta* production and accumulation of the mycotoxin by infecting fungi, a mycotoxin could function as an exposure-based biomarker if the compound is easily detected in plant tissues and correlated to exposure (Figure 2.2). The same may be true for metabolites of the mycotoxin formed by the plants, such as glucoside conjugates.

Given that the proximate causes and mechanisms of action of mycotoxins often involve disruption of universal cellular processes (e.g. protein synthesis or sphingolipid metabolism), individual mycotoxins may have phytotoxic properties in addition to their animal toxicity. Disruption of plant cellular processes and the potential accumulation of associated cellular metabolites could be assessed and quantified for use as mechanism-based biomarkers correlated to specific mechanisms of action and potential adverse effects on plant health and development (Figure 2.2). Our knowledge of mycotoxin effects on animal systems is therefore a valid and useful starting point for evaluating mechanisms of action in plants. An understanding of the mechanistic basis of phytotoxicity exhibited by fungal metabolites allows for in-depth studies into interactions between the fungi and host plants and increases the potential development of refined strategies to monitor or manage fungal virulence and plant disease.

Mycotoxins and phytotoxins have been defined historically as functionally separate fungal secondary metabolites, and in some cases the distinction is justified, for example the production of the host-selective toxin victorin by *Cochliobolus victoriae* (*Helminthosporium victoriae*) that causes Victoria blight of oats (Wolpert et al., 2002). In other cases the mycotoxin-phytotoxin distinction begins to blur, such as the phytotoxin produced by *Alternaria alternata* f. sp. *Lycopersici* (AAL) that causes Alternaria stem canker of tomato (Brandwagt et al., 2000; Gilchrist et al., 1992; Wang et al., 1996). The AAL phytotoxin is a sphinganine analogue very similar in structure to the fumonisin mycotoxins. In fact, FB_1 is able to cause disease symptoms on susceptible tomato that are similar to symptoms caused by AAL phytotoxin (Abbas et al., 1994; Brandwagt et al., 2000; Gilchrist et al., 1992). This review does not further address traditional phytotoxins such as victorin, but we suggest that the approach of biomarker development could be applied to these phytopathogenic systems. Use of exposure-based

biomarkers may be the most amenable approach since mechanistic biomarkers may be more difficult to validate given the nonspecific plant defense responses that occur in response to phytotoxins, such as formation of reactive oxygen species, ethylene signaling, and de novosynthesis of phytoalexins (Wolpert et al., 2002).

Currently, the only intensely studied mechanism-based biomarkers in plant systems are those used in studying fumonisin phytotoxicity. We outline below the *in planta* detection of sphingoid bases and sphingoid base 1-phosphates as mechanism-based biomarkers indicative of sphingolipid metabolism disruption in maize tissues and their correlation to foliar disease development in maize seedlings. While mechanism-based biomarkers may be applicable for studying phytotoxic effects of other important mycotoxins, such data are currently lacking for the most part. In contrast, numerous studies have quantified the *in planta* accumulation of mycotoxins or mycotoxin metabolites (i.e. glucoside conjugates), so we begin by outlining the utility of such assessments as exposure-based biomarkers.

EXPOSURE-BASED BIOMARKERS FOR *IN PLANTA* MYCOTOXIN ACCUMULATION

Many studies have quantified the production and accumulation of mycotoxins in different tissues of plants. This is to be expected since analytical assessment of mycotoxin concentrations in agricultural commodities is the basis for monitoring potential animal or human exposure from consumption of those products. For this review we are focusing on studies that have addressed the effects of mycotoxins on plant disease, growth, and development and suggest the utility of exposure-based biomarkers for such studies as a prelude to future development of mechanism based biomarkers.

AFB_1 production by *Aspergillus flavus* and *Aspergillus parasiticus* is a major concern due to the toxicity of the mycotoxin and the wide distribution of these fungi on various host plants. Despite near ubiquitous production of AFB_1, there have been few studies into the role of the mycotoxin in the biology of *Aspergillus* species (McLean, 1994b). AFB_1 was shown to have phytotoxic effects on maize embryos and tobacco (*Nicotiana tabacum*) seedlings. The plants exhibited a dose-dependent response to AFB_1 exposure, showing increasing inhibition of root elongation with increasing AFB_1 concentrations (McLean, 1994a; McLean et al., 1995). Such root inhibition has been noted in diseased peanuts infected with *A. flavus* and was deemed 'aflaroot' (Chohan and Gupta, 1968). Since the mode of action for aflatoxin phytotoxicity is unknown, mechanism based biomarkers are not currently attainable, yet detection of AFB_1 in different tissues of the plant can serve as an exposure-based biomarker for detailed examination of phytotoxicity. Detection of *in planta* derived metabolites may also be possible. For example, metabolism of AFB1to aflatoxicol was documented in parsley (Howes et al., 1991). Aflatoxicol is mutagenic and carcinogenic along with AFB_1 and has been detected as a metabolite of AFB_1 exposure in animals and humans (Kussak et al., 1998; Mariën et al., 1987; Wong and Hsieh 1978).

DON is one of the few mycotoxins definitely linked to fungal virulence and plant disease development. *Fusarium graminearum* and *Fusarium culmorum* produce. DON and other trichothecenes, including nivalenol and the acetyled DON derivatives, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol (Kimura et al., 2007). Evaluating the role of DON production on virulence was enhanced by the use of molecular genetics and creation of mutant strains. Specifically, deletion of Tri5, the gene encoding trichodiene synthase, the first committed enzyme in the DON biosynthetic pathway (Kimura et al., 2007), resulted in loss of DON

production by *F. graminearum* as well as reduced development of wheat head blight and maize ear rot (Bai et al., 2002; Desjardins et al., 1996; Harris et al., 1999; Proctor et al., 1995). They also were less effective at colonising wheat or barley heads (Boddu et al., 2007; Jansen et al., 2005). The same principles have been demonstrated in other DON-producing *Fusarium* species (Desjardins and Hohn, 1997). Additionally, DON was shown to inhibit root elongation in wheat (Masuda et al., 2007). DON elicited programmed cell death by inducing hydrogen peroxide production and was also linked to increased expression of defence related genes (Desmond et al., 2008). The acute phytotoxic effects of DON and other trichothecenes have been studied in-depth on the non-host *Arabidopsis thaliana* with similar symptoms noted (Masuda et al., 2007). Tolerance to DON exposure has been illuminated by studying the *Fhb1*gene associated with resistance to *Fusarium* head blight in wheat (Liu et al., 2006) and the DOGT1 homologue in *Arabidopsis* (Poppenberger et al., 2003). Over-expression of DOGT1, a UDP-glucosyltransferase, in *Arabidopsis* resulted in increased tolerance to DON due to detoxification by transformation to deoxynivalenol-3-Oglucoside (Poppenberger et al., 2003). Further studies in barley have demonstrated conversion to deoxynivalenol-3-O-glucoside even in susceptible barley varieties and a non-enzymatic formation of DON-glutathione adducts that may be suppressing the negative effects of DON (Gardiner et al., 2010). These metabolites as well as DON itself represent the available exposure-based biomarkers.

There has been little work thus far on determining the role, if any, of OTA in the pathogenicity *of Aspergillus ochraceus, Aspergillus niger,* and *Aspergillus carbonarius*. A recent study with *A. thaliana* has shown that detached leaves exposed to OTA produce a hypersensitive-like response, suggesting a defence response similar to the effects of DON (Peng et al., 2010). They demonstrated a biphasic oxidative burst resulting from exposure to OTA with

co-occurring down-regulation of antioxidant enzymes, suggesting that the phytotoxic effects of OTA may be associated with the generation of reactive oxygen species, as seen in animal systems (Baudrimont et al., 1994). Detection of OTA in plants is the only available exposure-based biomarker thus far.

Unlike the other mycotoxins, ZEA is not necessarily detrimental to plants and even has been suggested as a seed treatment for increased yield and seed viability since it increases the percentage of viable generative winter wheat embryos, reviving them from vernalisation (Biesaga-Kościelniak and Filek, 2010; Biesaga-Kościelniak et al., 2010; Kościelniak et al., 2009). The effects of ZEA resemble the effects of auxins, although auxins and ZEA share little structural similarity, unlike the animal associated similarity of oestrogen and ZEA. The stimulative effect of ZEA was shown on winter wheat, winter rape, soybean, and spring wheat (Biesaga-Kościelniak and Filek, 2010). Methods are available for detection of ZEA *in planta* that could facilitate its use as an exposure-based biomarker for more thoroughly evaluating its phytostimulative activity (Biesaga-Kościelniak and Filek, 2010). In animals, ZEA is a substrate for 3-alpha and 3-beta hydroxysteroid dehydrogenase (reviewed in Fink-Gremmels and Malekinejad, 2007), important enzymes in steroid metabolism. The ability of ZEA to interfere with hydroxysteroid dehydrogenases has not been investigated *in planta*. However, in *Arabidopsis* a putative hydroxysteroid dehydrogenase has been shown to be involved in regulating plant growth and development (Li et al., 2007).

FUMONISIN EXPOSURE AND MECHANISM-BASED BIOMARKERS IN PLANTS

The primary producers of fumonisin mycotoxins are *Fusarium verticillioides* and *Fusarium proliferatum*. The predominant cause of maize ear rot and fumonisin contamination in the United

States is *F. verticillioides*, while both species are common causes of ear rot in southern Europe (Logrieco et al., 2002; Munkvold, 2003). *F. proliferatum* is of additional concern due to its broad host range (e.g. banana, maize, fig, mango, pine, sorghum, wheat, and native prairie grasses) (Leslie, 1995; Leslie et al., 2004; O'Donnell et al., 1998) and production of additional mycotoxins (Glenn, 2007). With regard to human and animal exposure to fumonisins through maize and its processed products, *F. verticillioides* is generally regarded as the primary concern since it is the dominant *Fusarium* species commonly infecting maize kernels and stalks (Desjardins et al., 2000; Leslie et al., 1990).

In addition to ear rot, *F. verticillioides* is noted to cause seed rot, seedling blight, root rot, and stalk rot (Kommedahl and Windels, 1981; White, 1999). Development of disease will be dependent on genetic variability of *F. verticillioides* populations, the genetic resistance of maize hybrids, and environmental conditions (Desjardins et al., 1995; Duncan and Howard, 2010; Munkvold, 2003). Additionally, ear rot severity is positively correlated with insect feeding damage (Munkvold, 2003). As a result of such variability, symptomless *F. verticillioides* infections are common, and the fungus is generally considered an endophyte capable of systemic infection of maize (Bacon and Hinton, 1996; Foley, 1962). Also, contamination of symptomless kernels with significant levels of fumonisins can occur (Bush et al., 2004), indicating that visual scoring or sorting of symptomatic kernels may not be sufficient to effectively reduce fumonisin exposure to recommended levels. The role of fumonisin production in the development of F. verticillioides-associated maize diseases has been thoroughly assessed only for ear rot and seedling blight. Detailed studies with fumonisin-nonproducing strains have shown that fumonisin production is not necessary for development of ear rot (Desjardins and Plattner, 2000; Desjardins et al., 1998, 2002). In relation to maize seedling blight, fumonisins were suggested to increase

the virulence of *F. verticillioides* but were not necessary or sufficient for disease development (Desjardins et al., 1995). More recently a significant positive correlation was demonstrated between leaf lesion development on maize seedlings and the production of FB_1 by *F. verticillioides* (Williams et al., 2006, 2007). Also shown in these studies was a significant inverse correlation between root weight and stalk height and the amount of FB_1 associated with seedling roots. Most notably, fumonisin-nonproducing strains did not cause leaf lesions and had significantly less effect on root weight and stalk height. Similar to the inoculation studies, watering uninoculated maize seedlings with FB_1 caused leaf lesions and a significant dose-dependent reduction in root weight and stalk height. Glenn et al.(2008) provided conclusive evidence supporting the role of fumonisin production on maize seedling disease development. A fumonisin non-producing and non-pathogenic strain possessing a deletion of the fumonisin biosynthetic gene cluster was genetically complemented by transformation with the gene cluster. The complemented transformants were able to produce fumonisins and caused the full suite of seedling disease symptoms. Differences in disease development susceptibility were noted between varieties of maize, with sweet corn genotypes being more susceptible than dent genotypes (Glenn et al., 2008). Desjardins et al.(2005) noted differential sensitivity of maize genotypes to germination and growth on various concentrations of FB_1 and postulated that sensitivity is likely the ancestral trait in maize, with insensitivity being a derived and inheritable trait.

These studies have facilitated the development of both exposure and mechanism-based biomarkers for evaluating the role of fumonisin in maize-*Fusarium* interactions. For example, an important initial physiological process leading to foliar disease development, as well as adverse effects on plant growth, is the absorption and preferential accumulation of FB_1 by roots

(Williams et al., 2007; Zitomer et al., 2010). Approximately 10 times the amount of FB1accumulated in roots compared to fumonisin B2 when growing seedlings were watered with a combined solution of both fumonisins (Zitomer et al., 2010).

Such accumulation of FB_1 in maize roots was shown to cause disruption of sphingolipid metabolism. Building on the development of fumonisin-related biomarkers in animals and humans as outlined above, elevated concentrations of sphingoid bases (sphinganine and phytosphingosine) and sphingoid base 1-phosphates (sphinganine 1-phosphate and phytosphingosine 1-phosphate) due to inhibition of ceramide synthase by FB_1 was shown in both roots and leaves of maize seedlings (Williams et al., 2006, 2007; Zitomer et al., 2008, 2010). These analyses provided useful mechanism-based biomarkers for evaluating cellular effects of toxicity and were the first to document fumonisin related elevation of the 1-phosphates in plants. Elevated concentrations of sphinganine and phytosphingosine were previously observed in *Nicotiana*, Lemna, and AAL toxin susceptible varieties of *Lycopersicon* (tomato) species, with the increase in sphingoid bases occurring prior to the onset of disease symptoms (Abbas et al., 1994). Such increases were not observed in AAL toxin resistant varieties of tomato that were exposed to fumonisin. AAL toxin is a structural analogue of FB_1, and as with FB_1, the cellular effects of AAL toxin are associated with disruption of sphingolipid metabolism and accumulation of sphingoid bases in the tissues of susceptible varieties of tomato (Abbas et al., 1994). Pharmacological evidence supports the hypothesis that AAL phytotoxicity initially results from the accumulation of sphingoid bases and not the depletion of complex sphingolipids (Spassieva et al.,2002). Myriocin is an inhibitor of serine palmitoyltransferase, the first enzyme in the de novoceramide biosynthesis pathway that produces sphingoid bases, which are then utilised by ceramide synthase. Co-exposure of susceptible tomato plants to AAL toxin and

myriocin provided a protective effect, reducing phytotoxicity by preventing the buildup of sphingoid bases (Spassieva et al.,2002) and their subsequent metabolism to sphingoid base 1-phosphates.

Williams et al.(2006, 2007) and Zitomer et al.(2008, 2010) have examined the differential effects of FB_1 alone versus *F. verticillioides* infection of maize tissues and the *in planta* production of FB_1. Interesting distinctions were observed and are summarised in Figure 2.3. Both experimental treatments resulted in accumulation of fumonisin in the roots and inhibition of ceramide synthase as assessed from elevated sphingoid bases and sphingoid base 1-phosphates as mechanistic biomarkers. Yet, only the inoculation experiments detected fumonisin accumulation in the leaves (Zitomer et al., 2008); the fumonisin watering experiments indicated no fumonisin accumulation in the leaves (Zitomer et al., 2010). Furthermore, FB1was the predominant fumonisin found in the leaves from the inoculation experiments, with only trace amounts of fumonisin B2 and fumonisin B3 detected. Both treatments resulted in accumulation of sphinganine, phytosphingosine, and their 1-phosphates in the leaves. Collectively the data suggest that transpiration mediated shootward movement of fumonisins, sphingoid bases, and sphingoid base 1-phosphates from roots to leaves does not occur as chemically expected. The water-soluble fumonisins have limited movement while the much less soluble sphingoid bases and 1-phosphates appear to be readily translocated from roots to leaves, suggesting the need for some mechanism other than passive translocation through the plant, such as carrier molecules. Furthermore, while fumonisin is required for leaf lesion development in maize seedlings, such disease progression occurs via a mechanism that does not require either endophytic fungal colonisation or fumonisin accumulation in the leaves. Lesion development may occur as a consequence of disrupted sphingolipid metabolism in the roots and subsequent mobilisation of

signal molecules. The sphingoid bases and/or their 1-phosphates may be those molecules, as suggested for AAL toxin (Spassieva et al.,2002), but data are needed to support this hypothesis and the mechanisms leading to leaf lesion formation. Overall these studies demonstrate the information that can be gained using *in planta* biomarkers.

Numerous studies have utilised FB_1 as a research tool for studying the molecular events associated with pathogen induced programmed cell death in *A. thaliana* (Watanabe and Lam, 2006; Chivasa et al., 2005; Norholm et al., 2006; Kuroyanagi et al., 2005; Bindschedler et al., 2006; Lin et al., 2008; Shi et al., 2007). Collectively, these studies provide additional evidence for the FB1inhibition of ceramide synthase and disruption of sphingolipid metabolism as the proximate cause of *F. verticillioides* induced diseases in maize. Interestingly, these studies show that the response to FB_1, a fungal metabolite, mimics the responses of plants to infectious pathogens such as *Pseudomonas syringae* (Norholm et al., 2006). Not surprisingly, many of the affected systems in plants are similar to those in fumonisin exposed animals, such as induction of oxidative stress (Shi et al., 2007; Harvey et al., 2008; Bindschedler et al., 2006). One of the systems affected by FB_1 in *Arabidopsis* is depletion of extracellular ATP which could be expected given the high levels of sphingoid base 1-phosphates produced in maize plants when ceramide synthase is inhibited and given the fact that sphinganine production is uncoupled from ceramide production in fumonisin-treated cells.

Another potential downstream effect of the fumonisin induced accumulation of sphingoid bases in maize, particularly phytosphingosine, is disruption of stomatal regulation. As demonstrated in *Arabidopsis*, sphingosine kinase is an important component of abscisic acid induction of stomatal closure by phosphorylating sphingosine and phytosphingosine to their respective 1-phosphates (Coursol et al.,2003, 2005). These 1-phosphates were shown to both

induce stomatal closure and inhibit stomatal opening. As outlined above, phytosphingosine and phytosphingosine 1-phosphate significantly increase in concentration in maize leaf tissues when plants are inoculated with fumonisin producing *F. verticillioides* or when uninoculated plants are exposed to fumonisin alone. The potential therefore exists that FB_1-induced elevation in sphingoid base 1-phosphates may affect stomatal regulation as they accumulate in leaves.

CONCLUSIONS

The economic cost and health risks due to mycotoxin contamination has provided the impetus for research to better understand the dose-response relationships between mycotoxin exposure and increased risk of disease in humans and farm animals. Development and validation of exposure and mechanism-based biomarkers is critical for the accurate assessment of disease risk. This is best exemplified with aflatoxins, where biomarkers have been successfully used to establish the role of aflatoxins in human disease and to assess the effectiveness of interventions (Kensler et al., 2011). Development and validation of biomarkers for other mycotoxins has progressed considerably, but has not yet reached the same level of practical utility as the biomarkers developed for aflatoxin. For example, the extensive use of elevated sphingoid bases and their metabolites as biomarkers in farm and laboratory animals has validated the dose-response relationships between inhibition of ceramide synthase and the fumonisin-induced disruption of cellular processes leading to the various farm animal diseases and carcinogenicity of FB_1 in rodents. Progress has also been made to assess individual exposure in humans using urinary fumonisin, but definitively linking exposure to disease has been unsuccessful largely due to the lack of a validated mechanism-based biomarker in humans. Extension of this knowledge base to experiments addressing the effects of fumonisin on maize and other plants has demonstrated that

inhibition of ceramide synthase is the key event in the mechanism of action resulting in plant disease. This information has resulted in useful biomarkers for studies on disease development and the potential for altered physiological responses in maize that could contribute to susceptibility to other risk factors for diseases. The biomarkers may also prove beneficial to monitoring efforts since their early detection may provide management options for limiting plant disease and accumulation of fumonisin in maize products. Application of similar biomarker development strategies to other plant-fungal systems involving mycotoxigenic fungi could lead to a better understanding of the underlying phytopathology and also the potential of mycotoxins to modulate basic physiological processes that could contribute to the susceptibility of plants to environmental stress and disease.

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Table 2.1. Current Status of Biomarker Development in Animals and Humans. For each mycotoxin the likely mechanism of action is given followed by currently available exposure and mechanism-based biomarkers[1].

Mycotoxin	Likely Mechanism of Action	Biomarker	Location	Type	Species	Status
AFB	Metabolism of AFB_1 by cytochrome P450 to intermediates, namely AFB_1 - 8,9-epoxide, which bind to DNA and proteins forming adducts	AFB_1-DNA adducts	Urine Tissue	Exposure & mechanistic	Farm Lab Human	+++ +++ +++
		AFB_1-albumen adducts	Blood	Exposure	Farm Lab Human	+++ +++ +++
		DNA mutation in codon 249 (p53 tumor suppressor gene)	Blood Tissue	Mechanistic	Farm Lab Human	+++ +++ +++
		AFM_1	Milk Urine	Exposure	Farm Lab Human	+++ +++ +
DON	Induces proinflammatory cytokines via MAPK pathways, induces expression of suppressor of cytokine signalling (SOCS), impairs expression of hepatic insulin-like growth factor acid labile subunit (IGFALS) and insulin-like	DON and DON-glucuronide	Urine Tissue Feces	Exposure	Farm Lab Human	++ +++ +++
		Proinflammatory cytokines	Blood Tissue	Mechanistic	Farm Lab Human	+++ +++ ++
		Up-regulation of hepatic SOC3 and mRNA/protein	Tissue	Mechanistic	Farm Lab Human	+ +++ +
		Decrease of IGFALS and IGF1	Blood	Mechanistic	Farm Lab Human	+ +++ +

	growth factor 1 (IGF1) Ribotoxic stress response caused by DON-induced activation of p38, JNK and ERK via RNA-activated protein kinase (PKR)- and hematopoietic cell kinase (Hck)-dependent mechanisms					
FB	Disruption of sphingolipid metabolism though inhibition of ceramide synthase	Elevation of sphingoid base and sphingoid base 1-phosphate and depletion of more complex sphingolipids	Blood Tissue	Mechanistic	Farm Lab Human	+++ +++ +
		Levels of FB_1	Urine Feces Hair	Exposure	Farm Lab Human	+++ +++ +
OT	OTA disrupts phenylalanine related processes (i.e., phenylalanine metabolism, protein synthesis, etc. Disrupts signalling pathways and processes regulating cell growth and survival. OTA-DNA adducts	OTA or OTA metabolites/conjugates	Blood Urine Tissue	Exposure	Farm Lab Human	+++ +++ ++
		OTA-DNA adducts (controversial)	Tissue	Mechanistic	Farm Lab Human	++ ++ ++

		Metabonomic assessment of metabolite changes	Blood Urine Tissue	Mechanistic	Farm Lab Human	+++ ++ +
ZEA	Effects are based on similarities to estrogen. Conversion to alpha ZOL exacerbates estrogenic effects of ZEA. Both ZEA and alpha ZOL disrupt steroid metabolism.	ZEA, ZOL, and ZAL	Urine Feces	Exposure	Farm Lab Human	+++ +++ +
		Glucuronic acid-conjugates	Urine Feces	Exposure	Farm Lab Human	+++ +++ +
		Endocrine disruption	Tissue	Mechanistic	Farm Lab Human	+++ ++ +

[1.] The column labeled “location” indicates the biological material in which the biomarker is detected. Tissue is normally liver or kidney, but other organs are also possible. Blood includes serum, plasma and/or whole blood. For simplicity, the column labeled “Species” includes “Farm” (animals raised for commercial purposes), “Lab” (studies in laboratory animals), and “Human” (development of human biomarkers). Current status is given for “Species” and are indicated as follows; + = theoretically possible but not convincingly demonstrated, ++ = *in vitro* experimentation only or *in vivo* studies without dose-response relationships for the biomarkers, +++ = correlation demonstrated between exposure and/or disease incidence and the biomarker.

Figure 2.1. A generalised scheme for ranking the current status of biomarker discovery and validation for aflatoxins, fumonisins, deoxynivalenol, ochratoxin A, and zearalenone. This scheme is based on that of Groopmanet al. (2008) but focuses in a generic way on discovery, development and validation of both exposure biomarkers and mechanism-based biomarkers. Each box represents a step in the discovery and experimental validation of the biomarkers with the ultimate goal being the demonstration of the potential for the mycotoxin to be a cause or contributing factor to animal or human disease.

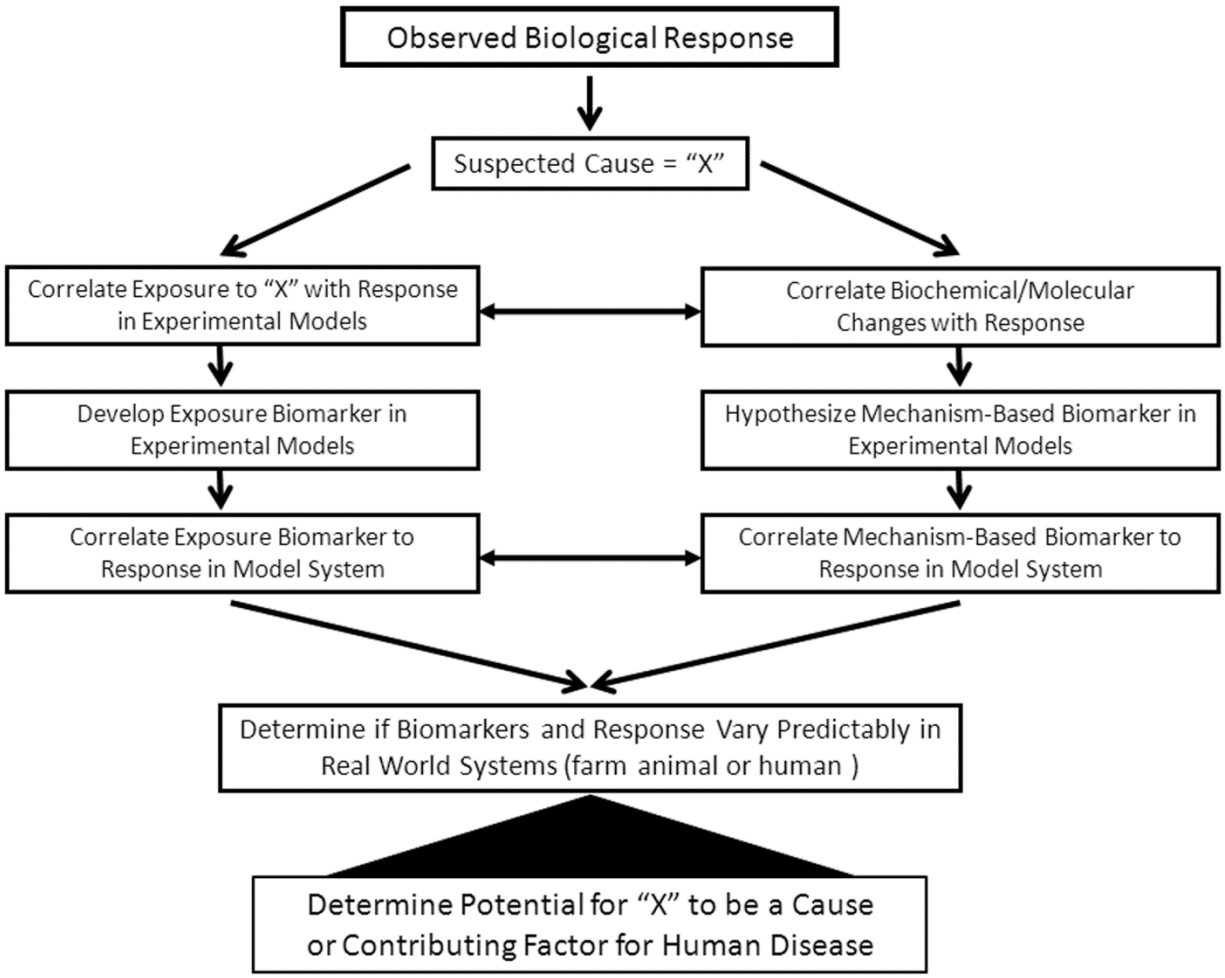

Observed Biological Response
Suspected Cause = "X"
Correlate Exposure to "X" with Response in Experimental Models
Correlate Biochemical/Molecular Changes with Response
Develop Exposure Biomarker in Experimental Models
Hypothesize Mechanism-Based Biomarker in Experimental Models
Correlate Exposure Biomarker to Response in Model System
Correlate Mechanism-Based Biomarker to Response in Model System
Determine if Biomarkers and Response Vary Predictably in Real World Systems (farm animal or human)
Determine Potential for "X" to be a Cause or Contributing Factor for Human Disease

Figure 2.2. Flow chart for development of *in planta* biomarkers resulting from exposure of plants to mycotoxins. Mycotoxins gain entry to the plant from either fungal infection or environmental exposure (e.g. uptake from the soil). The initial mechanism of action is the first specific interaction that is required for disruption of metabolic and cellular processes. The resulting changes in levels of metabolites and regulatory molecules directly linked to the mechanism of action are the best candidates for mechanism-based biomarkers (shaded boxes). The parent mycotoxin and/or its metabolites are the best candidates for exposure-based biomarkers (white boxes) and should be easily detected in plant tissues. The *in planta* accumulation of the parent mycotoxin, its metabolites, and the resulting evidence of disruption of metabolic and regulatory processes should correlate in a dose-dependent manner in order to be validated as biomarkers.

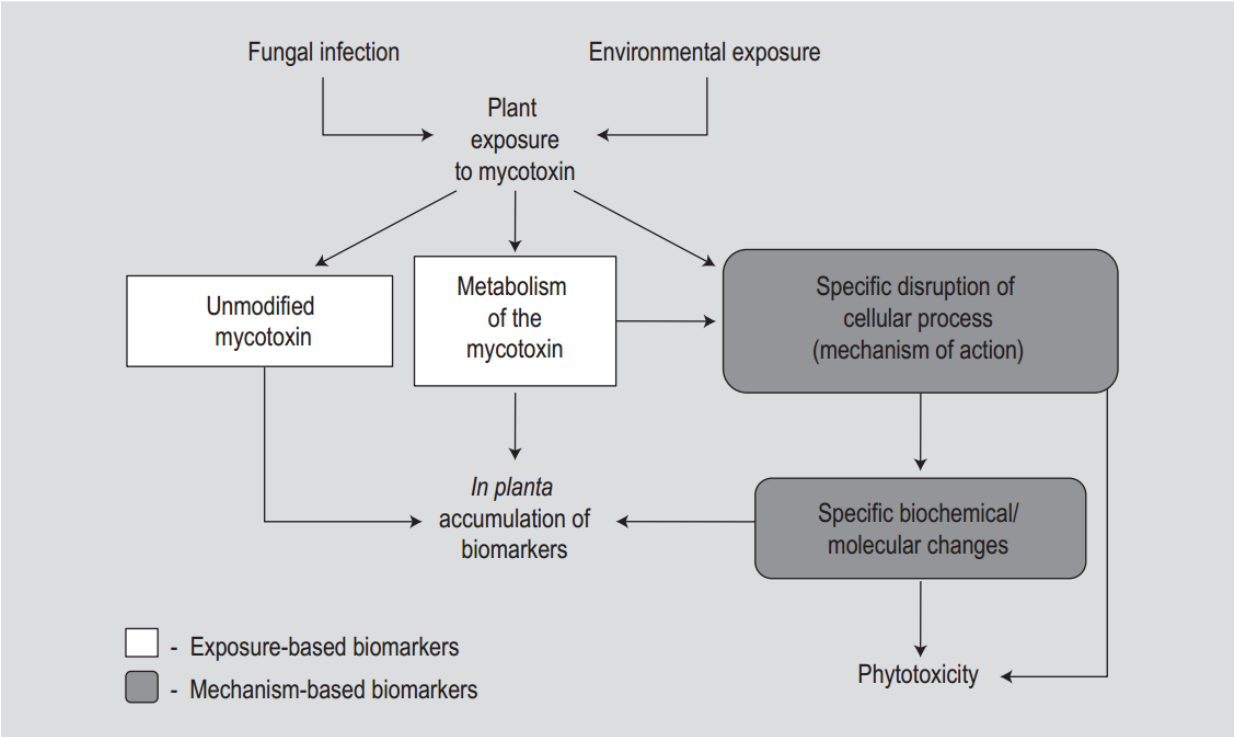
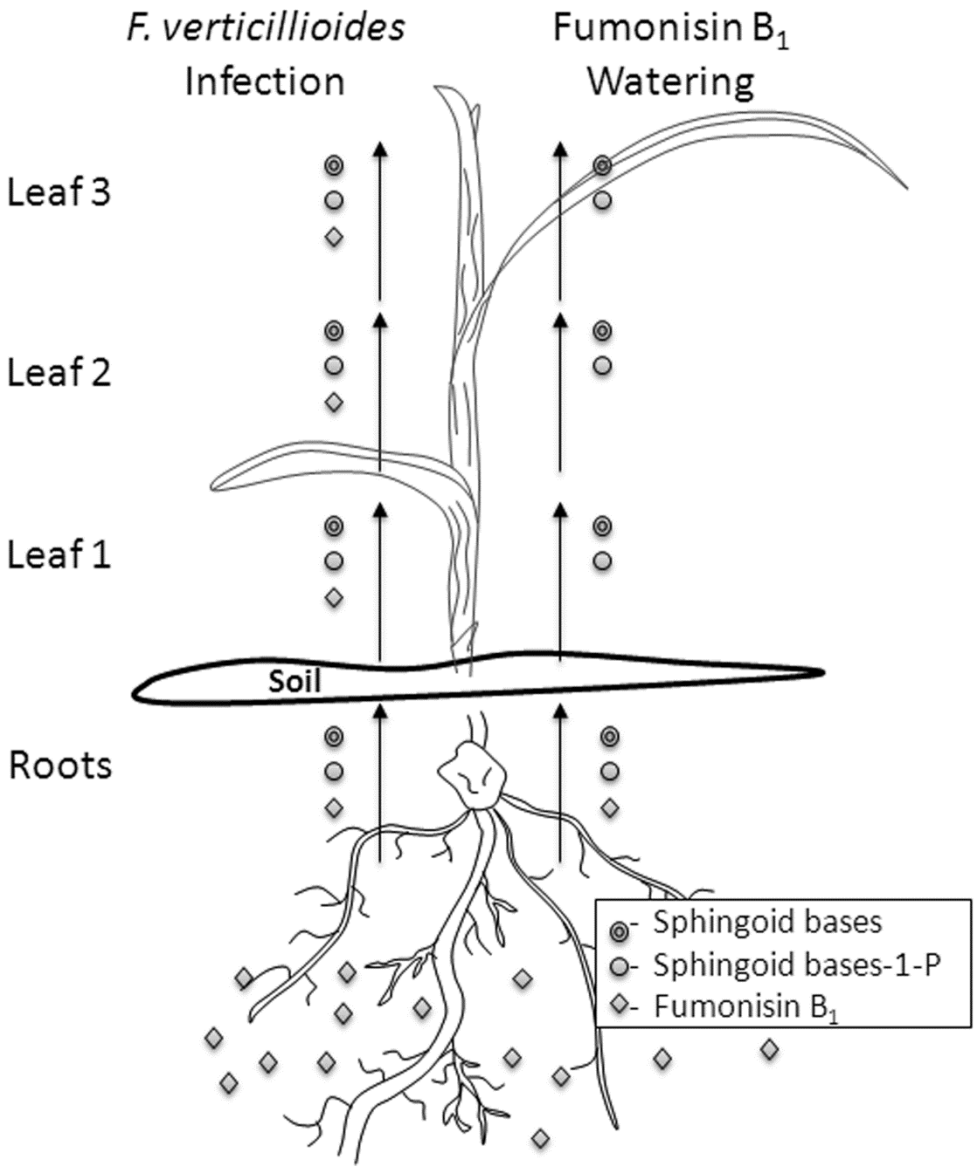

Fungal infection
Environmental exposure
Plant exposure to mycotoxin
Unmodified mycotoxin
Metabolism of the mycotoxin
Specific disruption of cellular process (mechanism of action)
In planta accumulation of biomarkers
Specific biochemical/ molecular changes
Phytotoxicity
- Exposure-based biomarkers
- Mechanism-based biomarkers

Figure 2.3. *In planta* Biomarker Detection in Maize Seedlings in Response to Fumonisin Exposure. The figure is based on data from Zitomer *et al.* (2008, 2010) and indicates detection of FB_1, sphingoid bases, and sphingoid base 1-phosphates in seedling tissues. Experiments consisted of either *F. verticillioides* infection of the seedlings (left) or exposure to only water-dissolved FB_1 (right). Infections resulted from planting seed inoculated with *F. verticillioides* (approx. 2500 conidia/seed). In the watering experiment, pots of 10 uninoculated seedlings were exposed to a total of 4.71 mg FB_1. Both treatments resulted in high accumulation of FB_1 in the soil. FB_1 also accumulated in the roots, causing inhibition of ceramide synthase and increased accumulation of sphingoid bases and sphingoid base 1-phosphates. Use of these exposure and mechanistic biomarkers highlights the different effects resulting from infection (presence of fungal hyphae producing FB_1 *in planta*) compared to presence of FB_1 solely. *F. verticillioides* infected seedlings accumulated noticeable levels of FB_1 in the 1st, 2nd, and 3rd leaves (and very little FB_2 or FB_3), while no FB_1 was detected in the any of the leaves from the FB_1-watering treatment.


F. verticillioides
Infection
Fumonisin B1
Watering
Leaf 3
Leaf 2
Leaf 1
Soil
Roots
- Sphingoid bases
- Sphingoid bases-1-P
- Fumonisin B1

CHAPTER 3

MAIZE SEEDLING BLIGHT INDUCED BY *FUSARIUM VERTICILLIOIDES* INVOLVES ACCUMULATION OF FUMONISIN B1 IN LEAVES WITHOUT COLONIZATION OF THE LEAVES

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ABSTRACT

Studies have shown that the fumonisin B_1 (FB_1) mycotoxin is necessary for development of leaf lesions and other disease symptoms during maize seedling infection by *Fusarium verticillioides*. Similarly, watering uninfected seedlings with an aqueous FB_1 solution also results in leaf lesions, yet the FB_1 was not detected in the leaves. The objectives of this study were to thoroughly address FB_1 mobility and accumulation in seedlings and determine if proximal infection by *F. verticillioides* is necessary for accumulation. Tissue and soil samples were analyzed to compare wild-type *F. verticillioides* against an aconidial mutant. Inoculation with either strain caused lesion development and accumulation of FB_1 in leaves, but the mutants were unable to colonize blighted leaves. FB_1, FB_2 and FB_3 were detected in the soil and seedling roots, but only FB_1 was detected in the leaves of any treatment. Such accumulation may be mediated by transpiration since abscisic acid-mediated stomatal closure reduced FB_1 uptake into the roots in an *in vitro* assay. Also, differential root accumulation of FB_1 was observed between maize genotypes. Our data suggest root infection by *F. verticillioides* is necessary for accumulation of FB_1 in leaves, and while the mechanism for accumulation is not yet clear, it does not require colonization of the leaves.

INTRODUCTION

The phytopathogenic and mycotoxigenic ascomycete *Fusarium verticillioides* is the primary fungal pathogen associated with maize cultivation worldwide. Maize can become infected with *F. verticillioides* at all growth stages, causing several types of disease, including seedling blight, stalk rot, seed rot, root rot, and kernel or ear rot (Kommedahl and Windels, 1981). Infection of maize often precipitates the production and accumulation of various mycotoxins, including fumonisins, fusaric acid, and fusarin C (Bacon and Hinton, 1996; Fotso et al., 2002; Giesbert et al., 2008; Marasas et al., 1986; Wiebe and Bjeldanes, 1981). Of these mycotoxins, fumonisins are the most environmentally common and can occur in high concentrations in maize. There are over 28 isomers of fumonisins, although most are rare and occur naturally in low concentrations (Bartok et al., 2010). Fumonisin B_1 (FB_1) is the most important of this group, making up a large portion of the total fumonisins, followed respectively by FB_2 and FB_3. FB_1 is also the most toxicologically active. FB_1 contamination of field-grown maize has been determined as the causal agent in farm animal diseases, including leukoencephalomalacia (Marasas et al., 1988) and porcine pulmonary edema (Colvin and Harrison, 1992), and human consumption of fumonisin-contaminated maize has been associated with human cancer (IARC, 2002) and neural tube defects (Marasas et al., 2004).

The mechanism of fumonisin toxicity involves inhibition of ceramide synthase and disruption of sphingolipid metabolism resulting in accumulation of toxic levels of sphingoid base metabolites and depletion of complex sphingolipids (Wang et al., 1991). Fumonisins are structural analogs of the sphingoid bases, and are also similar in structure to another well-studied fungal phytotoxin from *Alternaria alternata* f. sp. *lycopersici*, the AAL toxin. Previously demonstrated to inhibit ceramide synthase as well, the AAL toxin is necessary for the formation

of stem cankers on susceptible tomato (Abbas et al., 1994; Brandwagt et al., 2000; Spassieva et al., 2002). Functioning with effects similar to the AAL toxin, it has been shown that fumonisin is phytotoxic (Glenn et al., 2008) and may act to suppress plant defense mechanisms in *Nicotiana* and maize (Sanchez-Rangel et al., 2012; Vicente et al., 2013).

The role of fumonisins in the infection process is not fully understood and may play multiple and different roles depending on the mode of *F. verticillioides* infection. For example, fumonisins are not necessary or sufficient for ear rot development in maize (Desjardins et al., 2002; Desjardins et al., 1998). Yet, seedling blight development symptoms, including reduced root weight, stunting, and the appearance of leaf lesions and leaf abnormalities, require fumonisin production. This was demonstrated by Glenn et al. (2008) when seedling pathogenicity was successfully rescued from a non-fumonisin-producing strain by complementing the ability to produce fumonisin with insertion of the *FUM* gene cluster.

Furthermore, the correlation was determined between levels of fumonisin production by strains of *F. verticillioides* and occurrence of the leaf lesions in seedling blight infections (Williams et al., 2007). In that study, the aconidial strain AEG 3-A3-6 was noted to produce fumonisins and cause leaf lesions, but was inferred to lack the ability to colonize the aerial tissues of the host. Questions arose from that study on the correlation between leaf lesions and occurrence of fumonisins. It was assumed that leaf lesions were a result of fumonisin accumulation in the tissues, but this was unconfirmed. Subsequent investigation of the aerial tissues by analyzing the first, second, and third leaves determined the highest fumonisin accumulation occurred in the first leaf of maize seedlings grown from seed inoculated with *F. verticillioides* (Zitomer et al., 2008). Accumulation of fumonisins in these tissues is noted as containing a significantly higher ratio of FB_1 to FB_2 and FB_3 compared to the observed

accumulations in the roots (Zitomer et al., 2008), suggesting a mechanism of either greater accumulation of FB_1 over the other isomers by selective transport or by selective production of FB_1 by the fungus colonizing the leaf. Further questions arose about the mechanism of fumonisin accumulation when uninoculated seedlings were watered with FB_1 and FB_2 without detectable accumulation in the leaf tissues (Zitomer et al., 2010), thus suggesting the fungus is essential for accumulation of FB_1 in the leaves. In this study we address the colonization and fumonisin accumulation discrepancies of why FB_1 does accumulate in leaves infected with *F. verticillioides*, yet does not accumulate in leaves when uninfected seedlings are watered with an aqueous FB_1 solution. Additionally, we also address the potential necessity of conidia for colonization of the seedlings. A holistic sampling strategy was utilized to address the following questions: (i) what is the extent of colonization in maize seedlings when seeds are inoculated with fumonisin-producing and non-producing strains in combination with wild-type conidiation versus aconidial phenotypes, (ii) what is the resulting fumonisin accumulation in those seedlings, (iii) is the pattern of fumonisin accumulation and *F. verticillioides* colonization different in fumonisin-insensitive compared to -sensitive maize lines, and (iv) does transpiration have a possible role in the movement and accumulation of FB_1?

MATERIALS AND METHODS

Chemicals. Acetonitrile (MeCN) (Burdick & Jackson, Muskegon, MI, USA) and water (J.T. Baker, Phillipsburg, NJ, USA) were HPLC grade, and formic acid (>95%) (Sigma-Aldrich, St. Louis, MO, USA) was reagent grade. Fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) for standard preparation were provided as a gift from Ronald Plattner, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA. D-erythro-C_{17}-sphingosine-1-

phosphate (C_{17}-So 1-P), D-ribo-phytosphingosine-1-phosphate (Pso 1-P), D-erythro-sphingosine 1-phosphate (So 1-P), and D-erythro-dihydro-sphingosine-1-phosphate (Sa 1-P) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Phytosphingosine (t18:0) (Pso), DL-erythro-dihydro-sphingosine (d18:0) (Sa), and D-erythro-sphingosine (d18:1) (So) were purchased from Sigma-Aldrich. D-erythro-C_{16}-sphingosine (C_{16}-So) was from Matreya (Pleasant Gap, PA, USA). Stock solutions, working standards, and internal standards were prepared as previously described [22]. Sucrose (Sigma-Aldrich, St. Louis, MO, USA), Murashige and Skoog Basal Medium Macrosalts and Microsalts (Sigma-Aldrich, St. Louis, MO, USA) and Bacto™ Agar (Difco Lab., Sparks, MD, USA) were utilized for the plant nutrient agar.

Extraction and analysis of fumonisins, sphingoid bases, and sphingoid base 1-phosphates. The extraction procedure has been reported previously (Zitomer et al., 2008). Briefly, lyophilized maize leaf tissues were ground to a powder, spiked with C_{16}-So and C_{17}-So 1-P internal standards (10 µL each at 100 ng/µL), and then extracted with an aliquot of 1:1 MeCN:water + 5% formic acid (1 mL per 10 mg tissue). The filtered extracts (100 µL aliquots) were diluted into the initial mobile phase (900 µL) used for reverse phase-high performance liquid chromatography (RP-HPLC). Soils were extracted in a similar manner in 1:1 MeCN:water + 5% formic acid (25 mL per 2 g).

All analyses were performed as described previously (Zitomer et al., 2008). Analyses were conducted using a Finnigan Micro AS autosampler coupled to a Surveyor MS pump (Thermo-Fisher, Woodstock, GA, USA). Separation was accomplished using an Imtakt Cadenza 150×2 mm i.d., 3 µm CW-C18 column (Imtakt, Philadelphia, PA, USA). Column effluent was coupled to a Finnigan LTQ linear ion trap mass spectrometer (MS).

Fungal strains. The four strains of *F. verticillioides* used in this study were JFL-A04516 (JFL = John F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan, KS, USA), AEG 3-A3-2 (AEG = Anthony E. Glenn, Russell Research Center, USDA-ARS, Athens, GA, USA), AEG 3-A3-5, and MRC826 (Medical Research Council, Tygerberg, South Africa). Both AEG strains have an inability to produce conidia (Glenn et al., 2004). AEG 3-A3-5 produces fumonisins while AEG 3-A3-2 does not. Strain JFL-A04516 also does not produce fumonisins due to a mutation in *FUM1*, a polyketide synthase necessary for fumonisin production (Desjardins et al., 2002). MRC826, as our positive control, is wild type for fumonisin production and conidiation.

Maize line and maize seedling assay. The maize lines used were Silver Queen (Gurney's Seed & Nursery Co., Yankton, SD, USA), a sweet corn line that is susceptible to *F. verticillioides*-induced maize seedling disease (William et. al., 2007); W23 (The Germplasm Resources Information Network (GRIN), accession number NSL 30060), a resistant field corn inbred; and B73 (GRIN, accession number Bc5S4), a moderately resistant field corn inbred. Maize kernels were treated as described previously (William et. al., 2007). Briefly, kernels were surface-disinfected for 10 min in 100% commercial bleach (5.25% hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water. The seeds were then heat shocked by placing them in a 60 °C water bath for 5 minutes for internal sterilization (Bacon and Hinton, 1994). Seeds were inoculated with a 10^4 spores per mL suspension and incubated overnight at 27 °C. Three replicates of 10 seeds each were planted in sterile 10-cm plastic azalea pots (Hummert

International, Earth City, MO, USA) containing twice-autoclaved commercial potting soil mix (Conrad Faford Inc., Agawam, MA, USA).

The treatments were as follows: SQ/Control, SQ/MRC 826, SQ/AEG 3-A3-2, SQ/AEG 3-A3-5, SQ/JFL-A04516, W23/Control, W23/MRC826, B73/Control, and B73/MRC826. Pots were watered as needed throughout the duration of the assay. Assays were performed under aseptic conditions in a plant growth chamber at 30 °C under 14 h light (cool-white, high-output fluorescent tubes at an average of 254 µmol/m^2/s) and a 10-h dark regimen at 20 °C. Plant tissues were collected for analysis 14 days after planting. During harvest, the shoot portions of the seedlings were separated into first, second, and third leaves (numbered in order of their emergence and expansion) for each replicate pot. Leaves were excised approximately 5 mm from the ligule, such that all portions of the sheath were excluded from the analyses. After excision of the leaves, 2-3 cm section of the leaf was excised and stored for culturing. An approximate 3- to 4-cm section of the stem between the soil surface and the first leaf was also excised and stored for culturing. Root tissues were also collected and processed as described previously (William et. al., 2007). During root harvest, a section of the mesocotyl (as large as possible) was also excised and stored for culturing. All tissues removed for culturing were stored briefly at 4 °C. The root and leaf tissues to be analyzed for fumonisins, sphingoid bases, and sphingoid base 1-phosphates were immediately frozen at -20 °C.

***In planta* colonization.** Plant tissues reserved for culturing were surface sterilized according to tissue type. Leaves were sterilized in 10% bleach solution for 10 minutes followed by a 95% ethanol wash for 5 minutes. Stems and mesocotyls were sterilized in 20% bleach for 15 minutes followed by a 95% ethanol wash for 10 min. Tissues were rinsed 5 times with sterile distilled

water (dH_2O) then plated on potato dextrose agar (PDA, Sigma, St. Louís, MO, USA) and incubated at 27 °C to assess for internally colonizing fungal strains. Tissue infection was observed after 6 days and checked again after 10 days to allow for any slow-growing fungal strains. Fungal isolates were visibly identified to genus, and to species if possible. *Fusarium* isolates were microscopically examined for production of conidia, following culture on PDA.

Soil colonization. In the first trial, soil was collected from each individual pot and air dried for 2 weeks in open plastic bags. After 2 weeks, 1 g of soil was added to 100 mL of autoclaved dH_2O and placed on a 250 rpm rotary shaker for 10 minutes. A 100,000-fold dilution was made of each soil sample and 100 µL each of this dilution was plated in triplicate on PDA. Plates were incubated at 27 °C for 4 days. After 4 days, colony-forming units (CFU) were identified as *Fusarium* or other genera. The second trial was quantified in the same manner, but was performed using wet soil and sampled immediately at the end of the trial to detect fungi that may have been sensitive to desiccation in the first trial.

***In vitro* test tube exposure of FB_1.** Maize kernels were sterilized and imbibed as described previously (Williams et al., 2007). Lines utilized were Silver Queen and W23. For this trial only we also assessed Tama Flint (GRIN, accession number NSL 217411), which was previously noted to be very insensitive to FB_1 (Desjardins et al., 2005). Individual kernels were placed in culture test tubes (Kimble glass co., 25 x 150 mm) with 4 mL of plant agar media (Bohorova et al., 1999). Maize seedlings were grown for 7 days under the same growth chamber conditions noted above and culled for fungal contamination. Using a syringe, FB_1 (10 µM), abscisic acid (ABA; 200 µM), or FB_1 and ABA (10 µM and 200 µM) was injected into the media. Plants were

harvested after 24 and 48 hours by separating the leaves and stems from the roots. Care was taken not to allow the leaves or stems to have contact with the FB_1 containing agar media. Samples were lyophilized and extracted as mentioned above to assess FB_1 accumulation in the plant.

Statistical analysis. Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). When many groups were compared, one-way analysis of variance was used, followed by post-hoc multiple comparisons. Except where indicated otherwise, all data were expressed as mean ± standard deviation, and differences among means were considered to be significant if the probability (P) was ≤ 0.05. All results for plant tissues are expressed as dry weight.

RESULTS AND DISCUSSION

We hypothesized fumonisin is mobile *in planta*, accumulates in the leaves, and requires the presence of the fungus in the roots but doesn't require direct fungal colonization of the leaves. Thus it should follow that fumonisin accumulation in the leaves is likely via a plant derived mechanism impacted by the fungus in a manner other than direct colonization of the leaves. In order to assess colonization and co-localization of fumonisin accumulation during seedling infection, the sweet corn cultivar Silver Queen was inoculated with *F. verticillioides* strains MRC826, JFL-A04516, AEG 3-A3-2, or AEG 3-A3-5. Silver Queen is highly susceptible to fumonisin-related seedling blight (Glenn et al., 2008; Williams et al., 2006; Williams et al., 2007). Additional maize genotypes were assessed, and their differing features are summarized in Table 3.1. Results are presented separately for trial 1 and trial 2 since these data could not be

grouped statistically because of significant differences in colonization rates and fumonisin accumulation between trials. Both trials independently show patterns that support to the same conclusions despite the significant biological variation. Silver Queen seedlings demonstrated a clear correlation between seed inoculation with fumonisin-producing strains and development of disease symptoms (Table 3.2). We also assessed the fumonisin insensitive inbreds W23 and B73 in trial 1 only. These seeds were generally contaminated with several naturally occurring species, such as *Acremonium*, *Aspergillus*, *Penicillium*, and *Fusarium*, despite our attempts to sterilize the seed. When inoculated with MRC826, B73 showed no symptoms of seedling blight. Similarly, W23 had very few seedlings with disease symptoms.

Soil analysis was performed to correlate the ability of *F. verticillioides* to colonize the soil during seedling blight infection, with and without the ability to produced condia and/or fumonisins. Colonization of the dried soil from trial 1 was significant for MRC826 and JFL-A04516. In contrast, no colonization was observed for AEG 3-A3-2 and AEG 3-A3-5 (Figure 3.1A) even at low serial dilutions. For inbreds B73 and W23, soil colonization was observed in both control and MRC826 treatments, which was not surprising given the seed contamination noted above. When performing the second trial, wet soil was sampled to determine if the aconidial strains AEG 3-A3-2 and AEG 3-A3-5 were more susceptible to desiccation effects on viability (Figure 3.1B). Soil colonization was noted for AEG 3-A3-2 and AEG 3-A3-5, even though the CFU calculations were 250- to 500-fold less compared to the conidia-producing wild-type MRC826 (Figure 3.1B). The drying procedure in the first trial apparently did affect the viability of these aconidial strains, likely due to the lack of drought resistant structures. Other than this difference in how the soils were sampled for colonization (dry versus wet), all other experimental protocols were identical between Trials 1 and 2.

Figure 3.2 show fumonisin accumulation in the soil, and Figure 3.3 in the roots. Trials 1 and 2 provided similar results, where only fumonisin-producing strains resulted in fumonisin accumulation in the soil or the root tissues. Relatively small amounts of fumonisins were detected in soils from inbreds B73 and W23. Only a small amount of FB_1 (0.34 ± 0.2 nmol/g) was detected in the roots of W23 inoculated with MRC826. Despite the lower soil colonization detected for AEG 3-A3-5, the levels of fumonisin contamination in the soil were not statistically different from MRC826 (Figure 3.2). A similar trend was noted for fumonisin in roots (Figure 3.3). Statistical differences were noted for fumonisin accumulation in roots of MRC826 and AEG 3-A3-5 in trial 2 (Figure 3.3B), suggesting that fumonisin production may be tightly associated with the plant root and not bulk colonization of the soil.

Tissue colonization by the different strains of *F. verticillioides* is summarized in Table 3.3. Differences were noted between trials 1 and 2 for frequency of colonization, which suggested that colonization of the plant tissue can be highly variable. Some colonization of the mesocotyl and stem was observed in Silver Queen uninoculated controls in both trials and may be a testament to the sensitivity of the test and the difficulties of preventing cross contamination despite precautionary efforts. It should be noted that despite colonization of the Silver Queen control roots, there was no detection of fumonisins in the soil or roots, suggesting the background colonization had minimal effects on the parameters we assessed during the course of this study. The AEG 3-A3-2 and AEG 3-A3-5 strains had minimal colonization of the mesocotyl and no colonization of the stem in trial 1, but showed higher colonization rates in trial 2 (Table 3.3). Neither AEG 3-A3-2 nor AEG 3-A3-5 colonized the leaf tissues in trials 1 and 2. Trial 1 resulted in limited colonization of the mesocotyl, whereas trial 2 had 100% colonization of the mesocotyl and limited colonization of the stem. Interestingly, fumonisin accumulation in the

leaves (Figure 3.4) was clearly evident for AEG 3-A3-5 despite the inability to colonize the leaves. In trial 1, only MRC826 colonized the first leaf. In trial 2, colonization was noted for MRC826 and JFL-A04516 in all three leaves at varying rates. Although the trials indicate that leaf colonization is variable, it is clear that the aconidial strains AEG 3-A3-2 and AEG 3-A3-5 are greatly attenuated in their ability to infect the leaves of seedlings.

Only FB_1 was detected in the leaves, which supports the observations of Williams et al. (2007) and Zitomer et al. (2008). Trial 1 (Figure 3.4A) and trial 2 (Figure 3.4B) had similar accumulation of FB_1 in the first and second leaves for MRC826 and AEG 3-A3-5 inoculated Silver Queen despite the fact that AEG 3-A3-5 did not colonize leaf tissue (Table 3.3). The inbreds B73 and W23 had no detectable FB_1 in the leaves despite higher rates of colonization than in Silver Queen (Table 3.3). Colonization was counterintuitively higher in the controls in all leaf tissues for the inbreds B73 and W23 compared to the MRC826 inoculated plants. Perhaps inoculation triggered plant defenses, thus lowering colonization frequency. This observation requires further trialation.

Our data suggest *F. verticillioides* is necessary for accumulation of FB_1 in leaves, but that leaf colonization is not required for FB_1 accumulation in these tissues. Further investigations are necessary for understanding what this mechanism may be and how it preferentially targets FB_1. Furthermore, is this mechanism an important part of colonization of the leaves and progression of seedling blight symptom development? Perhaps of most interest is how the resistant inbreds seem to suppress growth of *F. verticillioides* and its capacity for fumonisin production and tissue colonization.

To refine our observations with inbred W23 and eliminate any influence of the fungi contaminating the seed, an experiment was performed on plant nutrient agar in test tubes to

quantify the uptake of FB_1 (10 μM) into the roots and aerial tissues when the FB_1 is added to the agar (Figure 3.5). Silver Queen and Tama Flint were included as well. The insensitivity of Tama Flint to FB_1 was noted previously (Desjardins et al., 2005). Germinated seeds were screened to eliminate any samples having fungal contamination.

Analysis of the FB_1 accumulation in the roots revealed a significant decrease in root uptake of FB_1 for W23 and Tama Flint compared to Silver Queen. No FB_1 was noted in the aerial tissues in all three maize lines (data not shown). As suggested by the seedling blight assays, the test tube assay indicated the FB_1-insensitive maize lines W23 and Tama Flint absorb less bioavailable fumonisin than does Silver Queen. The mechanism for absorption of fumonisin into the roots may explain differences in the FB_1-sensitive and -insensitive genotypes in this seedling model. Another possibility is that uptake of FB_1 may be driven by transpiration and bulk flow. The hormone abscisic acid (ABA) is known for triggering stomatal closure, thus limiting transpiration. Effectively, this function shuts down water loss in the plants (Schroeder et al., 2001). We hypothesized that if FB_1 uptake is dependent on transpiration and bulkflow, then the addition of ABA should limit uptake by closing stomata. To investigate this possibility, Silver Queen was co-exposed to ABA and FB_1 in test tube assays. FB_1 absorption was significantly less in ABA co-exposed roots after 48 hours, suggesting transpiration may have a role in FB_1 absorption into the roots (Figure 3.6).

In summary, local colonization of maize seedling leaf tissue is not necessary for accumulation of FB_1 in those leaves based on our Silver Queen model, but the mechanism for movement of FB_1 into the leaf tissues does require the presence of the fungus. Such movement has been elucidated for other mycotoxins. For example, wheat spikes infected with *Fusarium culmorum* were shown to have trichothecenes migrating beyond the infection hyphae into the

xylem and phloem sieve tubes where no fungal colonization was apparent (Kang and Buchenauer, 2002). Our trials would suggest a similar function of FB_1 moving ahead of colonization, at least in Silver Queen. Absorption of FB_1 was markedly decreased in the insensitive maize genotypes. The mechanism of FB_1 uptake into the roots is unknown; however, our study suggests ABA might modulate FB_1 uptake and alludes to a possible connection to bulk flow and transpiration. More research on the mechanism for FB_1 accumulation in the roots and how the fungus facilitates movement of FB_1 into the leaves will give us a better understanding of seedling blight disease progression and associated virulence of *F. verticillioides*, and perhaps whether such movement contributes to any food safety or exposure concerns. Also, the inbreds B73 and W23 seem to have inhibitory properties that decrease the growth and fumonisin production by *F. verticillioides* in the soil. This could be due to defense mechanisms expressed by the plant, such as fortifying the cell wall with callose deposits, activation of a hypersensitive response, expression of chitinases and beta-1,3-glucanases, and release of phytoprotective compounds such as the cyclic hydroxamic acids (DIMBOA and DIBOA) (Balasubramanian et al.; Frey et al., 1997; Greenberg et al., 1994; Gupta et al.; Østergaard et al., 2002). Another possibility is that the fungi naturally inhabiting the seed of B73 and W23, such as *Acremonium zeae*, may be antagonists suppressing the growth of *F. verticillioides* (Wicklow et al., 2005). Future research will investigate the role of plant defense mechanisms and competitive inhibition strategies for the control of *F. verticillioides*.

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Table 3.1. Characteristics of Maize Cultivars

Maize Line	Type	Fumonisin Sensitivity
Silver Queen (SQ)	White Sweet	Sensitive
B73	Dent Inbred	Moderately Insensitive [a]
W23	Dent Inbred	Insensitive
Tama Flint (TF)	Landrace	Highly Insensitive [a]

[a] Desjardins et al. (2005).

Table 3.2. Characteristics of *Fusarium verticillioides* Strains and Resulting Disease Incidence on Maize Lines Silver Queen (SQ), B73, and W23

			Disease Incidence (%)[a]		
	Conidia	Fumonisin	Sensitive	Insensitive	
Strain	Production	Production	SQ[b]	B73[c]	W23[c]
Water control	-	-	0 / 0	0	0
MRC826	Yes	Yes	59 / 92	0	4
JFL-AO4516	Yes	No	0 / 0	ND	ND
AEG 3-A3-5	No	Yes	76 / 87	ND	ND
AEG 3-A3-2	No	No	0 / 0	ND	ND

[a] Disease incidence is defined as the average percentage of seedlings that exhibit the symptoms associated with disease, such as stunting, leaf lesions, and leaf abnormalities.

[b] Disease incidence of trials 1 and 2 of the experiment are reported separately for SQ (trial 1/trial2).

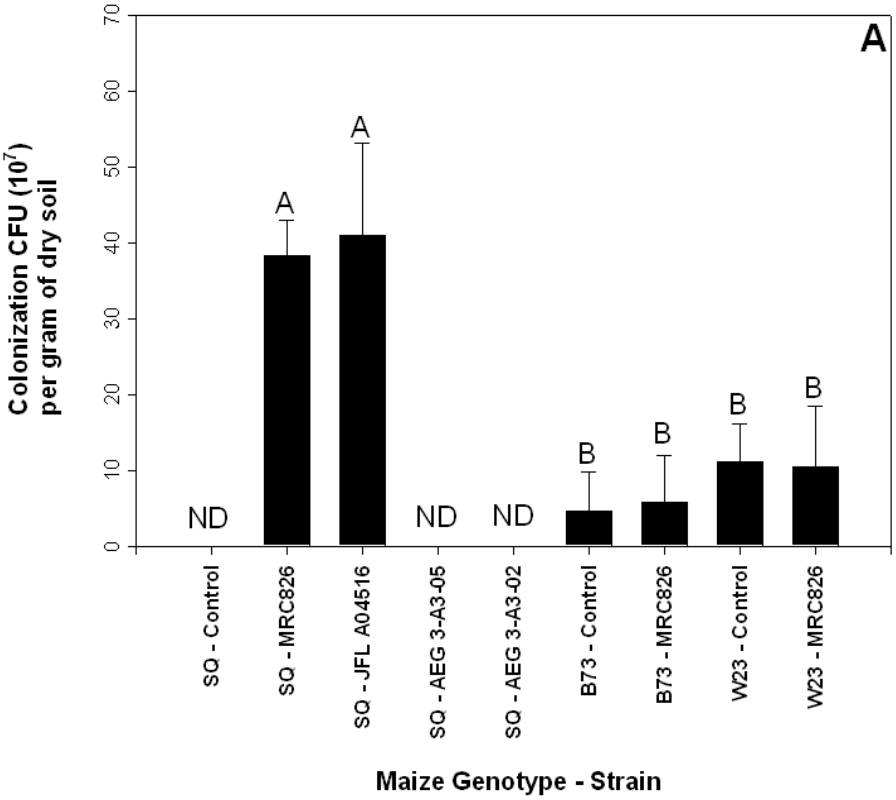
[c] B73 and W23 were included only in trial 1 and were inoculated only with MRC826. Disease incidence was not determined (ND) for other strains on B73 and W23.

Table 3.3. Percent Tissue Colonization by *Fusarium verticillioides* in Trial 1 and Trial 2. Values are presented as mean ± standard deviation of percent tissue from each treatment per pot (n = 3 pots per trial).

Trial 1	**Mesocotyl**	**Stem**	**Leaf 1**	**Leaf 2**	**Leaf 3**
SQ – Water control	38 ± 2	30 ± 44	0	0	0
SQ – MRC826	100	100	26 ± 25	0	0
SQ – JFL-A04516	100	100	0	0	0
SQ – AEG 3-A3-5	49 ± 29	0	0	0	0
SQ – AEG 3-A3-2	47 ± 45	0	0	0	0
B73 – Water control	95 ± 8	86 ± 15	33 ± 58	26 ± 45	15 ± 26
B73 – MRC826	51 ± 17	75 ± 7	0	0	0
W23 – Water control	59 ± 18	41 ± 37	31 ± 34	17 ± 29	19 ± 33
W23 – MRC826	100	95 ± 8	6 ± 11	0	0

Trial 2	**Mesocotyl**	**Stem**	**Leaf 1**	**Leaf 2**	**Leaf 3**
SQ – Water control	51 ± 38	43 ± 32	0	0	0
SQ – MRC826	100	100	17 ± 41	15 ± 24	36 ± 44
SQ – JFL-A04516	100	100	8 ± 20	11 ± 20	33 ± 52
SQ – AEG 3-A3-5	100	20 ± 18	0	0	0
SQ – AEG 3-A3-2	100	20 ± 13	0	0	0

Figure 3.1. Soil Colonization by Strains of *Fusarium verticillioides*. Values are mean ± standard deviation and letters indicate a statistically significant difference ($P \leq 0.05$, $n = 3$ pots per trial). Trial 1 (**A**) shows quantification of *F. verticillioides* strains in dried soil at the end of the 14-day experiment involving Silver Queen (SQ) grown from seed inoculated with the strains. Also shown are B73 and W23 water controls and MRC826 inoculations. Trial 2 (**B**) was similar but based on wet weight of soil. ND indicates no *Fusarium* colonies detected.


A
Colonization CFU (10^7) per gram of dry soil
0
10
20
30
40
50
60
70
ND
A
A
ND
ND
B
B
B
B
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
B73 - Control
B73 - MRC826
W23 - Control
W23 - MRC826
Maize Genotype - Strain

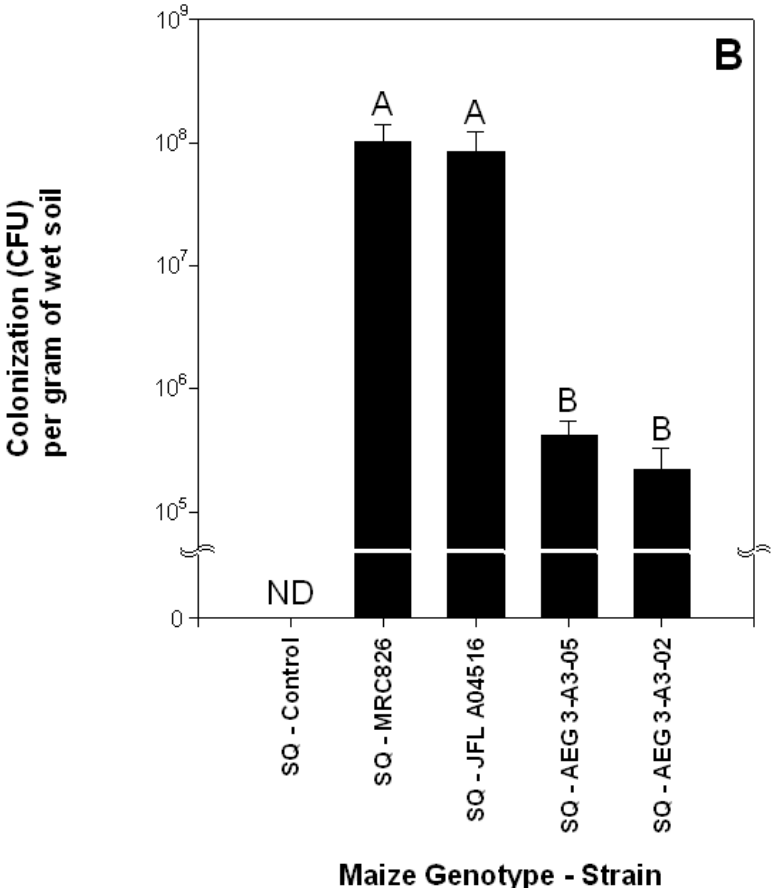
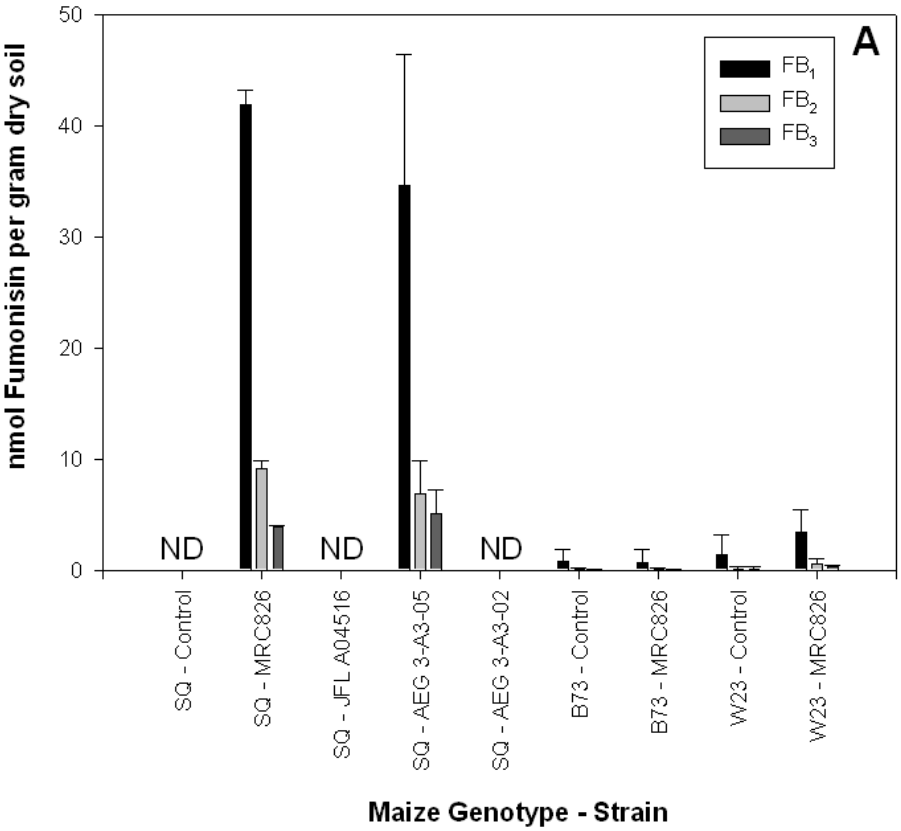

B
Colonization (CFU) per gram of wet soil
10^9
10^8
10^7
10^6
10^5
0
ND
A
A
B
B
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
Maize Genotype - Strain

Figure 3.2. Fumonisin Accumulation in Soil. Values are mean ± standard deviation of fumonisin B_1, B_2, and B_3 (respectively, FB_1, FB_2, and FB_3) accumulation in the soil for trial 1 (**A**) and trial 2 (**B**) (*n* = 3 pots per trial). ND indicates none detected.


A
FB1
FB2
FB3
nmol Fumonisin per gram dry soil
50
40
30
20
10
0
ND
ND
ND
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
B73 - Control
B73 - MRC826
W23 - Control
W23 - MRC826
Maize Genotype - Strain

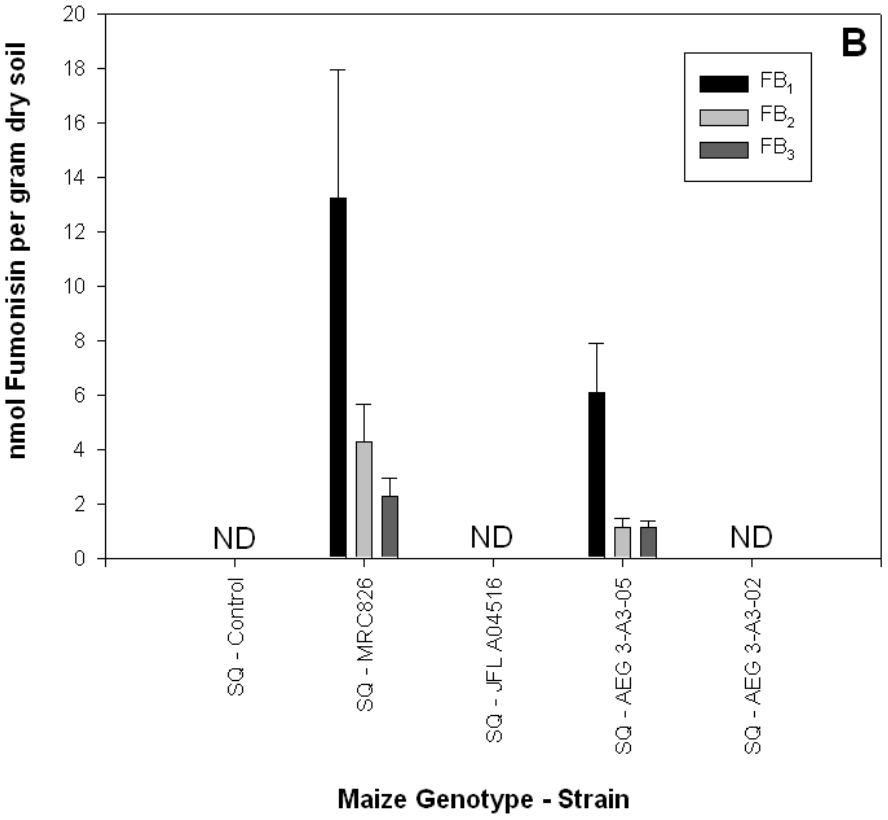
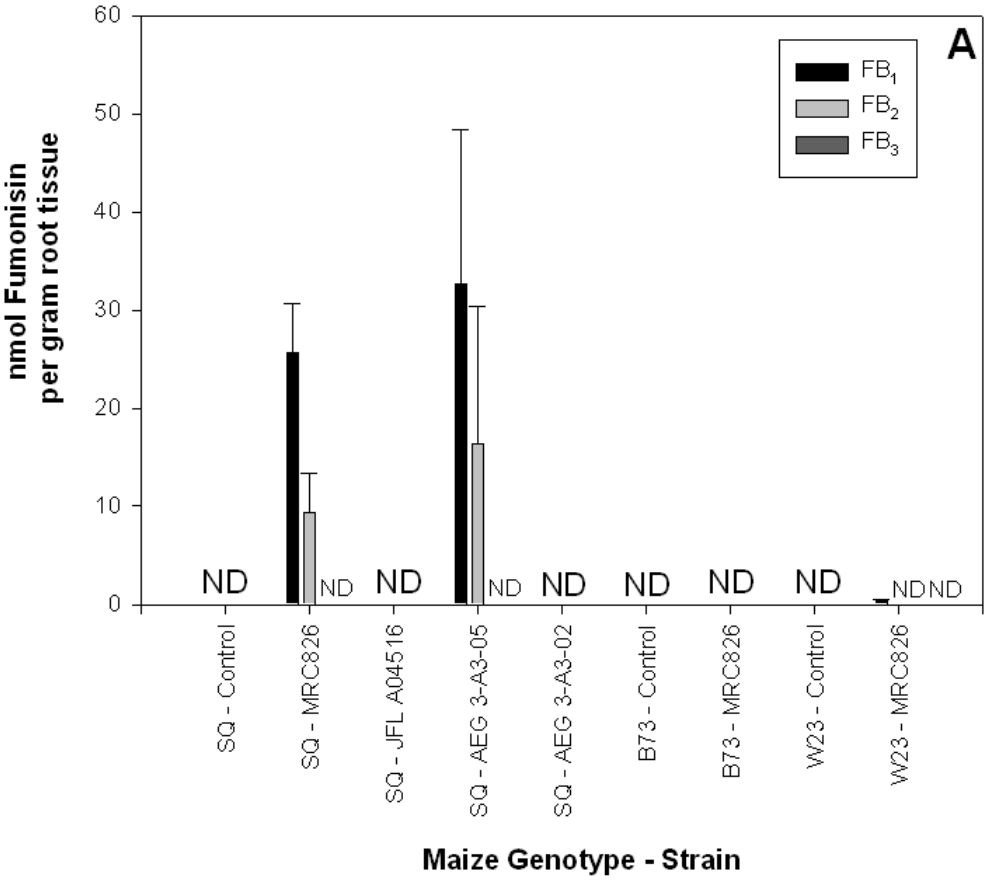

B
FB1
FB2
FB3
nmol Fumonisin per gram dry soil
20
18
16
14
12
10
8
6
4
2
0
ND
ND
ND
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
Maize Genotype - Strain

Figure 3.3. Fumonisin Accumulation in Root Tissue. Values are mean ± standard deviation of fumonisin B_1, B_2, and B_3 (respectively, FB_1, FB_2, and FB_3) accumulation in the root tissue for trial 1 (**A**) and trial 2 (**B**) (*n* = 3 pots per trial). ND indicates none detected.


A
FB1
FB2
FB3
nmol Fumonisin per gram root tissue
60
50
40
30
20
10
0
ND
ND
ND
ND
ND
ND
ND
ND
ND
ND
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
B73 - Control
B73 - MRC826
W23 - Control
W23 - MRC826
Maize Genotype - Strain

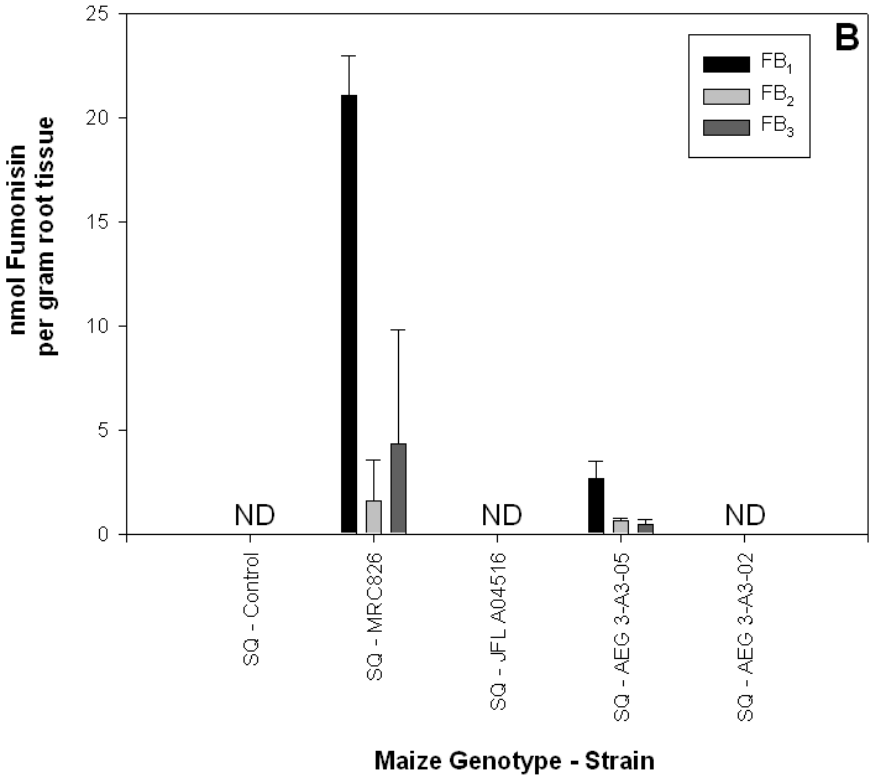
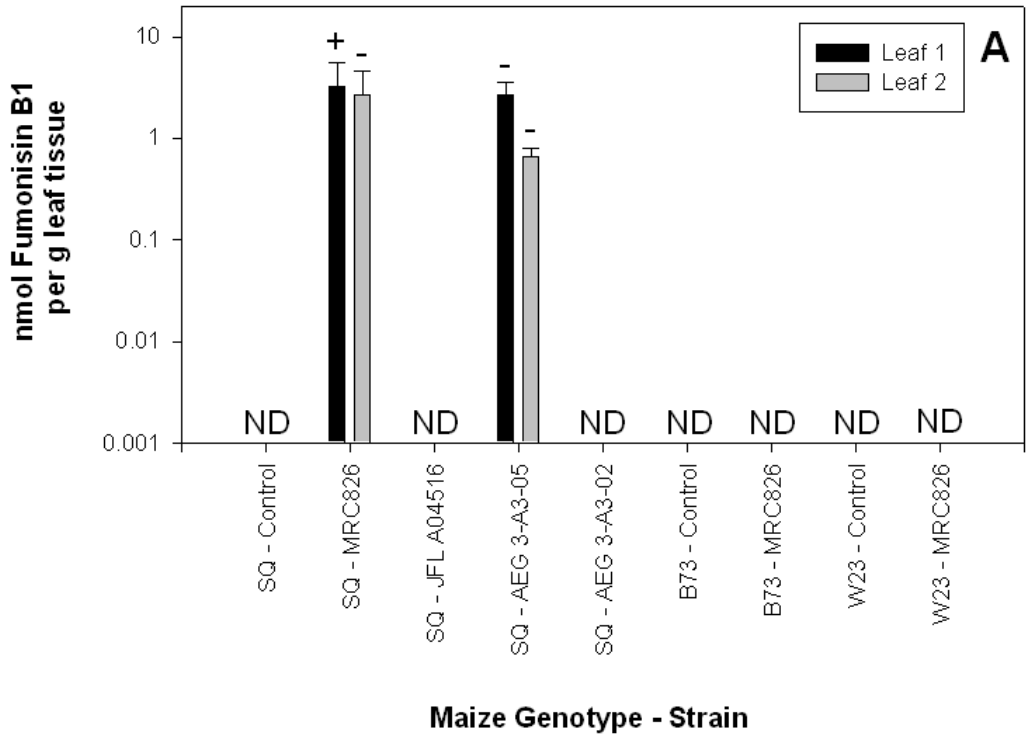

B
FB1
FB2
FB3
nmol Fumonisin per gram root tissue
25
20
15
10
5
0
ND
ND
ND
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
Maize Genotype - Strain

Figure 3.4. Fumonisin B1 Accumulation in the Leaves. Values are mean ± standard deviation of fumonisin B_1 detected in leaf 1 and 2. Fumonisin B_2 or B_3 was not detected in the leaves in trial 1 (**A**) and trial 2 (**B**) (*n* = 3 pots per trial). ND indicates not detected. +/- indicates colonization status of the leaf.


A
Leaf 1
Leaf 2
nmol Fumonisin B1 per g leaf tissue
10
1
0.1
0.01
0.001
+
-
-
-
ND
ND
ND
ND
ND
ND
ND
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
B73 - Control
B73 - MRC826
W23 - Control
W23 - MRC826
Maize Genotype - Strain

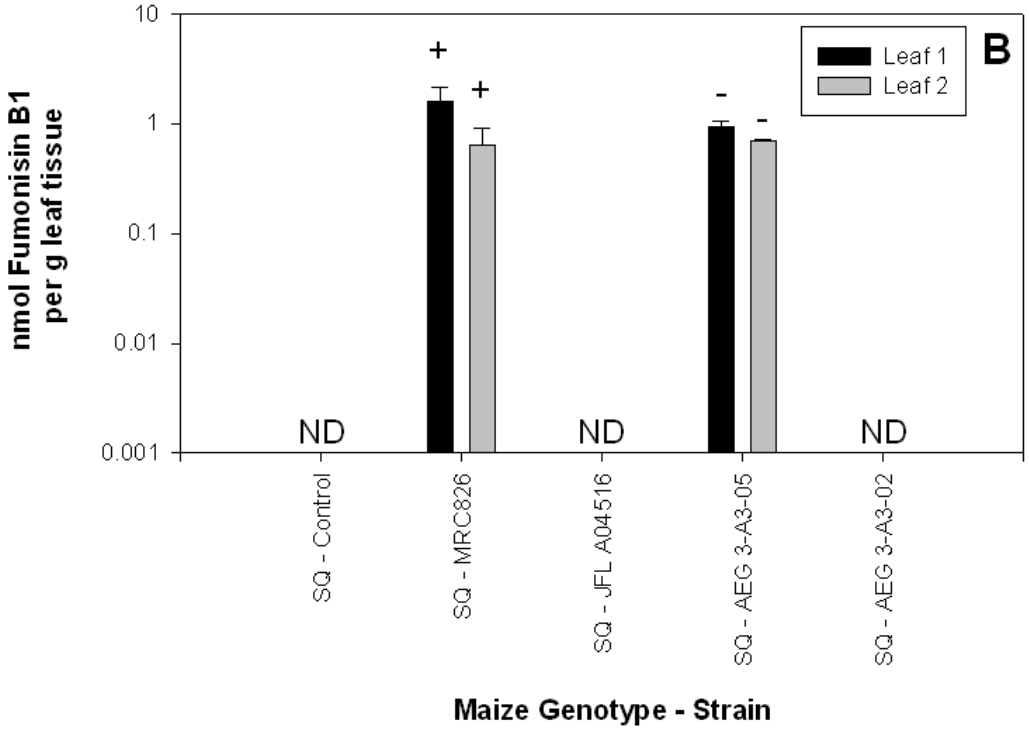

B
Leaf 1
Leaf 2
nmol Fumonisin B1 per g leaf tissue
10
1
0.1
0.01
0.001
+
+
-
-
ND
ND
ND
SQ - Control
SQ - MRC826
SQ - JFL A04516
SQ - AEG 3-A3-05
SQ - AEG 3-A3-02
Maize Genotype - Strain

Figure 3.5. Fumonisin B_1 (FB_1) Root Accumulation in Silver Queen (SQ), Tama Flint (TF), and W23. FB_1 was spiked into the agar of the test tubes containing the seedling for a final concentration of 10 μM and harvested 48 hours after exposure. Accumulation of FB_1 is reported as mean ± standard deviation from two biological replicates (n = 3 per replicate) and letters indicate a statistically significant difference ($P \leq 0.05$).

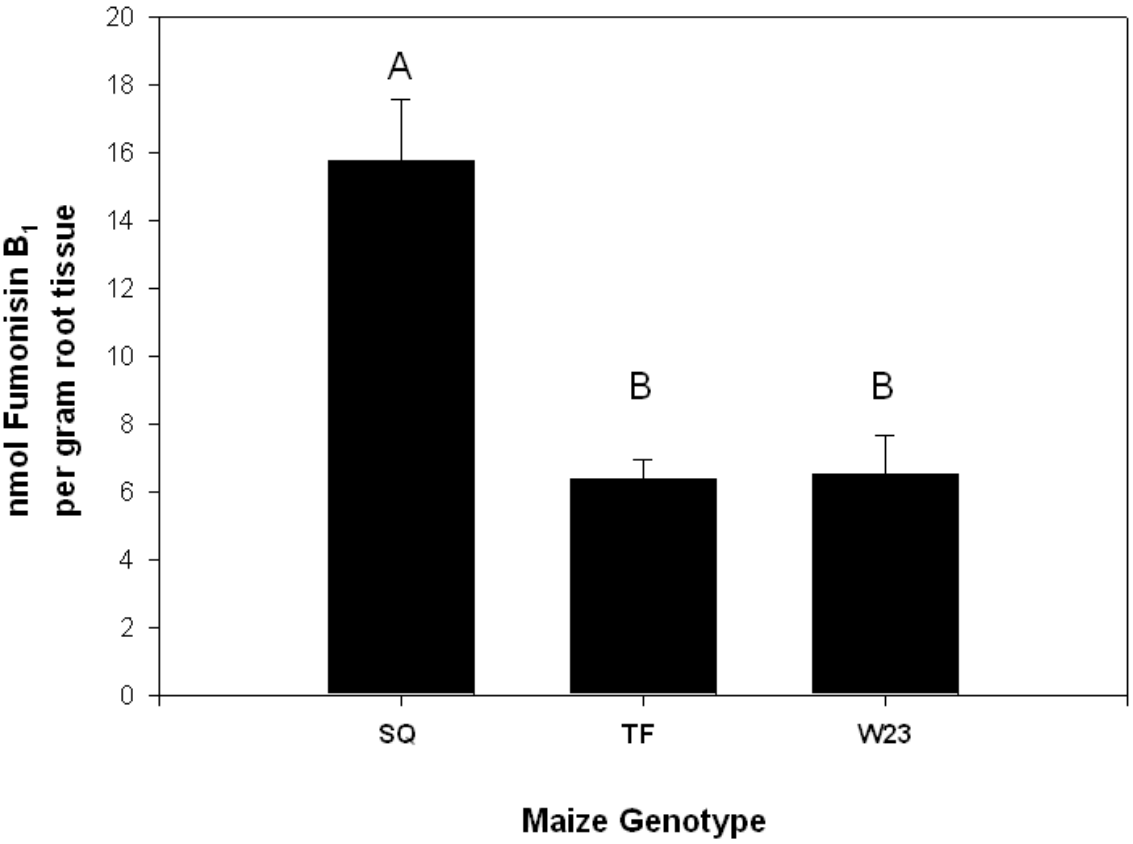
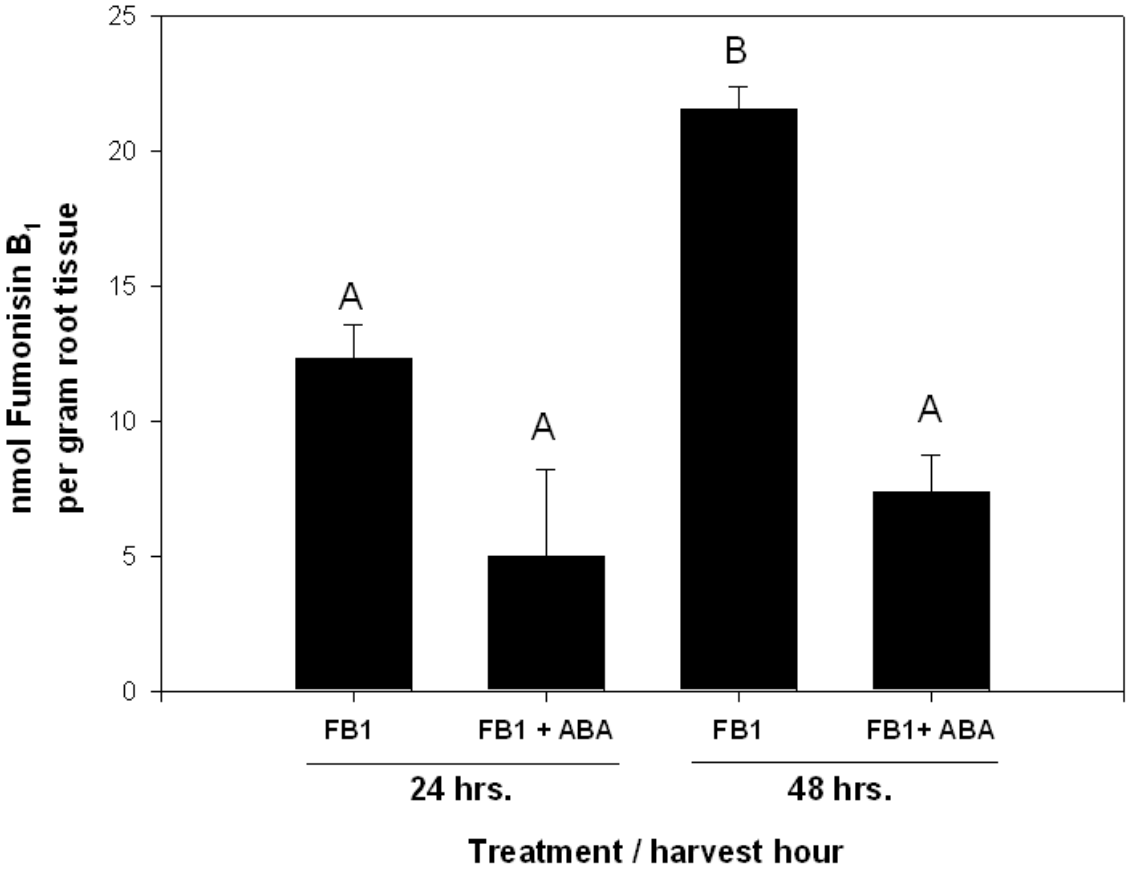

20
18
16
14
12
10
8
6
4
2
0
A
B
B
SQ
TF
W23
nmol Fumonisin B1
per gram root tissue
Maize Genotype

Figure 3.6. Fumonisin B_1 (FB_1) Accumulation in Seedlings Co-exposed with Abscisic Acid (ABA). Seedlings were assessed after 24 and 48 hours exposure to FB_1 (10 μM final concentration) or FB_1 plus ABA (200 μM). Accumulation of FB_1 reported as mean ± standard deviation from two biological replicates (n = 3 per replicate) and letters indicate a statistically significant difference ($P \leq 0.05$).


25
20
15
10
5
0
nmol Fumonisin B1
per gram root tissue
A
A
B
A
FB1
FB1 + ABA
FB1
FB1+ ABA
24 hrs.
48 hrs.
Treatment / harvest hour

CHAPTER 4

FUMONISIN B1 EFFECTS ON TRANSPIRATION AND STOMATAL APERATURE

Baldwin, T.T. and Glenn A.E. not submitted to a journal

INTRODUCTION

Infection of maize by *Fusarium verticillioides* precipitates the production and accumulation of various mycotoxins, including fumonisins, fusaric acid, and fusarin C (Bacon and Hinton, 1996; Fotso et al., 2002; Giesbert et al., 2008; Marasas et al., 1986; Wiebe and Bjeldanes, 1981). Of these mycotoxins, fumonisins are the most environmentally common and can occur in high concentrations in maize. In this group, fumonisin B_1 (FB_1) is the most toxic and makes up the greatest portion of the total fumonisins followed respectively by the FB_2 and FB_3 isomers. FB_1 contamination of field-grown maize has been determined as the causal agent in farm animal diseases, including equine leukoencephalomalacia (Marasas et al., 1988) and porcine pulmonary edema (Colvin and Harrison, 1992), and human consumption of fumonisin-contaminated maize has been associated with human cancer (IARC, 2002) and neural tube defects (Marasas et al., 2004). The proximate cause of fumonisin toxicity is inhibition of ceramide synthase (sphingoid base N-acyltransferase) a key enzyme in the *de novo* sphingolipid biosynthesis pathway (Wang et al., 1991). The major biochemical and cellular consequences subsequent to blockage of ceramide biosynthesis are accumulation of free sphingoid bases and sphingoid base 1-phosphates (Riley and Voss, 2006), depletion of more complex sphingolipids (Voss et al., 2009) and the global disruption of lipid metabolism (Merrill et al., 2001b).

Williams et al. (2007) discovered that watering maize seedlings with fumonisins resulted in reduced root weights, height, production of leaf lesions, and elevation of the free sphingoid bases and their phosphorylated metabolites. These effects along with the elevations were also seen when maize seedling were infected with *F. verticillioides* fumonisin-producing strains during seedling blight infection (Williams et al., 2007). Notably, the disease symptoms were more prevalent during inoculation with the fungus compared to watering solely with FB_1. The

free sphingoid bases include phytosphingosine, sphinganine, and their respective 1-phosphates (Figure 4.1) and are elevated because fumonisin disrupts plant ceramide synthase similar to animal systems (Abbas et al., 1994; Williams et al., 2006; Williams et al., 2007).

Exposure of free sphingoid bases alone are highly phytotoxic to *Lemna pausicostata* (Tanaka et al., 1993) and treatment with FB_1,caused significant elevation in sphinganine and phytosphingosine which occurred before the onset of toxicity (Abbas et al., 1994). Free sphingoid bases also invoke other physiological changes at lower concentrations. Recent articles have shown sphingosine-1-phosphate (S1P) is a calcium-mobilizing molecule in the plant and involved in abscisic acid (ABA) regulation of stomatal closure in *Commelina communis* (Ng et al., 2001). Others have shown that the S1P producing enzyme, sphingosine kinase (SphK), is stimulated by ABA in the stomata (Coursol et al., 2003; Coursol et al., 2005). Induction of sphingosine kinase (SphK) phosphorylates sphingosine to sphingosine-1-phosphate and results in the induction of stomatal closure and inhibition of stomatal opening. However, ABA induction of stomatal closure can occur independent of SphK induction through a possible unknown mechanism (Figure 4.2) (Coursol et al., 2003; Coursol et al., 2005). S1P and phytosphingosine-1-phosphate (Pso-1P) were also demonstrated to regulate stomata with the requirement of activity by a heterotrimeric G-protein coded by GPA1 (Figure 4.2). Heterotrimetric G-proteins are involved in regulation of stomatal aperture through slow anion channels and potassium channels (Figure 4.2) (Coursol et al., 2003; Coursol et al., 2005).

Certain secondary metabolites produced by pathogens are known to target the stomata and are important aspects of the pathogenic process. For example, coronatine produced by different strains of pathogenic *Pseudomonas syringae* triggers stomatal opening (Zhang et al., 2008). Alternatively, T toxin produced by *Cochliobolus heterostrophus* race T, blocks K^+, Na^+,

Cl^-, and PO_3^- channels in the guard cells and inhibits stomatal opening (Arntzen et al., 1973; Mertz and Arntzen, 1977). In this study we are interested in the physiological effects of the fumonisin mycotoxins on the transpiration and stomatal aperture of *Zea mays* and how that relates to *F. verticillioides* pathogenesis in maize seedlings. Our hypothesis is that fumonisins disrupt ceramide synthase in maize and the resulting elevation of phytosphingosine-1-phosphate and sphinganine-1-phosphate effects transpiration and stomatal aperture.

MATERIALS AND METHODS

Load Cell Measurement of Transpiration. Maize kernels were treated as described previously (William et. al., 2007). Briefly, kernels were surface-disinfected for 10 min in 100% commercial bleach (5.25% hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water. The seeds were then heat treated by placing them in a 60 °C water bath for 5 minutes for internal sterilization (Bacon and Hinton, 1994). Seeds were inoculated with either a 10^4 spores per mL suspension or sterile water. Three replicates of 10 seeds each were planted in sterile 10-cm plastic azalea pots (Hummert International, Earth City, MO, USA) containing twice-autoclaved commercial potting soil mix (Conrad Faford Inc., Agawam, MA, USA) Seedlings were grown.

Transpiration rates of Silver Queen seedlings inoculated with or without *F. verticillioides* were measured 9, 10, and 11 days after planting. Transpiration was calculated by measuring pot weight reduction with load cells (LSP-1 and LSP-2, Transducer Techniques, Temecula, CA) connected to a data logger (CR10, Campbell Scientific, Logan, UT) in a growth chamber (E-15, Conviron, Winnipeg, Manitoba, Canada). The weight of each pot was collected every ten

minutes and rate of transpiration was determined by slope of the regression of weight lost from the 16 hour light period.

Porometer Measurement of Transpiration. The effects of fumonisin B_1 on maize transpiration rates were tested using a leaf steady state porometer. Each treatment was comprised of 4 pots with 10 maize seedlings in each. The plants were grown in an environmental growth room (14hrs of light at 250 µmolar m^{-2} s^{-1} at 30˚ C and 10hrs of dark at 20˚ C). Seedlings were watered on days 9, 10, and 11 after planting with 100 ml of 0.5, 1.0, or 5.0 µg/mL FB_1 or 50, 250, and 500 µg/mL of ABA. FB_1 concentrations were based on previous experiments by Dr. Anne Marie Zimeri on phytotoxicity of FB_1 (data not shown). The transpiration rates were measured 12 days after planting on the second emerged leaf of three seedlings per pot for all three repetitions.

In Vitro Assays Using Epidermal Peels. Plants were grown in the growth chamber for three weeks in potting soil. Leaf samples at the second leaf were cut into 3 cm long strips, then the midvein was cut out. The abaxial leaf surface was peeled away from the leaf section, leaving intact tabs of full leaf on either end. Leaf incubation was based on Coursol et al. (2005). The leaf sections were incubated in the light (400 µmolar m^{-2} s^{-1} at 30˚ C) for 2 hours in the maize stomatal buffering solution (10 mM KCl_2, 7.5 mM potassium iminodiacetate, 10 mM MES, pH 6.15) to induce stomatal opening. The epidermal peels were incubated in the dark for 2 hours in maize stomatal buffering solution to induce stomatal closure. ABA treatments were added to open and closed stomata and left for a time course between 1, 2, 3 and 4 hours. The stomatal aperture was measured for each treatment at 400x using the Leica DM6000 B light microscope (Hamamatsu ORCA-ER, C4742-80 monochrome camera) and the Leica Application Suite (2.1.2

build 4530) to take accurate measurements of the stomatal opening. A total of 15 random stomata were selected for measurement of the stomatal opening.

Statistical Analysis: Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). Data was expressed as mean ± standard deviation or as a box plot (showing mean and 25% and 75% quartiles) and differences among means were considered to be significant if the probability (P) was ≤ 0.05. Statistical grouping was based on paired t-test or Mann-Whitney Test where specified.

RESULTS AND DISCUSSION

Transpiration experiments using load cells (Figure 4.3A) showed a significant difference on day 9 between *F. verticillioides* and control treatments based on a paired t-test (t=6.198, $P<0.05$). Similarly, day 10 and 11 showed significantly lower transpiration rates in the control (t=6.461 $P<0.05$, t=5.945 $P<0.05$, respectively). There was not a significant increase of transpiration rates between the days of each treatment. Transpiration experiments using a porometer (Figure 4.3 B) showed significantly higher transpiration rates in the control compared to *F. verticillioides* inoculated, as determined by a paired t-test (t=3.681, $P<0.05$, df=16). The FB_1 treatments (0.5, and 1.0 µg/ml) were not significantly different from the control rate of transpiration. However, at 5.0 µg/ml a significant difference did exist between the control and the ABA treatments of 50, 250, and 500 ppm (t=4.177 $P<0.05$, t=4.784 $P<0.05$, t=126 $P<0.05$, respectively). Notably, an increase in FB_1 concentration from 1.0 to 5.0 µg mL significantly decreased the rate of transpiration from 16.93 ± 4.250 mmol $m^{-2}s^{-1}$to 10.98 ± 3.16 mmol $m^{-2}s^{-1}$. The most diminished transpiration rate was observed at ABA 500 µg/ml at 1.27 ± 2.52 mmol $m^{-2}s^{-1}$.

The sphingoid base 1-phosphates, sphingosine 1-phosphate and phytosphingosine 1-phosphate have direct involvement in guard cell response and stomatal aperture control (Coursol et al., 2005). This interaction could play a dramatic role in *Fusarium* pathogenesis as a consequence of the elevated levels of sphingoid base 1-phosphates in leaf tissues during pathogenesis by affecting plant physiological processes, such as transpiration. Our research shows a direct effect on transpiration by either inoculation of the fungus or by watering with FB_1 at 5.0 µg/ml. Our epidermal peel experiments showed a significant decrease in stomatal aperture in the 10 µM ABA treatment (t=2.910, $P<0.05$) (Figure 4.4) and demonstrates the need for further investigation into possible effects of FB_1 on stomatal aperture.

ACKNOWLEDGMENT

The authors wish to thank Jency Showker and Britton Davis for their outstanding technical assistance. Special thanks to Dr. Marc van Iersel, The University of Georgia, Department of Horticulture for allowing us to utilize his load cell apparatus.

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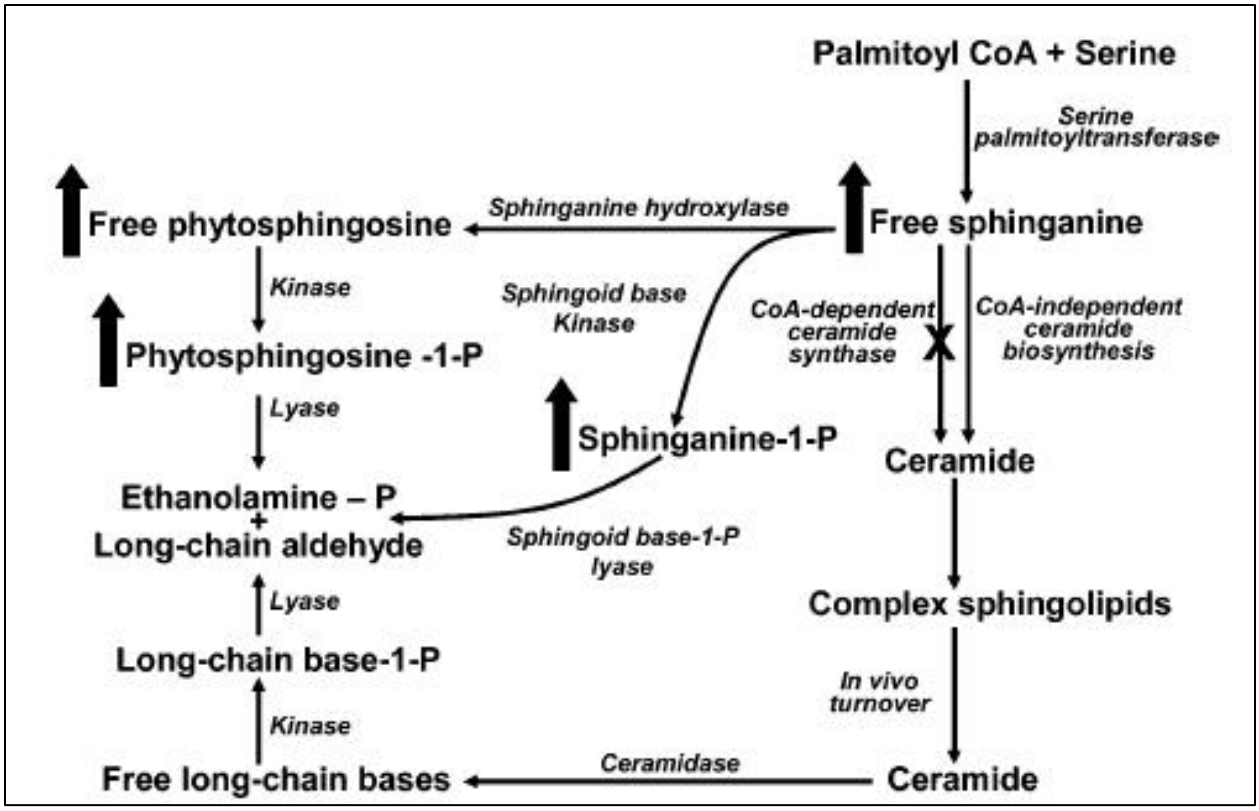
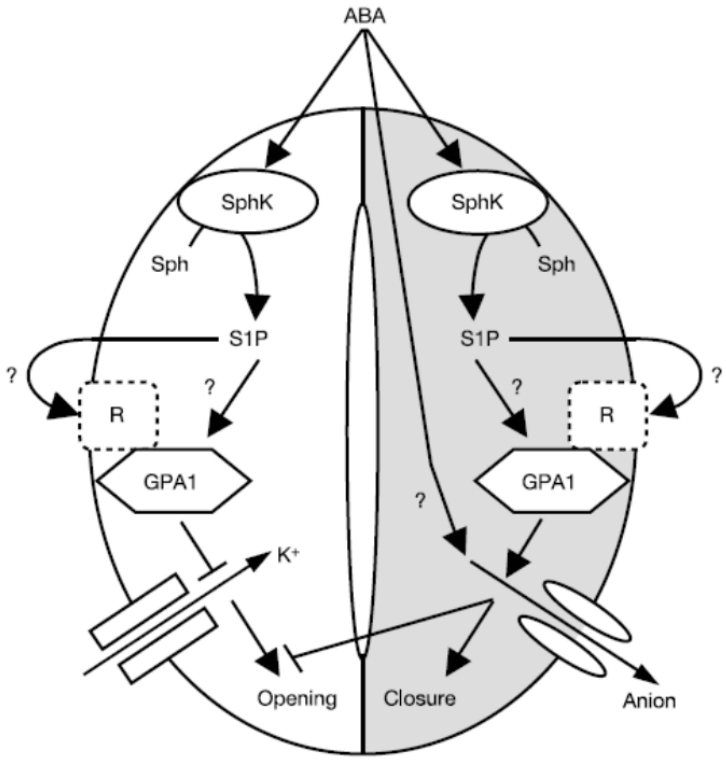
Figure 4.1. De *novo* Biosynthesis and Turnover Pathway of Sphingolipids in Plants (from Williams et al., 2007). Fumonisin disruption of ceramide biosynthesis is by inhibition of coenzyme A dependent ceramide synthase (X) and results in the increase of phytosphingosine, sphinganine, and their respective 1-phosphates, marked by (↑).



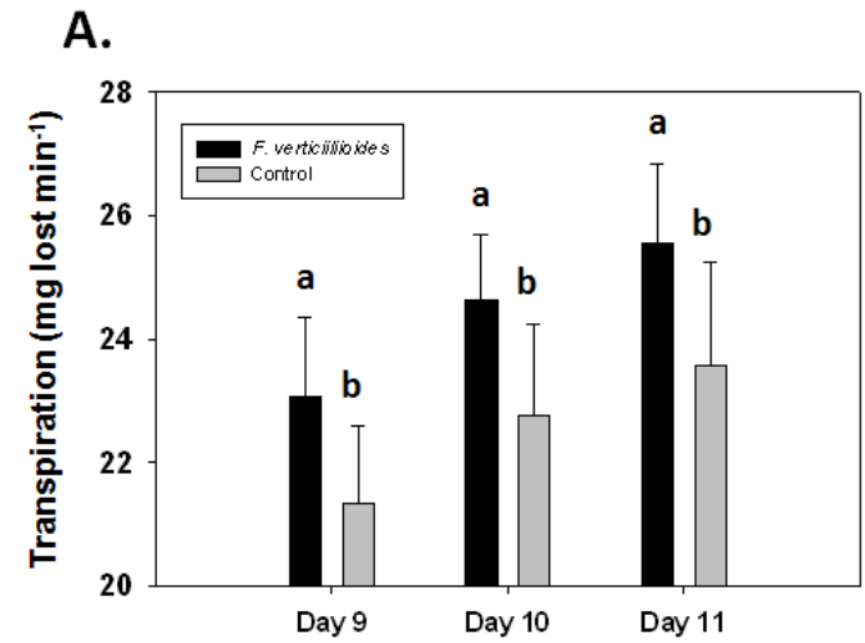
Williams et al., 2007

Figure 4.2. Diagram of a Proposed Stomate Abscisic Acid/Sphingosine-1-Phosphate (ABA/S1P) Signaling Pathway (from Coursol et al., 2003). Signaling pathway for stomatal aperture in Arabidopsis is by S1P production by ABA activated SphK. Stomatal closure in *gpa1* knockouts is induced by ABA, believed to be through a different signal pathway with slow anion channels. Interactions not well understood are marked (?). This model is depicting induction of sphingosine kinase (SphK) that phosphorylates sphingosine to sphingosine-1-phosphate resulting in the induction of stomatal closure and inhibition of stomatal opening. The model also depicts the fact that ABA induction of stomatal closure can occur independent of SphK induction through a possible unknown mechanism.



Coursol et al., 2003

Figure 4.3. *Fusarium verticillioides* and Fumonisin B1 (FB_1) Effects on Maize Leaf Transpiration Rates. (**A**) Transpiration rates were taken on days 9, 10, and 11 of inoculated and non-inoculated seedlings. Data were taken on load cells and the transpiration was determined by slope of water loss (mg). (**B**) Box plot of transpiration rates measured using a Decagon™ porometer. Non-inoculated seedlings were watered on days 9, 10, and 11 with 0.5, 1.0, or 5.0 µg/ml FB_1 or 50, 250, and 500 µg/ml of ABA or ddH_20. On day 12, mid-day, the transpiration rates were measured on the second emerged leaf from 3 seedlings per pot across 3 pots.


A.
Transpiration (mg lost min-1)
28
26
24
22
20
F. verticillioides
Control
a
b
a
b
a
b
Day 9
Day 10
Day 11

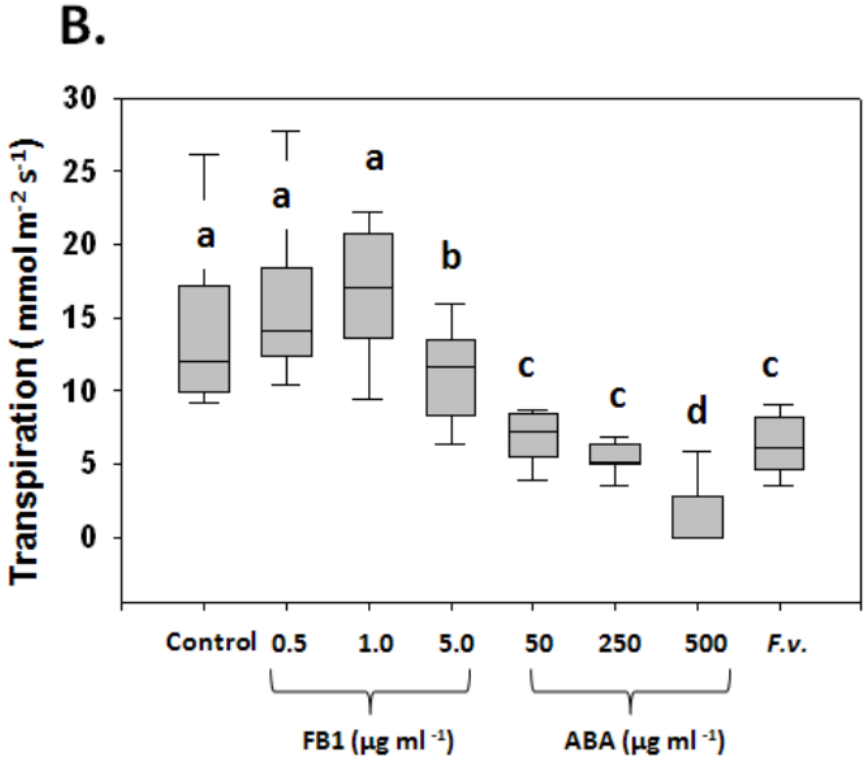
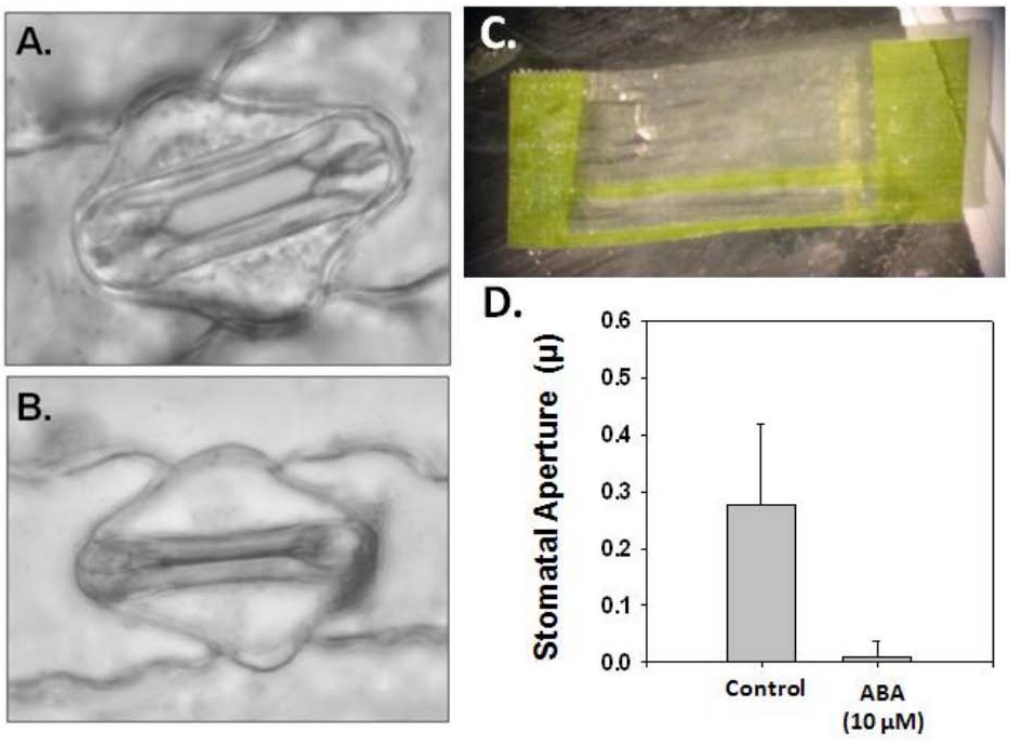

B.
Transpiration (mmol m-2 s-1)
30
25
20
15
10
5
0
a
a
a
b
c
c
d
c
Control
0.5
1.0
5.0
50
250
500
F.v.
FB1 (µg ml -1)
ABA (µg ml -1)

Figure 4.4. Maize Stomatal Aperture Assay. (**A**) Open stoma. (**B**) Closed stoma responding to ABA treatment. (**C**) An example of a prepared epidermal peel used in experiment. (**D**) Stomatal apertures measured from the control buffer and ABA treatments. A total of 15 stomata were measured using SimplePCL™ (C Imaging system) on three epidermal peels for each treatment. Treatments included ABA (10 µM) in opening buffer, or just buffer for the control. Incubated under high intensity light (450 µmol $m^{-2}s^{-1}$) for 2.5 hours before making measurements.


A.
B.
C.
D.
Stomatal Aperture (µ)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
ABA
(10 µM)

CHAPTER 5

FLAVOHEMOGLOBIN AND THE DENITRIFICATION PATHWAY IN DETOXIFICATION OF NITRIC OXIDE BY *FUSARIUM VERTICILLIOIDES*: IMPLICATIONS FOR PATHOGENICITY AND MYCOTOXIN PRODUCTION

Baldwin, T.T., Gold, S.E., Covert, S.F., Riley, R.T., and Glenn A.E. Submitted to *Molecular Plant-Microbe Interactions*

ABSTRACT

Nitric oxide (NO) is a highly mobile and potent signaling molecule in organisms, yet it can also cause nitrosative stress to cells. To alleviate negative effects from excessive accumulation of NO from endogenous or potential exogenous sources, flavohemoglobin proteins can convert NO into nontoxic nitrate ions. We have investigated the flavohemoglobins in *Fusarium verticillioides*, a mycotoxigenic plant pathogen of maize. Two genes encoding putative flavohemoglobin homologs, denoted *FHB1* and *FHB2*, were identified. Microarray analysis revealed a 17-fold induction of *FHB2* transcription and a 2-fold induction of *FHB1* when the fungus was exposed to exogenous NO (1.5 mM of NO-donor DETA NONOate). In addition these conditions caused a 246-fold increase in transcription of the dissimilatory nitrite reductase (*NIR1*) gene and high induction of other genes from the denitrification pathway. Deletion mutants (*fhb1*, *fhb2*, and *nir1*) challenged with NO (1.5 mM of NO-donor) showed *fhb1* and *fhb2* mutants had increased sensitivity to NO with reduced growth compared to wild type, and growth of the *fhb1*/*fhb2* double deletion was completely inhibited when challenged with NO. The *nir1* mutant exhibited no decrease in growth. All mutants were able to grow on nitrate medium. However, mutants *fhb2*, *nir1*, and *nor1* were unable to grow on nitrite under hypoxic conditions, whereas only the *fhb1*/*fhb2* double deletion had markedly decreased growth on nitrite under normal atmospheric oxygen. Our results suggest the flavohemoglobin FHB2 is linked to the denitrification pathway, as its function is indispensible in nitrite metabolism under hypoxic conditions. Maize seedling blight assays showed that the *fhb1*/*fhb2* double deletion was more virulent than wild type. The double deletion also had increased production of fumonisin B_1. Our results demonstrate a significant role of the denitrification pathway in NO detoxification and link such detoxification to virulence and fumonisin production.

Key Words: Nitric oxide, flavohemoglobin, denitrification, dissimilatory nitrite reductase, P450 nitric oxide reductase, FHB1, FHB2, fumonisin,

INTRODUCTION

Nitric oxide is a small ephemeral molecule having importance in cellular signaling from abiotic and pathogen stress, often occurring simultaneously with reactive oxygen species, such as hydrogen peroxide. NO at low concentrations has cellular protective effects, whereas high levels are considered toxic (Kröncke et al., 1997). NO is a Reactive Nitrogen Species (RNS) free radical and a source of nitrosative stress. Highly reactive with an unpaired electron, NO can exist in a variety of reduced states including the NO^- (nitroxyl ion) and NO^+ (nitrosonium ion), each having their own unique reaction capability giving NO exceptionally diverse biochemistry (Gow and Ischiropoulos, 2001). Moreover, some important physical aspects of NO include its low solubility in most aqueous solutions and small size, enabling near instantaneous mobility through membranes (Ignarro et al., 1987; Palmer et al., 1987).

NO was first elucidated as a messenger in the immune, nervous and cardiovascular systems in mammals and is the most powerful endogenous signal for vasodilatation yet discovered (Anbar, 1995; Grisham et al., 1999; Lundberg et al., 2008; Pfeiffer et al., 1999). The direct mechanism of NO signaling stems from its redox-based post-translational modification of proteins, including S-nitrosylation, which is a covalent bonding of an NO molecule to a sulfhydryl group on cysteine. This modification affects the protein's activity, localization, interactions, and folding conformation (Yun et al., 2012). This form of amino acid modification can also occur on carrier molecules such as glutathione, forming S-nitrosoglutathione by reacting with cysteine residues, and is the reason why a 5 to 15 s half-life signaling molecule can ramify a signal lasting for days. This carrier molecule can perform protein trans-nitrosylation and acts as a bio-reserve for NO (Kettenhofen et al., 2004).

Recently, NO has been demonstrated as an important signaling compound in plant development, such as plant germination, growth, stomatal closure, and abiotic stress (Besson-Bard et al., 2008; Delledonne et al., 1998; Moreau et al., 2010; Neill et al., 2002; Wilson et al., 2008; Zhang et al., 2008). Phytopathological studies have demonstrated NO signaling as an important aspect of plant defense against a variety of pathogens. Similar to the oxidative burst, a widely studied reactive oxygen species (ROS) event that marks the beginning of pathogen recognition, there is also an early NO burst and may even precede the oxidative burst (Asai et al., 2008). This has been seen in barley epidermal cells infected with powdery mildew *Blumeria graminis* f. sp. *hordei* (Prats et al., 2005) and in *Pelargonium* infected with the necrotrophic pathogen *Botrytis cinerea* (Floryszak-Wieczorek et al., 2007). Also, NO is an integral part of pathogenesis in *Arabidopsis* challenged with the bacterium *Pseudomonas syringae* pv. *tomato* (Bennett et al., 2005), the tobacco mosaic virus in tobacco (Durner et al., 1998), and the oomycete pathogen *Bremia lactucae* on *Lactuca* (Sedlarova et al., 2011). Additionally, cryptogein, an elicitor from *Phytophthora cryptogea* induced the NO burst in tobacco epidermal cells as detected via DAF-2 fluorescence probe. Support for the fluorescence measurements was based on its elimination by the addition of the NO-scavenger, cPTIO (Foissner et al., 2000).

Induction of elevated levels of NO results in many downstream effects in plants. For instance, the formation of structural barriers by cell wall modifications in the initial phases of the *Colletotrichum coccodes* – tomato interaction (Wang and Higgins, 2005b). Similarly, Prats et al. (2005) observed significant papilla deposition co-occurring with the NO burst in the barley- *B. graminis* f. sp. *hordei* interaction. Another well-studied effect of the induction of NO is production of defense secondary metabolites by the plant. This induction is usually mediated by fungal elicitors and include metabolites of interest because of their pharmaceutical importance,

including saponin, hypericin, puerarin, catharanthine, and taxol (Hu et al., 2003; Wang et al., 2006; Xu et al., 2005; Xu et al., 2006; Xu and Dong, 2005; Zheng et al., 2008)

There is some conflicting evidence about the function of NO in pathogenicity. Exposure of the susceptible tomato cultivar *Lycopersicon esculentum* Mill. cv. "Perkoz" to the pathogenic *Botrytis cinerea* demonstrated an increase in NO levels paired with decreased H_2O_2 levels in the compatible interaction (Malolepsza and Rozalska, 2005). Also, *B. cinerea* produces endogenous NO that diffuses into the host plant, triggering hypersensitive response (HR), which causes cell death and seems to benefit the infecting necrotrophic pathogen (Turrion-Gomez and Benito, 2011). In contrast, infection by the pathogen *Fusarium oxysporum* f. sp *lupini* on yellow lupine embryo axes with co-exposure to exogenous nitric oxide suggested cross talk between nitric oxide and resulted in the activation of defense genes (i.e. the PAL genes) and production of isoflavonoids to mount a successful defense against the pathogen (Morkunas et al., 2013).

NO activating defense genes and the NO burst as part of pathogen defense makes detoxification of NO during pathogenicity crucial for an effective pathogen. Detoxification of NO for successful pathogenicity has been studied in *Candida albicans* virulence on mice (Ullmann et al., 2004) and in the bacterial pathogen *Erwinia chrysanthemi* on *Saintpaulia ionantha* (Favey et al., 1995). Both systems utilize the highly conserved flavohemoglobins, which are comprised of a globin domain fused to a ferredoxin reductase like FAD- and NAD-binding modules (Gardner et al., 1998). Flavohemoglobins are sometimes referred to as nitric oxide dioxygenases (NOD) in bacterial systems because of their ability to detoxify NO by oxidation to nitrate (NO_3^-). Flavohemoglobins are prolific and conserved throughout all life (Gardner, 2005).

Mutating the *Erwinia chrysanthemi HmpX* flavohemoglobin resulted in a significant increase in NO level accumulation, disease resistance, and an HR response in the *S. ionantha* host (Favey et al., 1995). Interestingly, insertion of *hmpx* into the avirulent *Pseudomonas syringae* pv. tomato avrB suppressed the HR response in *Arabidopsis*, reverting the *Pseudomonas syringae* pv. tomato avrB back to a competent pathogen (Boccara et al., 2005). Similarly, transformation of *Arabidopsis* with the flavohemoglobin from *Escherichia coli* also suppressed the NO burst and HR response when challenged with *Pseudomonas syringae* pv. tomato avrB (Zeier et al., 2004). The function of flavohemoglobins in detoxification of NO is a virulence factor for a number of pathogens. *Cryptococcus neoformans* was also shown to require a flavohemoglobin, gene *FHB1*, for both virulence on mice and NO detoxification as found by insertional mutagenesis (Idnurm et al., 2004).

NO represents a complex and potent signaling and antimicrobial compound in pathogenic interactions and the role of NO in the *F. verticillioides* – maize interactions is yet to be explored. This study addressed the following questions on NO in *F. verticillioides*: (*i*) what genes are induced with exposure to exogenous NO? (*ii*) What is the role of the two *F. verticillioides* flavohemoglobins in NO detoxification? (*iii*) How does nitric oxide detoxification effect pathogenicity and mycotoxin production? The *F. verticillioides* genome contains two genes coding for flavohemoglobins (*FHB1*) and (*FHB2*). Double deletion of both flavohemoglobin genes led to greater virulence on maize seedlings and a greater accumulation of fumonisin mycotoxins. Moreover, *FHB2* is linked to nitrite/nitrate metabolism under hypoxic conditions through the denitrification pathway. This pathway is considered a hypoxic pathway, yet we show that under oxygenated conditions the genes in this pathway are activated to detoxify NO.

MATERIALS AND METHODS

Fungal strains, culture conditions, and bioinformatic tools. Other than the strains created during this study, *Fusarium verticillioides* strain M3125 was utilized for MAT1-1 wild type and strain A00999 was utilized for MAT1-2 wild type for transformation. Strains were grown in potato dextrose broth (PDB) for 3 days at 200 rpm or on potato dextrose agar (PDA) for 7 days and incubated at 27°C in the dark unless stated differently. Additionally, shaking (250 rpm) was necessary for PDB cultures in 3 mL snap cap tubes. All strains were kept frozen in 10% glycerol at -80°C for long term storage. Gene and protein sequences were attained using BROAD Institute via the *Fusarium* Comparative Database (www.broadinstitute.org) or National Center for Biotechnology (www.ncbi.nlm.nih.gov) database for BLAST and BLASTP to mine and compare sequences. Gene sequences were analyzed via Sequencher (Version 4.10.1, Gene Codes Corp., Ann Arbor, MI) and peptide alignment and neighbor joining were analyzed via Geneious (Version 7.0.4, Biomatters, San Francisco, CA). Signal peptides predictions were made using TargetP (http://www.cbs.dtu.dk/services/TargetP/), MitoProt II (http://ihg.gsf. de/ihg/mitoprot.html), and WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html). Primers for all constructs and PCRs were designed and ordered via Integrated DNA Technologies, Inc. (http://idtdna.com/).

Nitric Oxide exposure and bioscreen experiments. DETA NONOate (Cayman Chem., Ann Arbor, MI) was used as a NO donor in the following experiments. It has a half-life of 56 hours at pHs about 7.4 at 27ºC (Hrabie et al., 1993; Keefer et al., 1996). Dilutions of this NO donor were made in 0.01 M NaOH to keep solution basic and prevent premature breakdown. *F. verticillioide*s acidifies the growth media to pHs of 3 or 4. Therefore, all experiments involving

growth and exposure to NO were conducted using PDB buffered with 0.1 M sodium phosphate buffer to maintain culture growth at pH 7.0 for the duration of the experiment. No physiological changes were noted in growth of the fungus under the microscope attributable to growing on buffered media. However, the color of the both culture had changed from maroon to a milky off-white.

Growth rates during exposure to NO were determined on the automated turbidometer, Microbiology Bioscreen C Reader (Labsystems, Helsinki, Finland). Each well of the two 100 well microplates were filled with 200 μL of PDB buffered with 0.1 M sodium phosphate and inoculated with 3 day old *F. verticillioides* adjusted to a final concentration of 10^4 spores per mL Cultures were incubated at 25 ºC with constant medium shaking on the Bioscreen C for 100 hours with OD_{600} measurements every 30 min. Each strain was replicated in 10 wells. Experiment was repeated with 3 biological replications.

Quantitative real-time PCR (qPCR) of *FHB1* and *FHB2* genes. *Fusarium verticillioides* strain M3125 was adjusted to 10^3 spores per mL into 3 mL PDB buffered with 0.1 M sodium phosphate media in 15 ml snap cap tubes and grown at 27 ºC at 240 rpm. After 3 days, 100 μL of DETA NONOate was added for a final concentration of 1.5 mM. M3125 was run alongside controls for 15, 45, and 360 min before harvesting. Upon harvest, cultures were immediately centrifuged (1,500 x g for 5 min at 15 ºC) and the fungal material was collected ground under liquid nitrogen. RNA extraction and purification was performed using PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA). DNase cleanup was achieved through TURBO™ DNase (Life Technologies, Carlsbad, CA). Ground tissue and RNA extracts were stored at -80°C for long term storage. cDNA synthesis was quantitative real-time PCR measuring relative gene

expression was performed using the Express- one shot (Life Technologies, Carlsbad, CA) for cDNA creation and quantitative real-time. Normalization and fold change were calculated using the tubulin gene TUB2 for analysis as a constitutive standard. See Table 5.1 for primers used in this experiment. All reactions were performed on a Cepheid Smartcycler I (Cepheid, Sunnyvale, CA) with the following thermal cycling parameters: Stage 1, 50 ºC for 20 min – reverse transcriptase cDNA creation, Stages 2- 4 denaturing step 95 ºC for 3 min followed by 45 cycles of two-step PCR, 95ºC for 15 s, and 65ºC for 60 s. A melting temperature analysis was run at the end from 60 ºC to 95 ºC at 0.2 degrees/second. The relative expression level of each target gene was calculated by $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001). The fold changes values were average Ct values of three biological replicates with at least three technical replicates per experiment.

Microarray experiment. Microarray analysis was used to compare gene expression in M3215 exposed and unexposed to 1.5 mM DETA NONOate for 45 min. Experiments were performed with the same procedure used for quantitative real-time PCR. DNase cleanup was achieved through TURBO™ DNase (Life Technologies, Carlsbad, CA). RNA sample quality was analyzed on a Roche Bioanalyzer and was prepared for cDNA creation using the cDNA synthesis systems (Roche NimbleGen Inc., Madison, WI, USA) before sending to Roche Nimblegen for processing on a *Fusarium verticillioides* microarray chip (Brown et al., 2005). Comparative analysis of normalized data was accomplished using Arraystar 5 software (DNASTAR, Madison, WI).

Gene deletion construction and fungal transformation. Gene deletion constructs from *fhb1*, *fhb2*, *nir1*, and *nor1* strains were accomplished using the DelsGate deletion construct creation

method for homologous recombination transformation (García-Pedrajas et al., 2008). Hygromycin was utilized as a selectable marker. See Tables 5.3 and 5.4 for sequence and description of primers. Briefly, a 5' flanking and 3' flanking DNA fragment of approximately 1.0 – 1.5 kb surrounding the ORF was amplified via PCR using primers 1 and 2 or primers 3 and 4, respectively. BP clonase Life Technologies, Carlsbad, CA was utilized to clone the fragments into a pDONR-A-Hyg deletion plasmid and was transformed into One Shot TOP10 Electrocompetent *E. coli* cells (Life Technologies, Carlsbad, CA, USA) using electroporation (BioRad micropulser, Ec1 setting, 1.8kV, 3.6ms). Transformants were selected for on LB + kanamycin (50µg/ml) and incubated at 37°C then screened via PCR using primer sets I-SceI F (5'-TAGGGATAACAGGGTAAT-3') and Donr F (5'-ATCAGTTAACGCTAGCATGGATCTC-3') for the 5' flank, and *I-SceI* R (5'-ATTACCCTGTTATCCCTA-3') and Donr R (5'-GTAACATCAGAGATTTTGAGACAC-3') were used to test the 3' flank.

Plasmids were digested with *I-SceI* to linearize the deletion construct for fungal homologous transformation. Digestion was incubated overnight at 37°C and cleaned using Qiagen PCR Purification Kit (Qiagen Sciences, Maryland, USA). Fungal homologous transformation was performed according to the procedures used by Glenn et al. (2008). In a 1.5 mL microcentrifuge tube, 20 µL of prepared protoplast were added to 100 µL STC solution (1.2M Sorbitol, 50mM $CaCl_2$-$2H_2O$, 10mM Tris-HCl), 50µl 30% PEG solution, and 8 -10 µg of DelsGate construct. After 20 min, 2 mL of STC was added followed by an additional 2 mL of 30% PEG. After gentle mixing, the solution was poured into 55 ºC molten overlay medium, which was then poured on to regeneration plates (Glenn et al., 2008). Finally, 1% agar with 515

μg/ml hygromycin B (Roche) was overlaid the following morning at a final concentration of 150 μg/ml.

Transformants were screened for the chimeric insert with the outer primers (5 or 6, respectively) in combination with either the pDONR-A-Hyg specific HygF (5'-ATCGCGGCCTCGACGTTTCC-3') for the 5'flank or the KanR (5' – TTATCGTGCACCAAGCAGCA-3') for the 3' flank. Selected transformants were placed on hygromycin B PDA (50 μg/ml) to maintain after the initial screen. Transformants that showed amplification of the ORF using primers 7 and 8 (Table 5.3 and 5.4) were culled. Finally, a Southern blot was performed for all deletion transformants using a DIG labeled probe (Roche) according to the manufacturer's directions. Genomic digestion was performed via *HpaI* for *FHB1*, *EcoRI* for *FHB2*, *EcoRV* for *NIR1*, and *XbaI* for *NOR1*. Digested DNA (1 μg) from a 1.0% Tris-acetate-EDTA agarose gel was blotted onto a Hybond H+ nylon membrane (Amersham Biosciences, Buckinghamshire, England) according to standard procedures (Glenn et al., 2008). Gene-specific probes were generated using a digoxigenin (DIG) PCR labeling kit (Roche, Indianapolis, IN, U.S.A) off of the 5' or 3' flanking PCR primers. Hybridization was detected on an the Alpha Innotech FluorChem 8000 digital imaging system (San Leandro, CA, USA) for 50 min. Protoplasting was performed as in Glenn et al. (2008) for wild type and deletion mutant strains.

Mating A00999 *fhb2* and M3125 *fhb1* for double deletions. A00999 *fhb2* and M3125 *fhb1* created by DelsGate were mated to generate a *fhb1/fhb2* double deletion. Mating was performed as reported on carrot agar media (Leslie and Summerell, 2006). A00999 *fhb2* was utilized as the female. Individual columns of ascospores were picked each placed in 1 mL ddH2O in a 1.5 ml

centrifuge tube and vortexed. A 10–fold dilution was plated on hygromycin B PDA (50 μg/ml) and incubated at 27 ºC for 2 days. Germinating ascospores were transferred and the progeny were later tested by multiplex PCR for the *FHB1*, *FHB2*, and *TUB2* ORFs (Table 5.2). Progeny that amplified the *TUB2* ORF and not the *FHB1* and *FHB2* ORFs were considered *fhb1/fhb2* double deletions. Double deletions were tested for the *MAT1-1* and *MAT1-2* mating type by duplex PCR modified from Steenkamp et al. (2000) changing GFmat2c to the following (5'-AGCGTCACCATTCGATCAAG-3'). Modifications were based on noted sequence differences in *F. verticillioides*.

Creation of add-back strains, transformation, and selection. PCR was performed using primers 5 and 6 (Table 5.3 and 5.4) to amplify the entire ORF and checked on a 1% gel for predicted size. *FHB1, FHB2, NIR1,* and *NOR1* constructs were created using M3125 genomic DNA template. Add back constructs were purified via Qiagen PCR Purification Kit (Qiagen Sciences, Maryland, USA). Co-transformation of equal parts 0.5 μg PCR amplicon and 0.5 μg pGEN-AscI vector containing the geneticin resistance vector (Received from P.R. Proctor, Peoria, IL) on protoplasted deletion strains was performed. The transformation protocol was the same as above, except selection was performed using and overlay to generate a final concentration of 150 μg/ml hygromycin and 200 μg/ml geneticin.

Nitrite and nitrate growth under hypoxic and normal atmospheric conditions. Strains of *F. verticillioides* were first grown for 7 days on PDA. A 4.0 mm diameter core was transferred to the center of the nitrate or nitrite minimal media. Minimal media was made with nitrate (NO_3^-) in accordance to Leslie and Summerell, 2006. Nitrite (NO_2^-) minimal media was made by

substituting in nitrite for nitrate. Both nitrate and nitrite minimal media were made at a 40 mM concentration. After the core was added, plates were incubated in the dark at 27 ºC for 7 days in either normal atmospheric conditions or under hypoxic conditions. Hypoxic conditions were attained by loading plates into Oxoid anaerobic chambers (Oxoid Limited, Hampshire, UK) that were flooded with hypoxic gas mixture 5.0% O_2, 10.0% CO_2, and balanced with N_2 (Airgas, Radnor, PA).

Pathogenicity on maize seedlings. The maize cultivar Silver Queen (Gurney's Seed & Nursery Co., Yankton, SD, USA), a sweet corn line susceptible to *F. verticillioides*-induced maize seedling disease, was used in this study. Maize kernels were treated as described by William et. al. (2007). Briefly, kernels were surface-disinfected for 10 min in 100% commercial bleach (5.25% hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water. The seeds were then heat treated by placing them in a 60 °C water bath for 5 min for internal sterilization (Bacon and Hinton, 1994). Seeds were inoculated with a 10^4 spores per mL suspension. Three replicates of 10 seeds each were planted in sterile 10-cm plastic azalea pots (Hummert International, Earth City, MO, USA) containing twice-autoclaved commercial potting soil mix (Conrad Faford Inc., Agawam, MA, USA).

The treatments were as follows: M3125 (wt), *fhb1*, *fhb2*, *fhb1/fhb2*, *nir1*, *nor1*, and respective add-backs strains and compared against non-inoculated seedlings. Pots were watered as needed throughout the duration of the assay. Assays were performed under aseptic conditions in a plant growth chamber at 30 °C under 14 h light (cool-white, high-output fluorescent tubes at an average of 254 µmol/m^2/s) and a 10-h dark regimen at 20 °C. Plant tissues were collected for

analysis 14 days after planting. During harvest shoots and roots were saved and dried in a lyophilizer to measure biomass. Soil was saved for analysis of fumonisin accumulation.

Quantification of Soil Fumonisins. Fumonisin quantification and data analyses were performed as previously described by Zitomer *et al.*(2008). Soil from seedlings infected with deletion mutants and wild-type *Fusarium verticillioides* was air dried before extraction. Soil samples were extracted in a 1:1 MeCN:water + 5% formic acid (25 mL per 2 g). Extracts were filtered and diluted 1:10 into the initial mobile phase used for reverse phase-high performance liquid chromatography (RP-HPLC). Analyses were conducted using a Finnigan Micro AS autosampler coupled to a Surveyor MS pump (Thermo-Fisher, Woodstock, GA, USA). Separation was accomplished using an Imtakt Cadenza 150×2 mm i.d., 3 μm CW-C18 column (Imtakt, Philadelphia, PA, USA). Column effluent was coupled to a Finnigan LTQ XL linear ion trap mass spectrometer (MS).

Assessment of endogenous nitric oxide production. The fluorescent probe DAF-FM DA (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate) (Life Technologies, Carlsbad, CA) was utilized to visualize the endogenous production of NO in *F. verticillioides* mutants and M3125. A working stock of 5mM in dimethyl sulphoxide (DMSO) was diluted to 20 μM in Tris-HCl (10 mM, pH 7.5). Ten microliters of 3 day old *F. verticillioides* PDB culture was added into 250 μl of Tris- HCl (10 mM, pH 7.5), 20 μM DAF-FM DA and incubated in the dark at room temperature for 2 hrs. Fluorescence was detected with 495 nm excitation and 515 nm emission. DAF-FM DA was cell permeable and detected. Each experiment contained at least three biological replicates. Either 4,4,5,5-Tetramethylimidazoline -l-oxyl3-oxide (PTIO) or carboxy-

PTIO (cPTIO) was utilized to demonstrate specificity of DAF-FM DA and loss of fluorescence was observed with increasing concentrations of PTIO. Images were taken with a Leica DM6000 B light microscope (Hamamatsu ORCA-ER, C4742-80 monochrome camera). Quantification was performed using the Leica Application Suite (2.1.2 build 4530) software.

Statistical analysis. Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). When many groups were compared, analysis of variance (ANOVA) was used followed by post-hoc multiple comparisons. Unless specified, all data were expressed as mean ± standard deviation and differences among means were considered significant if the probability (P) was ≤ 0.05.

RESULTS

Induction of *FHB1* and *FHB2* by NO detected by qPCR

A BLASTP search of the *Fusarium* comparative genomics website hosted by the Broad Institute was performed using the *Candida albicans* YHB1 and revealed *F. oxysporum* has four flavohemoglobins, whereas *F. graminearum* and *F. verticillioides* each contain only two. Neighbor-joining analysis of the flavohemoglobin protein sequences from filamentous ascomycetes recovered from NCBI indicated the two FHB homologs in *F. verticillioides* clearly belong to two distinct sister clades and each pair with a *F. oxysporum* ortholog (Figure 5.1) homolog. Notably, the FHB1 and FHB2 flavohemoglobins from *F. verticillioides* were in separate clades from the well characterized *Aspergillus* flavohemoglobins (Zhou et al., 2010).

In order to test the induction of the flavohemoglobin genes *FHB1* and *FHB2* upon exposure to NO, a growth curve analysis was first performed to view the effect of increasing NO concentrations on the growth of *F. verticillioides* strain M3125 (Figure 5.2). As expected, the highest concentration of DETA NONOate had the greatest effect on growth and all levels of DETA NONOate had a dose-dependent restrictive effect on the growth of *F. verticillioides*. It should be noted that after approximately 55 hours of NO exposure additional growth recovered and all cultures quickly gained turbidity to match the growth of the unexposed control. This recovery is likely due to the half-life of the spontaneously dissociating DETA NONOate for releasing 2 moles of NO per mole of parent compound at 22-25°C at pH 7.4. Furthermore, there were observed morphological effects of 6 mM DETA NONOate on spore germination, including thin, elongated hyphal-like germ tubes with limited or no branching (data not shown). Based on the growth of wild type, 1.5 mM of DETA NONOate was chosen to perform subsequent experiments, as that level we observed limited growth with no observable defects in morphology.

Induction of the *FHB1* and *FHB2* genes was observed with exposure at 1.5 mM DETA NONOate. The highest induction was seen at 45 min with a 149-fold induction of *FHB2* and a 48-fold induction of *FHB1* (Figure 5.3). The initial time point produced no induction of *FHB1* and *FHB2* (~1 fold). After 360 min the expression levels of *FHB1* and *FHB2* return to base expression levels (1- and 3-fold, respectively) (Figure 5.3). β-tubulin (*TUB2*) was used to normalize the data. Also notable is that across all treatments and time points *FHB1* had higher expression than *FHB2* (data not shown). So while *FHB2* exhibited greater induced expression to the NO treatment, *FHB1* had a higher level of constitutive expression.

Microarray analysis of NO challenged *Fusarium verticillioides*

Roche NimbleGen microarrays were used to assess global changes in gene expression in response to NO exposure using 1.5 mM DETA NONOate. *F. verticillioides* strain M3125 was exposed to NO for 45 min and tested against an unexposed control. Reported fold increase is the average of two biological replicates. The flavohemoglobin gene *FHB2* showed a 17-fold induction compared to the 3-fold induction by *FHB1*. Similar to the qPCR data, *FHB1* had a high level of normalized gene expression in the controls as well as the NO treatment (data not shown).

Listed in Table 5.2 are 30 of the genes with the greatest induction in gene expression. Many of the genes induced by NO exposure are currently uncharacterized. *FHB1* induction was minimal but is listed in Table 5.2 for comparison. The greatest fold change (246-fold) was observed for FVEG_08676. NCBI BLASTP search suggests this gene encodes a copper-containing nitrite reductase. Current literature places this dissimilatory nitrite reductase (dNir) in the denitrification pathway in *F. oxysporum*, a pathway which also utilize a dissimilatory nitrate reductase (dNar) and a P450 nitric oxide reductase (Nor) (Shoun et al., 2012; Takaya and Shoun, 2000). These enzymes correspond to FVEG_01721 (NAR1) and FVEG_10773 (NOR1) in *F. verticillioides*, which were also noticeably induced by NO treatment (16- and 22-fold, respectively).

Down regulation of some genes was noted following exposure to NO, but these were not linked to a single pathway. An allantoate transporter protein (FVEG_00232) was down regulated 19-fold. An x pro-dipeptidyl peptidase (FVEG_00224) and an amino acid permease (FVEG_11569) were also notably down regulated 17- and 15-fold, respectively.

Deletion of *FHB1*, *FHB2*, *NIR1*, and *NOR1* from *Fusarium verticillioides*

Selected genes FVEG_11186 (*FHB1*), FVEG_13827 (*FHB2*), FVEG_08676 (*NIR1*), and FVEG_10773 (*NOR1*) that either produce or detoxify NO were deleted using the DelsGate method (García-Pedrajas et al., 2008). See Tables 5.3 and 5.4 for primers used for deletion construct creation and screening of transformants. All transformants were screened first by testing the 5' and 3' flank chimera and the absence of the ORF by PCR. Southern blot analysis was used to verify gene knockouts. All deletions were in the M3125 background unless specified.

FVEG_11186 (*FHB1*) Southern analysis using a 5'flank probe produced a ~1.3 kb knockout fragment with genomic digestion by *HpaI*, as compared to the ~3.9 kb fragment detected in wild type (Figure 5.4 A). Transformants *fhb1* -1, 2, and 3 were identified as true knockouts and one ectopic was identified (Figure 5.4 B). FVEG_13827 (*FHB2*) Southern analysis using a 3'flank probe produced a ~1.7 kb wild-type allele fragment with genomic digestion by *EcoRI*, as compared to the ~5.4 kb fragment detected for the deletion allele (Figure 5.4 A). Transformants *fhb2* 1-6 were identified as true knockouts, and three ectopic transformants identified (Figure 5.5 B). Additionally, knock k-outs *fhb2* 1-3 and one ectopic transformant were identified in strain A00999 (Figure 5.4 C).

To generate the *fhb1/fhb2* double mutant, M3125 *fhb1* was mated to A00999 *fhb2*. After 4-5 weeks approximately 20 to 30 perithecia were observed per plate. Single ascospore progeny were cultured and tested by multiplex PCR for the *FHB1* and *FHB2* ORFs. Of the 30 progeny tested, 9 were identified as *fhb1/fhb2* double deletion, 2 were *fhb1* deletion, and 19 were *fhb2* (Figure 5.6 A). Wild-type *FHB1/FHB2* was not detected since progeny were selected on hygromycin B media. Progeny were further tested for the *MAT1-1* and *MAT1-2* mating types. Of

the 8 strains tested, 4 were determined to be *MAT1-1*, and 4 were determined to be *MAT1-2* (Figure 5.6 B). For further experiments *fhb1/fhb2 MAT1-1* was used.

FVEG_08676 (*NIR1*) Southern analysis using a 5'flank probe produced a ~8.5 kb knockout fragment with genomic digestion by *EcoRV* compared to the ~1.9 kb fragment detected in wild type (Figure 5.7 A). Transformant *nir1* 4 was identified as a knockout and two ectopic transformants were identified. Transformants *nir1* 1*,2*, and 3* were utilized for supporting data as deletion strains since they contain multiple insertions (Figure 5.7 B). FVEG_10790 (*NOR1*) Southern analysis using a 5'flank probe produced a ~6.5 kb knockout fragment with genomic digestion by *XbaI* compared to the ~2.5 kb fragment detected in wild type (Figure 5.8 A). Transformant *nor1* 2 was identified as a true knockout and one ectopic transformant was identified. Transformants *nor1* 1* and 3* were also utilized for supporting data due to the multiple insertion (Figure 5.8 B).

Growth rate of mutants on nitric oxide (NO) compared to wild type

Growth of the deletion mutants and M3125 challenged with 1.5 mM DETA NONOate (NO donor) demonstrated significant differences in growth. Experiments had at least 3 biological repetitions with comparable trends. The flavohemoglobin *fhb1* and *fhb2* deletions each showed decreased growth with initiation of growth starting at ~30 hrs compared to ~20 hrs for wild type (~20 hrs). Notably *fhb2* deletion had a greater effect on growth than *fhb1* (Figure 5.9 A). Similarly, *nor1* deletion also produced a decrease in growth comparable to wild type, however, the *nir1* deletion exhibited an increase in growth (Figure 5.8 B). The unexposed growth in PDB observed tight grouping of growth curves of the deletion mutants with wild type (Figure 5.8 C). Also, there was relatively tight grouping noted for the ectopic strains to the wild-type M3125

when exposed to NO (Figure 5.9 D). Comparison of all growth curves demonstrated a significant difference between NO exposed and unexposed treatments (Figure 5.9 E) The most notable growth effect was seen with exposure of the *fhb1/fhb2* double deletion strains to NO, in which no growth was detected (Figure 5.9 A). At the end of the bioscreen experiment, wild-type M3125 and *fhb1/fhb2* double deletion were sampled and plated onto PDA to determine if NO was biostatic or toxic (Figure 5.9 F). A survival rate of 7% was calculated and indicates the toxic effects of NO without the protection of the flavohemoglobins.

Growth rate of mutants on nitrate (NO_3^-) and nitrite (NO_2^-) compared to wild type

To understand the role of *FHB1*, *FHB2*, *NIR1*, and *NOR1* in nitrate and nitrite metabolism, deletion strains were challenged on minimal media with either 40 mM of nitrate or nitrite under both hypoxic and atmospheric conditions. Hypoxic conditions were relevant given that the denitrification pathway was previously described as a hypoxic pathway in *F. oxysporum* (Shoun et al., 2012; Takaya and Shoun, 2000). All strains grew on nitrate minimal media under both hypoxic and normal atmospheric conditions (Figure 5.10 A and B, respectively). Interestingly, under hypoxic conditions the *fhb1/fhb2* and *nir1* deletions had slightly increased growth over most other strains and the wild type. No strains suffered a significant decrease in growth over wild type (Figure 5.10 A). No differences in growth were noted under normal atmospheric conditions (Figure 5.10 B).

Growth on nitrite media produced the most insightful results for the role of these genes in denitrification. Mutants *fhb2*, *fhb1/fhb2*, *nir1*, and *nor1* were unable to grow on nitrite minimal media under hypoxic conditions and are hence required for denitrification (Figure 5.11 A). These

data suggest *FHB2* is directly linked to denitrification and plays an important role during nitrite metabolism. All strains were able to grow on nitrite media under normal atmospheric conditions, although *fhb1/fhb2* double deletion had an observable reduction of growth.

Virulence of mutants during seedling infection and fumonisin production

Seedling blight virulence of deletion mutants was tested in comparison to wild type. Seedling height and biomass were assessed 14 days after inoculation and all single gene deletions had similar effects on seedling growth comparable to wild type (Figure 5.12 A). In contrast the *fhb1/fhb2* double deletion reduced seedling height and biomass. Thus, the *fhb1/fhb2* mutant exhibited greater virulence on susceptible maize cultivar Silver Queen (Figure 5.12 B and C). Likewise, and linked to the increased virulence of *fhb1/fhb2* on Silver Queen, soil sample analysis revealed a significant increase in fumonisin B1 (FB_1) accumulation in the soil (128.03±38.73 nmol/g soil FB_1) in the double deletion compared to wild type (57.98±13.06 nmol/g soil FB_1)

Morphology and endogenous production of nitric oxide by *F. verticillioides* in wild type and mutants.

Endogenous production of NO in *F. verticillioides* was explored with the use of the NO probe DAF-FM DA. The diacetate (DA) conjugate of DAF-FM allows for entry into cell where DA is cleaved off by esterases and traps the probe intracellularly (McQuade and Lippard, 2010). All strains demonstrated significant probe staining. Wild-type endogenous production of NO was observed mostly in the cytosol in most conidia and hyphae. It is unclear what the biological

implications are for the conidia and hyphae that did not stain in these samples and might be an artifact of asynchronized growth of the culture. Staining equivalent to wild type was seen in *fhb2* and *nir1* mutants (Figure 5.12 A). The *fhb1/fhb2* and *nor1* stains also demonstrated staining similar to wild type (data not shown). However, there were morphological differences noted in *fhb1*, including increased phialide formations and greater intensity of DAF-FM DA (Figure 5.12 B).

DISCUSSION

In this study we investigated the role of the flavohemoglobins FHB1 and FHB2 in detoxification of nitric oxide in *Fusarium verticillioides*. These flavohemoglobins were demonstrated to be the primary detoxifying agents when challenged with exogenous NO. Moreover, we have placed these enzymes into a perspective of nitrate and nitrite metabolism. Somewhat surprisingly, genes from the denitrification pathway were highly induced as well as the flavohemoglobins when challenged with exogenous NO. As denitrification was previously characterized as a hypoxic pathway (Shoun et al., 1992; Takaya and Shoun, 2000), our experiments extend the functionality of this pathway to included detoxification of NO under oxygenic conditions in *F. verticillioides*. Through our nitrate and nitrite media experiments *FHB2* was linked to denitrification, as deletion of that gene renders the fungus incapable of growing on nitrite media under hypoxic conditions and suggests NO detoxification by *FHB2* is important in hypoxic nitrogen metabolism of denitrification.

The ability to detoxify NO is a necessity for microorganisms across different environmental niches. NO is involved in most pathogenic interactions and its detoxification by flavohemoglobins has been implicated as a virulence factor in *Candida albicans*, *Cryptococcus*

neoformans, and the bacterial plant pathogen *Erwinia chrysanthemi* during infections (Favey et al., 1995; Gardner, 2005; Idnurm et al., 2004; Ullmann et al., 2004). Organisms can contain one or many copies of the flavohemoglobins in their genome with perhaps differing functional roles. *Saccharomyces cereviseae* has two and *Candida albicans* has four (Lushchak et al., 2010; Ullmann et al., 2004). However some important pathogens, such as *Cryptococcus neoformans*, *Magnaportha oryzae*, and *Botrytis cinerea* have a single flavohemoglobin gene (Idnurm et al., 2004; Luis Turrion-Gomez et al., 2010; Samalova et al., 2013).

Certain *Aspergillus* species, including *A. oryzae* and *A. nidulans*, contain two paralog flavohemoglobins that function in different physiological roles. In *A. oryzae*, *Fhb1* is a cytosolic flavohemoglobin that is inducible by exogenous NO, whereas *Fhb2* is a mitochondrial locating flavohemoglobin and does not respond to exogenous NO (Zhou et al., 2010). *Fhb2* likely functions to detoxify nitric oxide produced in the mitochondria by respiratory chain reactions. A similar function was noted in *A. nidulans* (Schinko et al., 2010). In *F. oxysporum* there are orthologs of *FhbB* and *Fhb2*, FOXG_06984 and FOXG_15840 that contain the mitochondrial transpeptide sequence (Figure 5.1). However, *F. graminearum* or *F. verticillioides* seem to lack this type of flavohemoglobin. Instead, the *F. verticillioides FHB2* does not contain a mitochondrial transpeptide sequence and appears in a unique clade paired with orthologous FOXG_17028 from *F. oxysporum.* These *Fusarium FHB2* proteins show a common relationship with *Verticillium* flavohemoglobins. This clade represents significant soil-borne and vascular pathogens and thus may be relevant to their survival in that lifestyle (Figure 5.1). Interestingly, *F. graminearum* is not considered a significant or vascular soil-borne pathogen and lacks a *FHB2* ortholog. However, it does share an *FHB1* flavohemoglobin with *F. verticillioides*, *F. oxysporum* and *Nectria haematococca*. The *FHB1* orthologs also lack a mitochondrial signal peptide and are

predicated to be cytosolic with *FHB2*. Notably, another flavohemoglobin homolog exists in *F. graminearum* (FGSG_00765), which groups with different ascomycetes, including *Nectria*, *Myceliophthora*, *Thielvaia*, *Trichoderma*, and *Sordaria* (Figure 5.1). Further investigation into this protein could provide interesting results on how *F. graminearum* detoxifies NO in its specific lifestyle.

We hypothesized that flavohemoglobins were the primary mode of NO detoxification and thus would have the highest induction by exogenous NO in *F. verticillioides*. However, microarray analysis revealed *NIR1*, a dissimilatory nitrite reductase and an integral part of the denitrification pathway, to have 14 times higher expression than *FHB2*. Also, the high expression of the other denitrification genes *NAR1* and *NOR1* were observed during NO exposure. Denitrification is the process of reverting fixed nitrogen back to gaseous nitrogen and is a substantial source of N_2O emissions common among soil bacterial species. This process robs the soil of nutrients and contributes to the release of a powerful greenhouse gas that's nearly 300 times more effective than CO_2 (EPA, 2010). Limiting this microbial process in agricultural soils would be beneficial as agricultural soil is the main contributor of N_2O and this process removes nitrogen from soil (Long et al., 2013). Denitrification involves a 4 step process reducing nitrate (NO_3^-) to atmospheric nitrogen (N2) (Zumft, 1997). Catalysis at each step involves conversion of NO_3^- to NO_2^- to NO to N_2O, and finally to N_2 with the respective enzymes being dissimilatory nitrate reductase (dNar), dissimilatory nitrite reductase (dNir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) (Zumft, 1997). In fungi, denitrification is rare and lacks the final nitrous oxide reductase (Nos), ending with N_2O instead of N_2. *Fusarium oxysporum* and *Cylindrocarpon tonkinense* denitrification has been modeled and demonstrated to produce energy under hypoxic conditions in the mitochondria (Kobayashi et al., 1996; Shoun et al., 1992; Shoun

and Tanimoto, 1991). Ours is the first molecular genetic analysis to thoroughly characterize through mutagenesis of the genes in the denitrification pathway.

F. verticillioides clearly activates the denitrification pathway for detoxification of NO. Interestingly, this hypoxic pathway was activated during aerobic conditions with NO exposure. Furthermore, we demonstrate *FHB2* has an important function for recycling NO to NO_3^- in the denitrification pathway as this flavohemoglobin was necessary for *F. verticillioides* to grow on nitrite under hypoxic conditions, where denitrification is the sole pathway for nitrite metabolism. Additionally, of the two flavohemoglobins *FHB2* is postulated to be primarily responsible for detoxifying exogenous NO and likely acts as an extra flavohemoglobin for additional detoxification capabilities. Furthermore, we demonstrate the importance of the flavohemoglobins for protection of *F. verticillioides* against NO stress with the *fhb1/fhb2* double deletion in NO exposure experiments and on nitrite media. Furthermore, the NO stress leading to nitrosative stress is potentially triggering increased fumonisin production in *F. verticillioides* and, by extension, virulence.

Our model suggests different functions of the two flavohemoglobins in *F. verticillioides*. Assessment of gene expression with qPCR and microarrays indicated *FHB2* is generally expressed at low levels and activated upon induction of the denitrification pathway, during NO stress or in nitrite metabolism. However, the *FHB1* paralog is a constitutively expressed gene that likely provides consistent background scavenging of NO produced by normal cellular metabolism and by diffuse environmental NO. This would explain why the NO probing exhibited an abundance of NO probe fluorescence in *fhb1* mutants. We hypothesize that these cases of greater fluorescence is caused by higher levels of NO in the cytosol not being detoxified in fhb1 deficient mutants, and these NO levels might be below the threshold for activation of

FHB2. Proliferation of phialides was also noted with increased NO detection in *fhb1* mutants and may be attributable to morphological effects of increased background NO in the *fhb1* mutants (Figure. 5.13 B). Understanding the function NO in *F. verticillioides* may help control *F. verticillioides* pathogenicity and even fumonisin production in maize seedling disease.

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Table 5.1. Quantitative Real-Time PCR (qPCR) Primers for FVEG_11186 (*FHB1*) and FVEG_13827 (*FHB2*). Primers are listed for FVEG_04081 (TUB2), which was utilized for normalization of qPCR.

Gene	Set	Tm (C°)	gc%	Product Size (bp)	Sequence -3’
FHB1	Forward	55.6	55	100	CCAAGCTCCAGAAGGAACAG
FHB1	Reverse	55.1	50	100	AGGAATGCCCTGACATTGAC
FHB2	Forward	54	50	154	CTTTATCAACCGCGAGAAGC
FHB2	Reverse	56.5	55	154	ATACTGACCAGGACGGAACG
TUB2	Forward	61.7	56	151	CAGCGTTCCTGAGTTGACCCAACAG
TUB2	Reverse	63.4	60	151	CTGGACGTTGCGCATCTGATCCTCG

Table 5.2. Top 30 Genes by Fold Increase Expression After Nitric Oxide Exposure to Wild-type M3125 Based on Microarray Analysis. EST number is represented in the first column and the corresponding genome number is in the second column. Putative function is listed in the third column. The last column gives the fold changes of gene expression in wild type (M3125) treatment of nitric oxide compared to non-treated wild type (M3125) from microarray analyses. Genes of interest are bolded in the table.

#	Fold Change	Genome ID	Transcript ID	Broad Description	NCBI pBLAST Closest Match
1	**246**	**FVEG_08676**	**FVET_08691**	**hypothetical protein**	**copper-containing nitrite reductase (*NIR1*)**
2	160	FVEG_10494	FVET_10511	hypothetical protein	aminotransferase
3	67	FVEG_00007	FVET_00007	hypothetical protein	choline transport protein ; GABA permease
4	47	FVEG_00006	FVET_00006	hypothetical protein	MFS Maltose permease MAL31
5	26	FVEG_13151	FVET_13170	hypothetical protein	transcription factor WhiB
6	23	FVEG_11970	FVET_11988	hypothetical protein	putative 4-hydroxyacetophenone monooxygenase
7	23	FVEG_01722	FVET_01724	hypothetical protein	transcriptional regulator
8	22	FVEG_03704	FVET_03708	hypothetical protein	formate/nitrite transporter family protein, putative
9	22	FVEG_10401	FVET_10418	hypothetical protein	transcriptional regulator, putative
10	**22**	**FVEG_10773**	**FVET_10790**	**cytochrome P450 55A1**	**cytochrome P450 (*NOR1*)**
11	21	FVEG_06152	FVET_06160	hypothetical protein	hypothetical protein
12	21	FVEG_07246	FVET_07256	hypothetical protein	hydrolase, lipase, esterase
13	19	FVEG_07808	FVET_07819	hypothetical protein	hypothetical protein
14	19	FVEG_09104	FVET_09119	hypothetical protein	hypothetical protein
15	19	FVEG_11973	FVET_11991	aldehyde dehydrogenase	aldehyde dehydrogenase
16	18	FVEG_09103	FVET_09118	hypothetical protein	Arrestins (regulates G protein-coupled receptors)
17	18	FVEG_10772	FVET_10789	hypothetical protein	asparagine synthase, glutamine-hydrolyzing
18	**17**	**FVEG_13827**	**FVET_13847**	**hypothetical protein**	**flavohemoglobin (*FHB2*)**
19	16	FVEG_12433	FVET_12452	hypothetical protein	glutathione s-transferase
20	16	FVEG_03329	FVET_03333	hypothetical protein	P450 oxidoreductase
21	**16**	**FVEG_01721**	**FVET_01723**	**hypothetical protein**	**Nitrate reductase (*NAR1*)**
22	15	FVEG_08677	FVET_08692	hypothetical protein	hypothetical protein
23	15	FVEG_01600	FVET_01602	hypothetical protein	aromatic and neutral aliphatic amino acid permease
24	14	FVEG_03315	FVET_03319	acetyl-CoA acyltransferase	3-ketoacyl-CoA thiolase, peroxisomal
25	13	FVEG_11142	FVET_11159	hypothetical protein	peroxisomal catalase
26	13	FVEG_03361	FVET_03365	hypothetical protein	short-chain dehydrogenase
27	12	FVEG_08629	FVET_08644	hypothetical protein	hypothetical protein
28	12	FVEG_09401	FVET_09417	hypothetical protein	zinc-binding oxidoreductase
29	11	FVEG_10493	FVET_10510	hypothetical protein	Beta-glucosidase
30	9	FVEG_06902	FVET_06911	hypothetical protein	C6 transcription factor
31	**3**	**FVEG_11186**	**FVET_11203**	**nitric oxide dioxygenase**	**flavohemoglobin (*FHB1*)**

Table 5.3. DelsGate Primers Used to Generate Deletion Constructs for FVEG_11186 (*FHB1*) and FVEG_13827 (*FHB2*). Primers 1, 2, 3, and 4 include extensions from DelsGate construct development for homologous replacement of the gene of interest. The underlined text in primers 1 and 4 are complementing *I-SceI* sequences that overlap. Primers 2 and 3 include the *attB1* and *attB2* sequences (bold), respectively for homologous integration into the pDONR vector. Primers 5 and 6 are utilized with HygF and KanR (from García-Pedrajas et al., 2008), respectively, to detect the chimeric inserts. Primers 7 and 8 were used to detect the open reading frame (ORF).

Gene	Primer	Location/Addition	Sequence
FHB1	1	5'Fw w/ I-SceI	TAGGGATAACAGGGTAAT TGAGCAATGCCGGGTACACT
	2	5'Rv w/attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT** AAGCACCTGGGATCTTGATG
	3	3'Fw w/attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA** AGGCAAGGCTTGAGTTACGA
	4	3'Rv w/ I-SceI r/c	ATTACCCTGTTATCCCTA TCGACCATGTGGTGCTACATGAGG
	5	5'Fw outer	GCTGGAGGAGCTCAAAGATG
	6	3'Rv outer	TCGTCGTGAGACCTTGTCAG
	7	Fw - ORF	AGGTTCTCGGTTCTGCTTTGACGA
	8	Rv - ORF	TGGCGGTGATATTCTCGTGCTTCT
FHB2	1	5'Fw w/ I-SceI	TAGGGATAACAGGGTAAT TTGGTCGCGAAATGATGTTA
	2	5'Rv w/attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT** GCGTGCTTTCTGAAGATTCC
	3	3'Fw w/attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA** CGTAACCTCGTACCGCATCT
	4	3'Rv w/ I-SceI r/c	ATTACCCTGTTATCCCTA TTCAACGCCTCGGTACAAGT
	5	5'Fw outer	CCCGAATCAATTCAGCAAGT
	6	3'Rv outer	AGGGCTGACAAGGTAGCAGA
	7	Fw - ORF	GGCGAGCAGCTTATCAAAGCCATT
	8	Rv - ORF	AAGCCAGAATTCGCCACGAGGATA

Table 5.4. DelsGate Primers Used to enerate Deletion Constructs for FVEG_08676 (*NIR1*) and FVEG_10773 (*NOR1*). Primers 1, 2, 3, and 4 include extensions from DelsGate construct development for homologous replacement of the gene of interest. The underlined text in primers 1 and 4 are complementing *I-SceI* sequences that overlap. Primers 2 and 3 include the *attB1* and *attB2* sequences (bold), respectively for homologous integration into the pDONR vector. Primers 5 and 6 are utilized with HygF and KanR (from García-Pedrajas et al., 2008), respectively, to detect the chimeric inserts. Primers 7 and 8 were used to detect the open reading frame (ORF).

Gene	Primer	Location/Addition	Sequence
NIR1	1	5'Fw w/ I-SceI	TAGGGATAACAGGGTAAT CCTCCGACATATCTATTGTGAC
	2	5'Rv w/attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT** GAAGGAGTTGGCTGGTTATT
	3	3'Fw w/attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA** CAGAATCCTGGCGTTTATCA
	4	3'Rv w/ I-SceI r/c	ATTACCCTGTTATCCCTA TTATCCTCAACTACCGCAAAG
	5	5'Fw outer	AGCTTACTTCAGCATCTTCTC
	6	3'Rv outer	TACTCACGTAGCGGAGATAG
	7	Fw - ORF	TGACTCAGGCTCAGGTATAG
	8	Rv - ORF	CGAAGACCATTGGGATATGAG
NOR1	1	5'Fw w/ I-SceI	TAGGGATAACAGGGTAAT CTTGCTTCAAAACCCTTTCG
	2	5'Rv w/attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT** GCCATGGTGATGGGAATAAC
	3	3'Fw w/attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA** TATATCAACCCGCCAACCAT
	4	3'Rv w/ I-SceI r/c	ATTACCCTGTTATCCCTA CAGTCTGGGCTTTCTCCTTG
	5	5'Fw outer	CGCTTGTTGCGTCTTTACAA
	6	3'Rv outer	GCGTACTCACAGCCTTCACA
	7	Fw - ORF	ACCTCGGAAGCAGTCAAGAA
	8	Rv - ORF	TAGTCGTAGGCACCCCAAAC

Figure 5.1. Neighbor-Joining Analysis of the Conserved Flavohaemoglobins Identified in Filamentous Fungi. NCBI and Broad Institute BLASTP search of the protein homologs of filamentous Ascomycetes are presented. *Fusarium verticillioides* contains two FHB paralogs (in red) and are in separate clades paired with a *Fusarium oxysporum* ortholog. Published flavohemoglobins in *Aspergillus* are listed name first. FHB homologs containing mitochondrial targeting signal peptide sequence are marked †. Predictions were made using TargetP, MitoProt II, and WoLF PSORT.

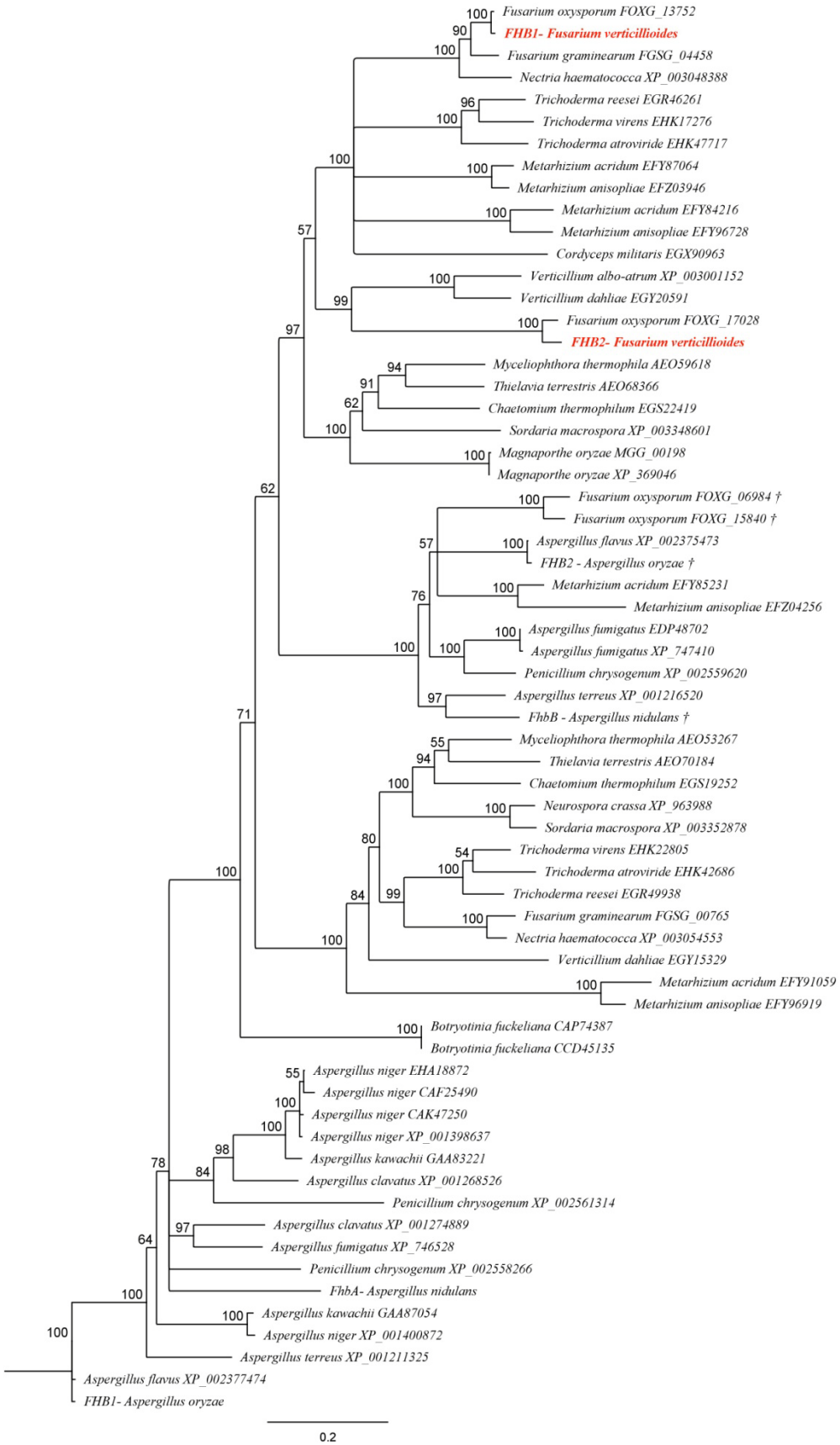

Fusarium oxysporum FOXG_13752
FHB1- Fusarium verticillioides
Fusarium graminearum FGSG_04458
Nectria haematococca XP_003048388
Trichoderma reesei EGR46261
Trichoderma virens EHK17276
Trichoderma atroviride EHK47717
Metarhizium acridum EFY87064
Metarhizium anisopliae EFZ03946
Metarhizium acridum EFY84216
Metarhizium anisopliae EFY96728
Cordyceps militaris EGX90963
Verticillium albo-atrum XP_003001152
Verticillium dahliae EGY20591
Fusarium oxysporum FOXG_17028
FHB2- Fusarium verticillioides
Myceliophthora thermophila AEO59618
Thielavia terrestris AEO68366
Chaetomium thermophilum EGS22419
Sordaria macrospora XP_003348601
Magnaporthe oryzae MGG_00198
Magnaporthe oryzae XP_369046
Fusarium oxysporum FOXG_06984 †
Fusarium oxysporum FOXG_15840 †
Aspergillus flavus XP_002375473
FHB2 - Aspergillus oryzae †
Metarhizium acridum EFY85231
Metarhizium anisopliae EFZ04256
Aspergillus fumigatus EDP48702
Aspergillus fumigatus XP_747410
Penicillium chrysogenum XP_002559620
Aspergillus terreus XP_001216520
FhbB - Aspergillus nidulans †
Myceliophthora thermophila AEO53267
Thielavia terrestris AEO70184
Chaetomium thermophilum EGS19252
Neurospora crassa XP_963988
Sordaria macrospora XP_003352878
Trichoderma virens EHK22805
Trichoderma atroviride EHK42686
Trichoderma reesei EGR49938
Fusarium graminearum FGSG_00765
Nectria haematococca XP_003054553
Verticillium dahliae EGY15329
Metarhizium acridum EFY91059
Metarhizium anisopliae EFY96919
Botryotinia fuckeliana CAP74387
Botryotinia fuckeliana CCD45135
Aspergillus niger EHA18872
Aspergillus niger CAF25490
Aspergillus niger CAK47250
Aspergillus niger XP_001398637
Aspergillus kawachii GAA83221
Aspergillus clavatus XP_001268526
Penicillium chrysogenum XP_002561314
Aspergillus clavatus XP_001274889
Aspergillus fumigatus XP_746528
Penicillium chrysogenum XP_002558266
FhbA- Aspergillus nidulans
Aspergillus kawachii GAA87054
Aspergillus niger XP_001400872
Aspergillus terreus XP_001211325
Aspergillus flavus XP_002377474
FHB1- Aspergillus oryzae
0.2

Figure 5.2 Growth Curves of *Fusarium verticillioides* Wild Type (M3125) Challenged with Nitric Oxide via NO-donor DETA NONOate. Growth curve was analized a Bioscreen C challenging *F. verticillioides* with 0, 1.5, 3, and 6 mM NO-donor (DETA NONOate)

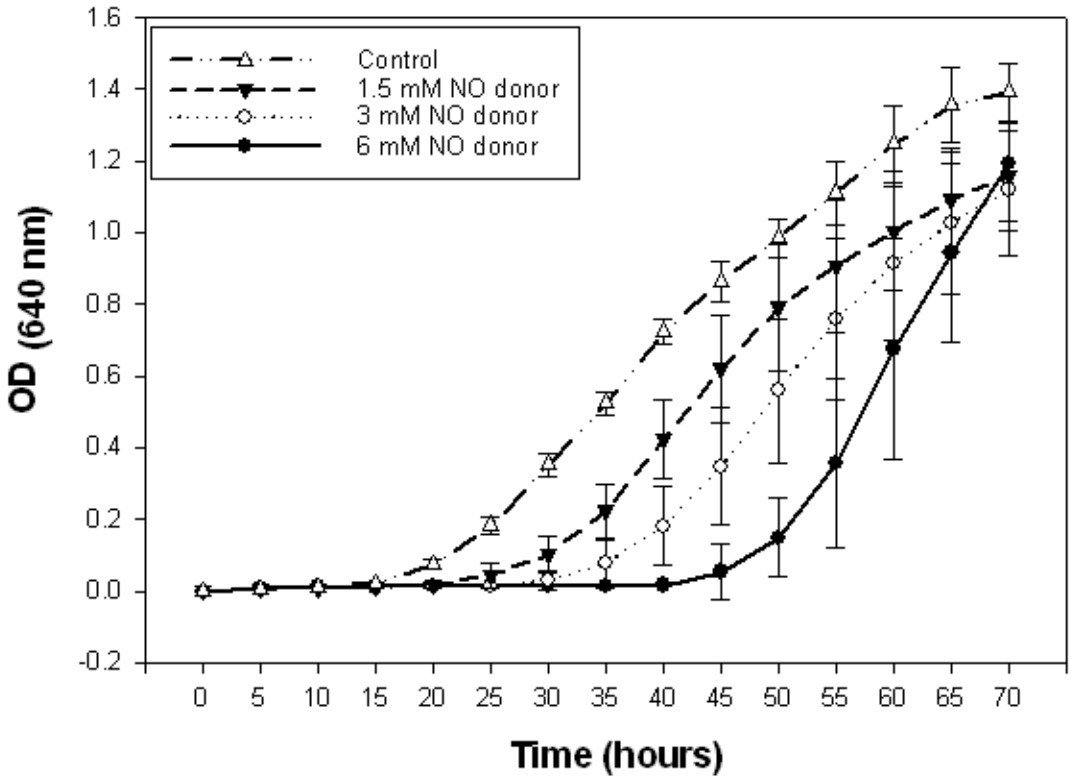

Control
1.5 mM NO donor
3 mM NO donor
6 mM NO donor
OD (640 nm)
Time (hours)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70

Figure 5.3. Quantitative Real time PCR of FVEG_11186 (*FHB1*) and FVEG_13827 (*FHB2*). Wild-type strain M3125 was exposed to 1.5 mM DETA NONOate (NO donor) for 15, 45, and 360 min. Gene transcription was noted for *FHB1* and *FHB2* at 45 min (approximately 50- and 150-fold, respectively). Transcription fold change is calculated via $2^{-\Delta\Delta Ct}$.

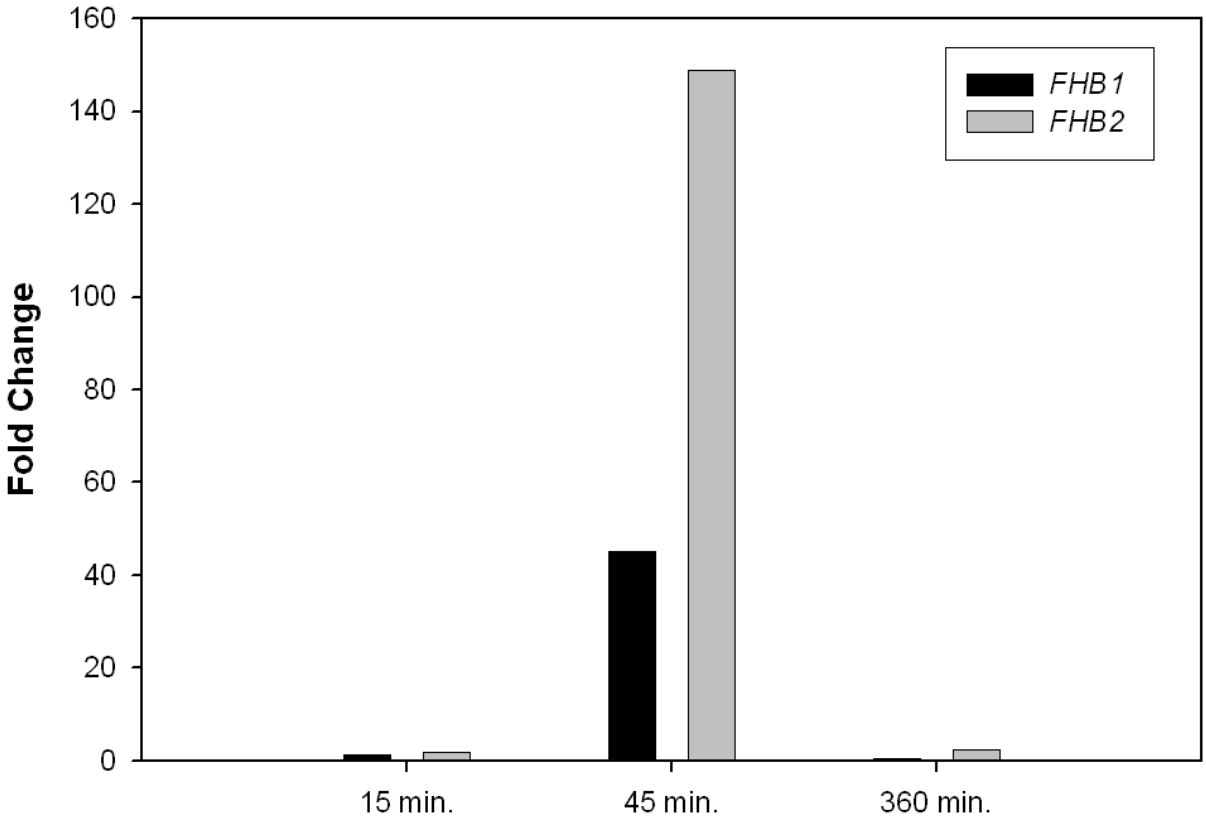
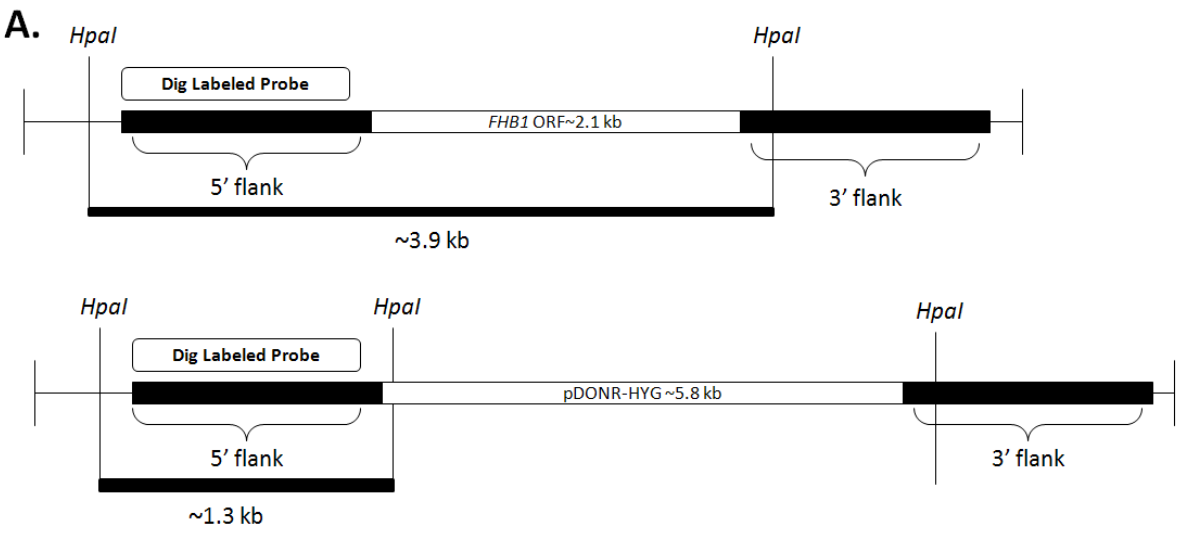

160
140
120
100
80
60
40
20
0
Fold Change
FHB1
FHB2
15 min.
45 min.
360 min.

Figure 5.4. Deletion Construct and Southern Blot to Confirm Deletion of *FHB1*. (**A**) The ~2.1 kb *FHB1* ORF was replaced with the ~5.8 kb DelsGate construct created with the 5' and 3' flank of the *FHB1* ORF. Genomic DNA was cut with *HpaI* and probed with a 5' flank DIG labeled probe. (**B**) WT allele was detected at ~3.9 kb and the deletion allele was detected at ~1.3 kb. Transformants were found to have the deletion allele. Ectopic transformant (*Ect.*) was utilized as a control in experiments.


A.
HpaI
HpaI
Dig Labeled Probe
FHB1 ORF~2.1 kb
5' flank
3' flank
~3.9 kb
HpaI
HpaI
HpaI
Dig Labeled Probe
pDONR-HYG ~5.8 kb
5' flank
3' flank
~1.3 kb

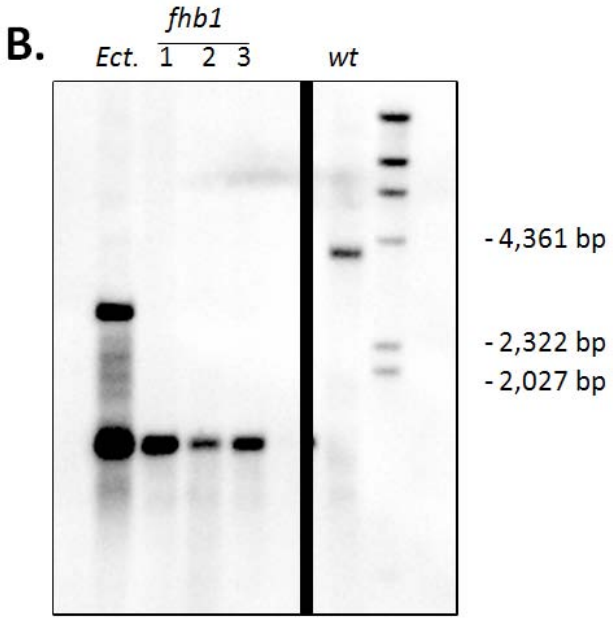
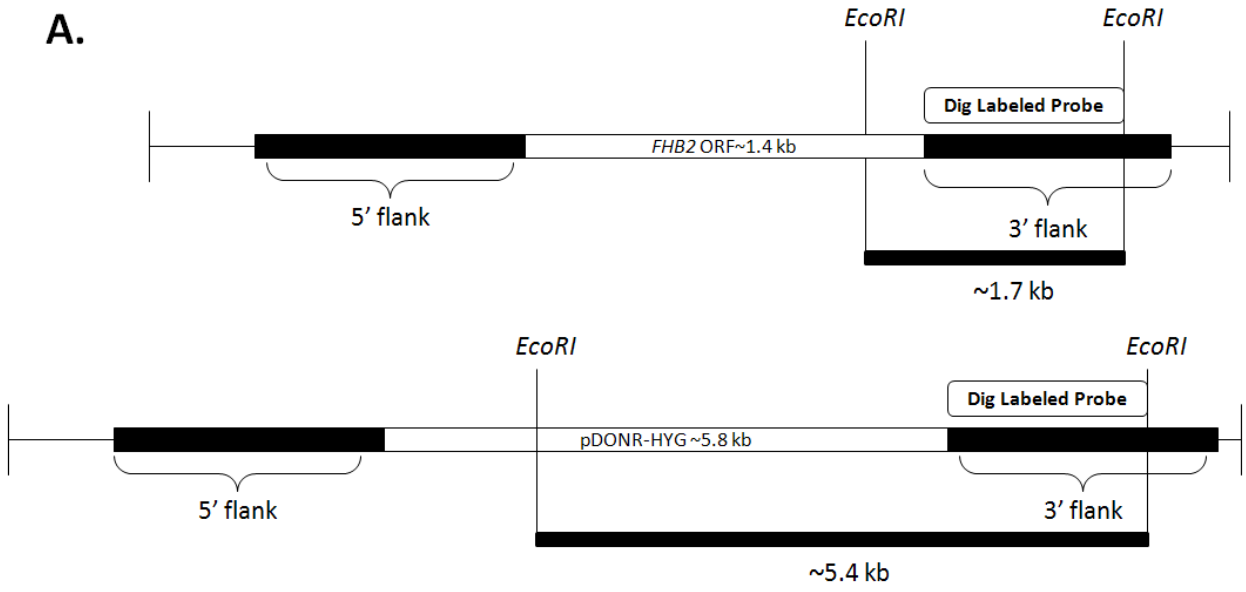
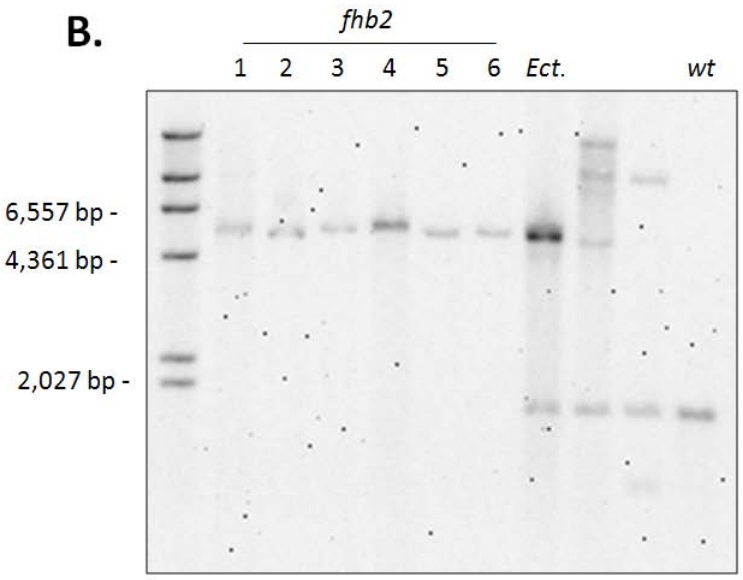

B.
fhb1
Ect. 1 2 3
wt
- 4,361 bp
- 2,322 bp
- 2,027 bp

Figure 5.5. Deletion Construct and Southern Blot to Confirm Deletion of *FHB2*. (**A**) The ~1.4 kb *FHB2* ORF was replaced with the ~5.8 kb DelsGate construct created with the 5' and 3' flank of the *FHB2* ORF. Genomic DNA was cut with *EcoRI* and probed for detection of the 3' flank. (**B**) M3125 background WT allele was detected at ~1.7 kb and the deletion allele was detected at ~5.4 kb. Transformants were found to have the deletion allele. Ectopic transformant (*Ect.*) was utilized as a control in experiments. (**C**) Southern of A00999 background used for mating with M3125- *fhb1*.


A.
EcoRI
EcoRI
Dig Labeled Probe
FHB2 ORF~1.4 kb
5' flank
3' flank
~1.7 kb
EcoRI
EcoRI
Dig Labeled Probe
pDONR-HYG ~5.8 kb
5' flank
3' flank
~5.4 kb


B.
fhb2
1 2 3 4 5 6 Ect. wt
6,557 bp -
4,361 bp -
2,027 bp -

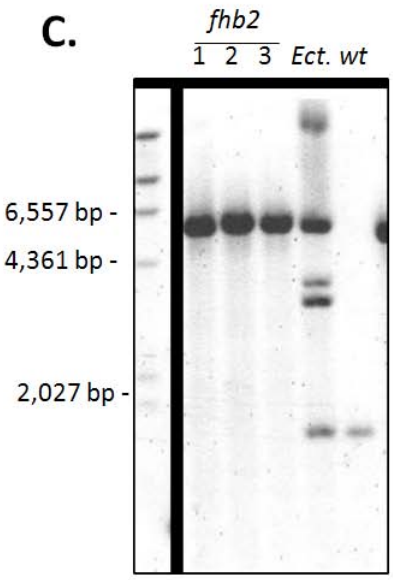
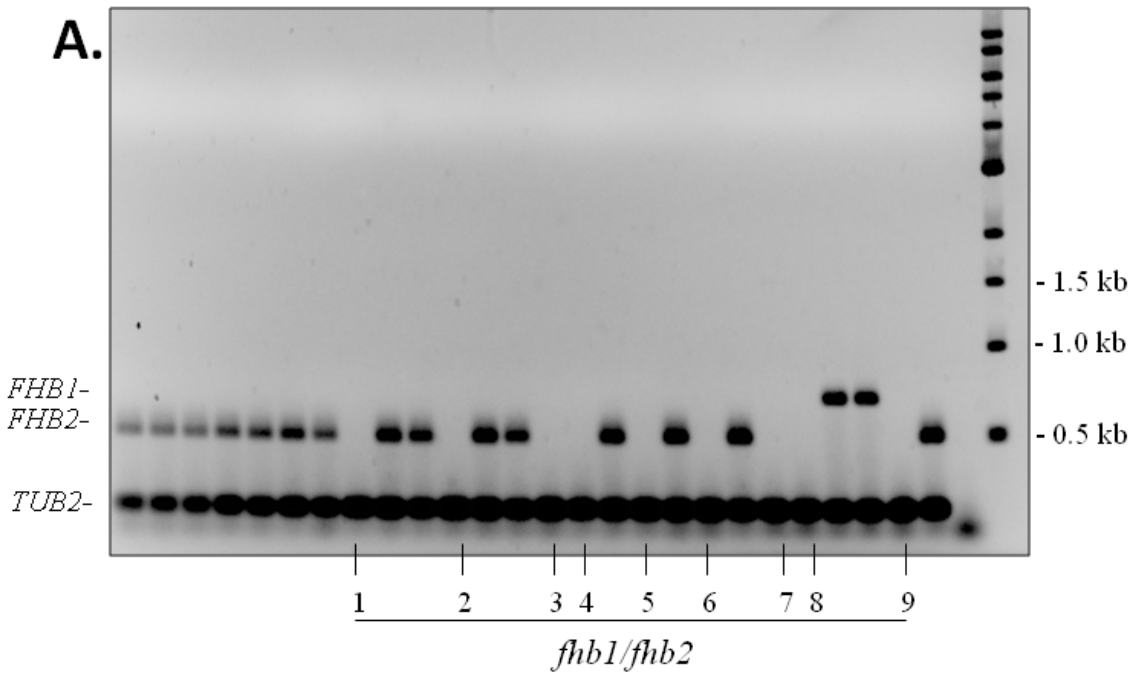

C.
fhb2
1 2 3 Ect. wt
6,557 bp -
4,361 bp -
2,027 bp -

Figure 5.6. Multiplex Screening for Identification of *fhb1/fhb2* Double Deletion Mutants. Progeny from a cross of *fhb1* (MAT1-1) to *fhb2* (MAT1-2) were screened to generate the *fhb1/fhb2* double deletion. (**A**) Double deletions were screened using ORF specific amplifying primers for *FHB1*, *FHB2*, and *TUB2*. There were 26 isolates screened and 9 of them lacked both the *FHB1* and *FHB2* ORF and were selected as the *fhb1/fhb2* double deletion. (**B**) Isolates were tested for mating type allele with 4 identified containing the *MAT1-1* loci and 4 identified containing *MAT1-2* loci.


A.
FHB1-
FHB2-
TUB2-
- 1.5 kb
- 1.0 kb
- 0.5 kb
1 2 3 4 5 6 7 8 9
fhb1/fhb2

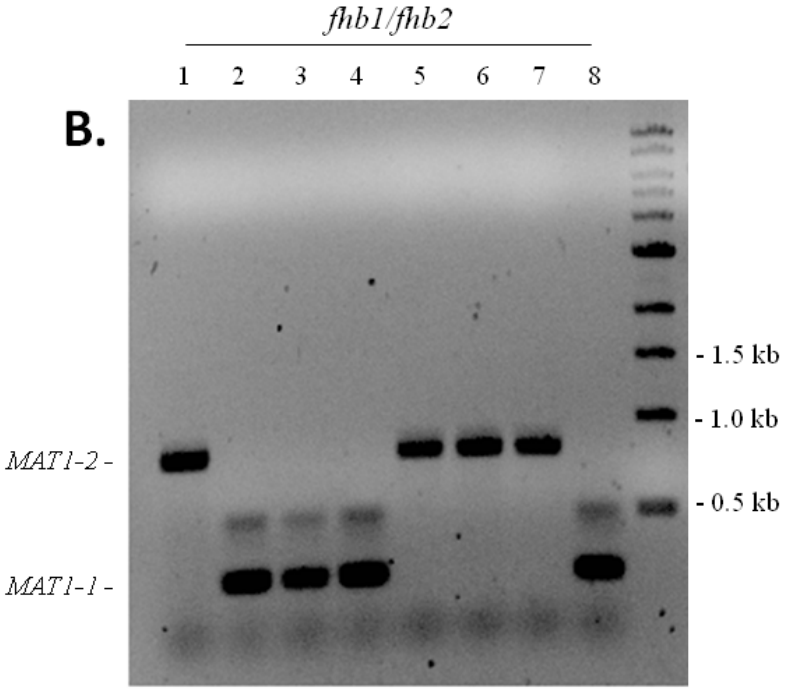
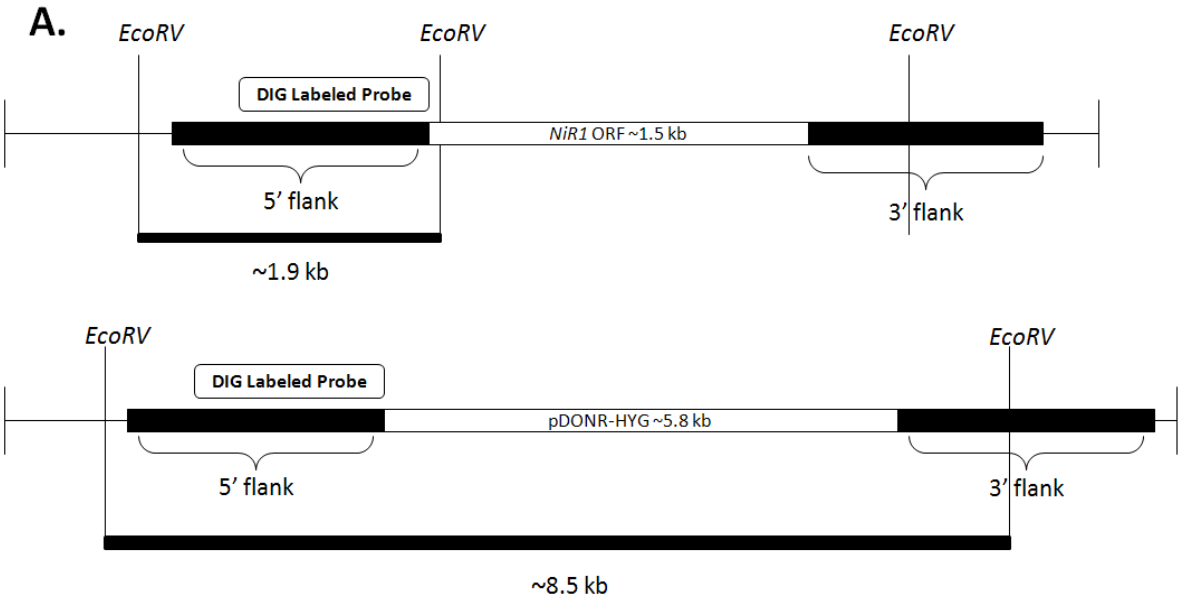

fhb1/fhb2
1 2 3 4 5 6 7 8
B.
MAT1-2 -
MAT1-1 -
- 1.5 kb
- 1.0 kb
- 0.5 kb

Figure 5.7. Deletion Construct and Southern Blot to Confirm Deletion of *NIR1*. (**A**) The ~1.5 kb *NIR1* ORF was replaced with the ~5.8 kb DelsGate construct created with the 5' and 3' flank of the *NIR1* ORF. Genomic DNA was cut with *EcoRV* and probed for detection of the 5' flank. (**B**) WT allele was detected at ~1.9 kb and the deletion allele was detected at ~8.5 kb. Transformant 4 was found to have the deletion allele. However, transformants 1, 2, and 3 were noted with an extra insertion and were marked *. Ectopic transformant (*Ect.*) was utilized as a control in experiments.


A.
EcoRV
EcoRV
EcoRV
DIG Labeled Probe
NiR1 ORF ~1.5 kb
5' flank
3' flank
~1.9 kb
EcoRV
EcoRV
DIG Labeled Probe
pDONR-HYG ~5.8 kb
5' flank
3' flank
~8.5 kb

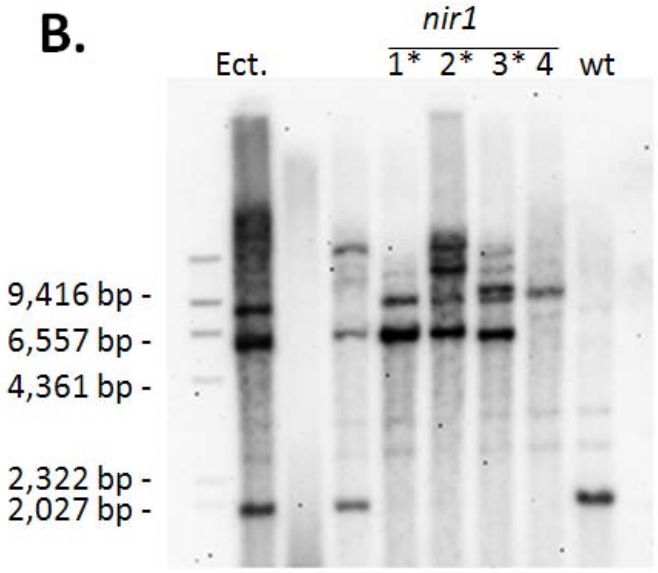
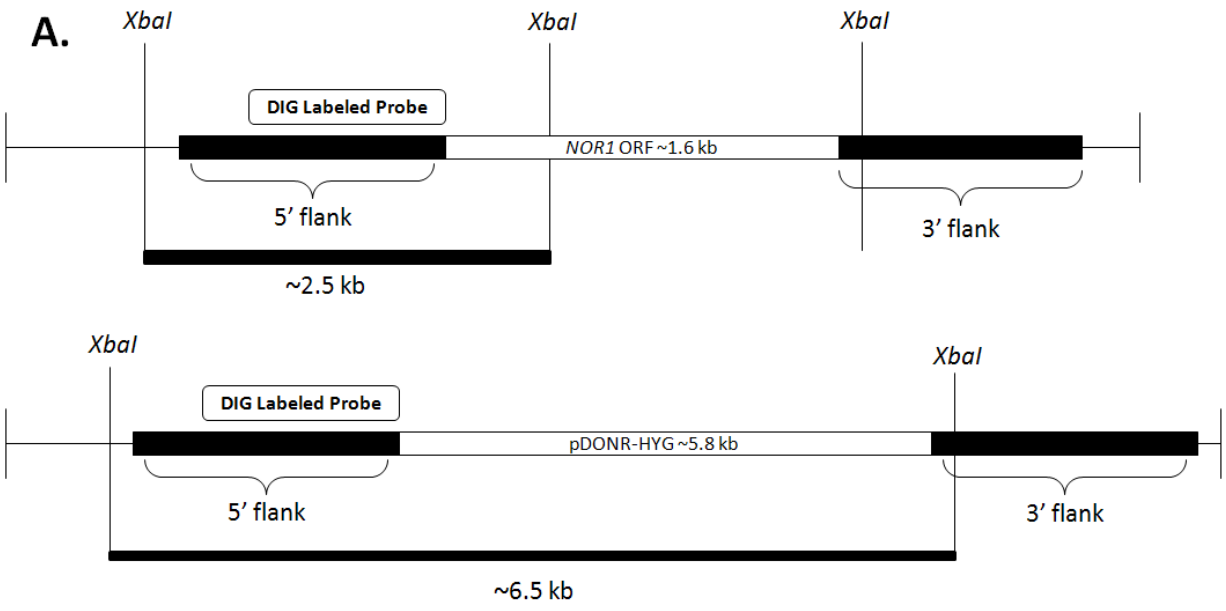

B.
nir1
Ect.
1*
2*
3*
4
wt
9,416 bp -
6,557 bp -
4,361 bp -
2,322 bp -
2,027 bp -

Figure 5.8. Deletion Construct and Southern Blot to Confirm Deletion of *NOR1*. (**A**) The ~1.6 kb *NOR1* ORF was replace with the ~5.8 kb DelsGate construct created with the 5' and 3' flank of the *NOR1* ORF. Genomic DNA was cut with *XbaI* and probed for detection of the 5' flank. (**B**) WT allele was detected at ~2.5 kb and the deletion allele was detected at ~6.5 kb. Transformant 2 was found to have the deletion allele. However, transformants 1 and 3 were noted with an extra insertion and marked with an *. Ectopic transformant (*Ect.*) was utilized as a control in experiments.


A.
XbaI
XbaI
XbaI
DIG Labeled Probe
NOR1 ORF ~1.6 kb
5' flank
3' flank
~2.5 kb
XbaI
XbaI
DIG Labeled Probe
pDONR-HYG ~5.8 kb
5' flank
3' flank
~6.5 kb

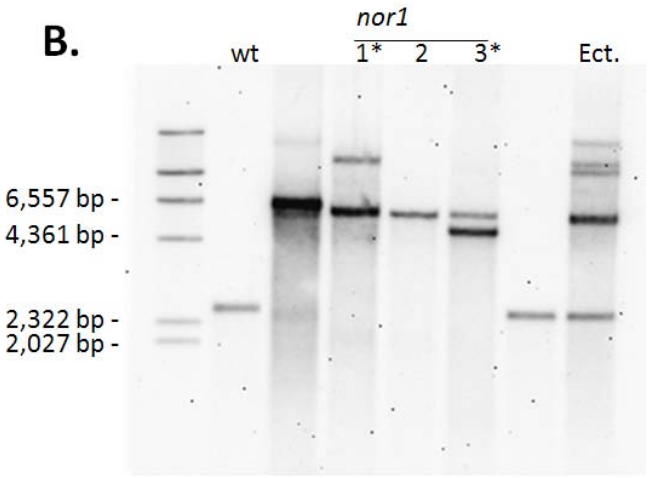

B.
nor1
wt
1*
2
3*
Ect.
6,557 bp -
4,361 bp -
2,322 bp -
2,027 bp -

Figure 5.9. Growth Curves *Fusarium verticillioides* Wild Type, Deletion Mutants, and Ectopic Controls Challenged with 1.5 mM DETA NONOate (NO Donor). (**A**) Flavohemoglobin mutants *fhb1*, *fhb2*, and *fhb1/fhb2* compared to M3125 (*wt*) following exposure to NO. (**B**) *nor1* and *nir1* mutants compared to M3125 (*wt*) following exposure to NO. (**C**) Mutant growth without exposure to NO showing the grouping with M3125 (*wt*). (**D**) Ectopic transformants growth patterns compared to M3125 (*wt*) following NO exposure. (**E**) The growth rates of all strains in one chart (unexposed control marked ^{C}). (**F**) NO exposed and unexposed M3125 (*wt*) and *fhb1/fhb2* regrown on PDA to view survival rate.

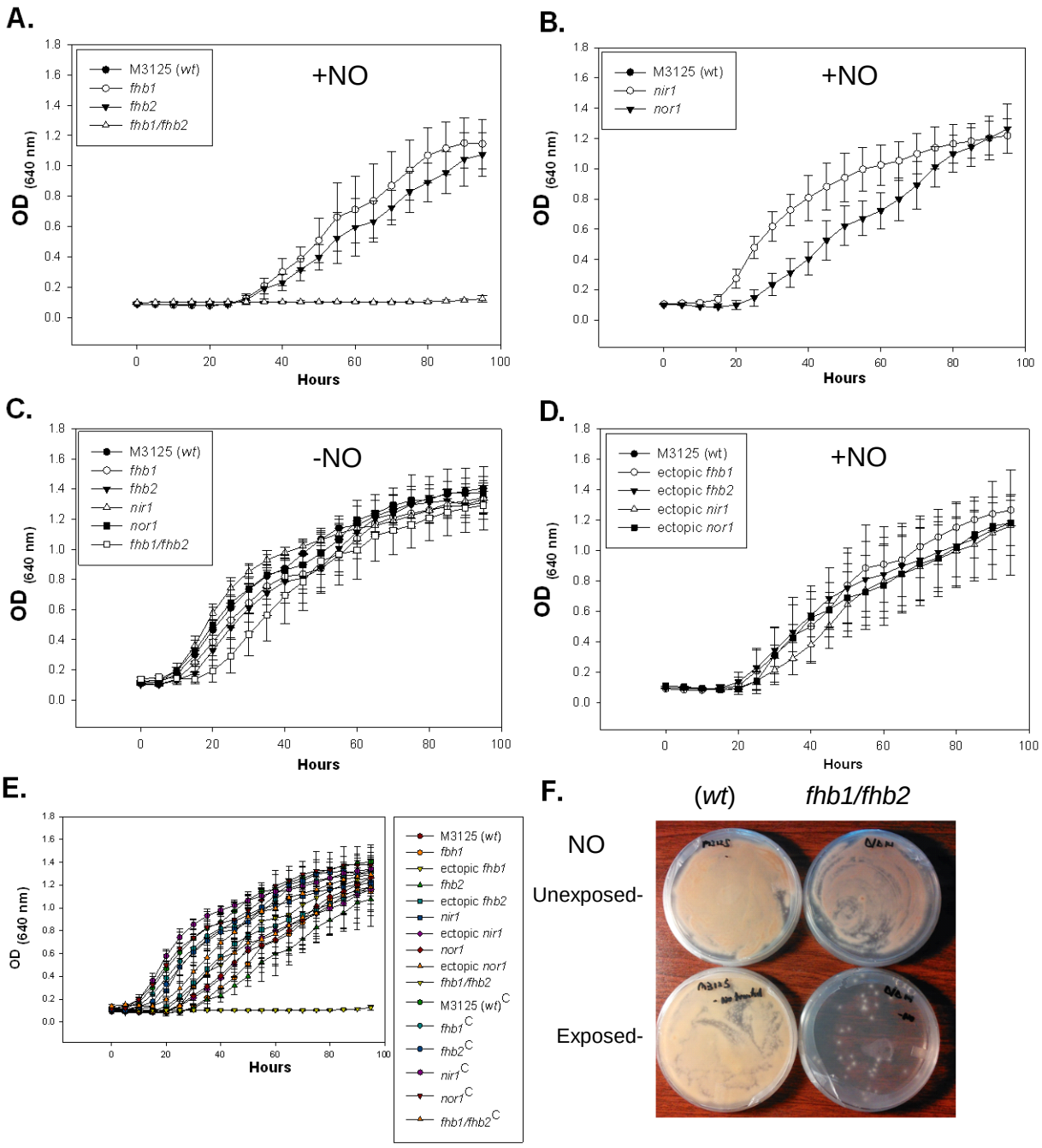

A.
+NO
M3125 (wt)
fhb1
fhb2
fhb1/fhb2
OD (640 nm)
Hours
B.
+NO
M3125 (wt)
nir1
nor1
OD (640 nm)
Hours
C.
-NO
M3125 (wt)
fhb1
fhb2
nir1
nor1
fhb1/fhb2
OD (640 nm)
Hours
D.
+NO
M3125 (wt)
ectopic fhb1
ectopic fhb2
ectopic nir1
ectopic nor1
OD (640 nm)
Hours
E.
OD (640 nm)
Hours
M3125 (wt)
fbh1
ectopic fhb1
fhb2
ectopic fhb2
nir1
ectopic nir1
nor1
ectopic nor1
fhb1/fhb2
M3125 (wt)C
fhb1C
fhb2C
nir1C
nor1C
fhb1/fhb2C
F.
(wt)
fhb1/fhb2
NO
Unexposed-
Exposed-

Figure 5.10. Growth of Wild Type and Mutants on Minimal Media Containing Nitrate (40 mM) Under Oxygenic and Hypoxic Environments. (**A**) Oxoid chambers were utilized for generating the hypoxic conditions. (**B**) Normal atmospheric conditions. Radial growth (mm) was measured after 7 days (n=3, $P < 0.05$)

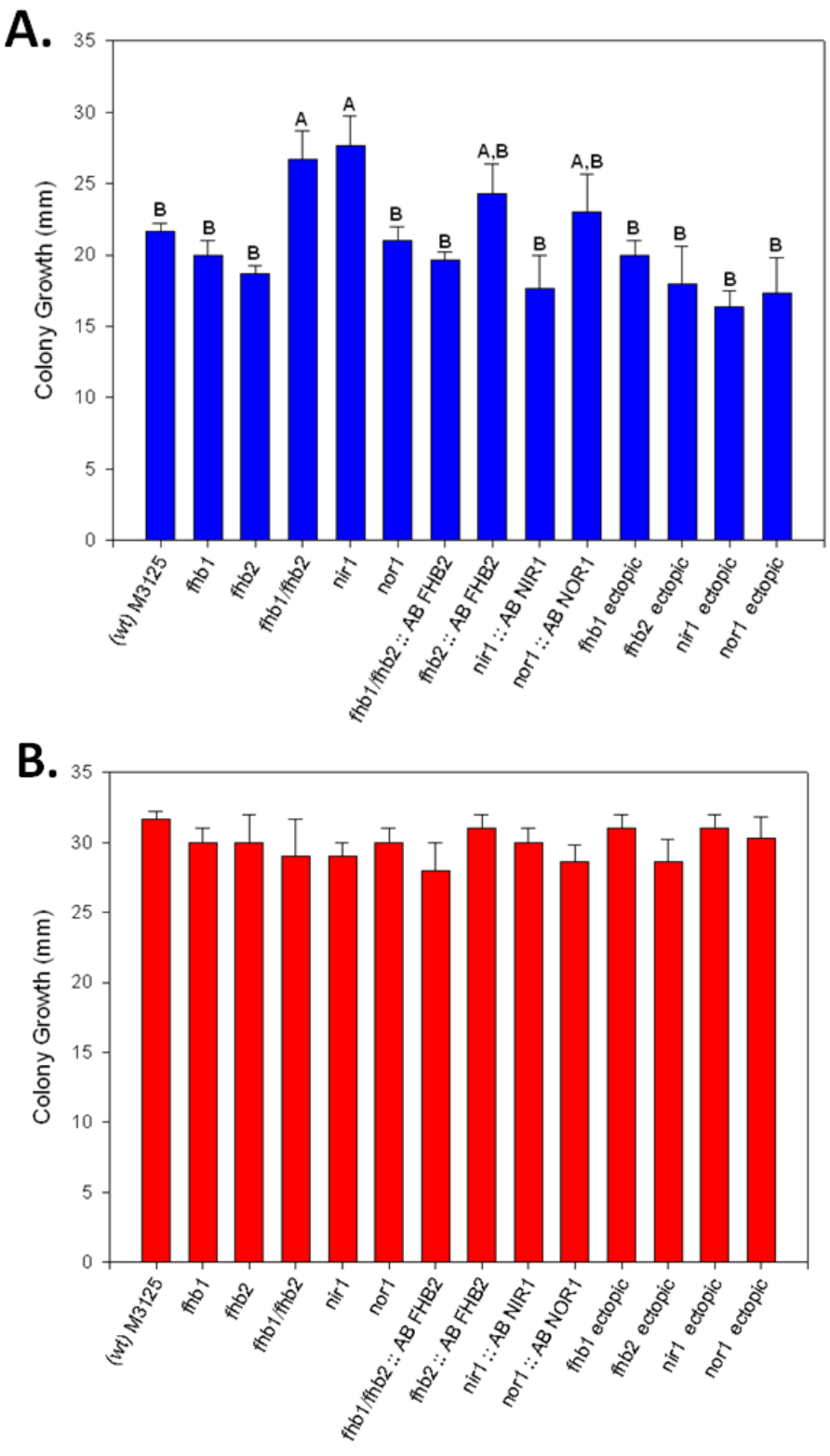
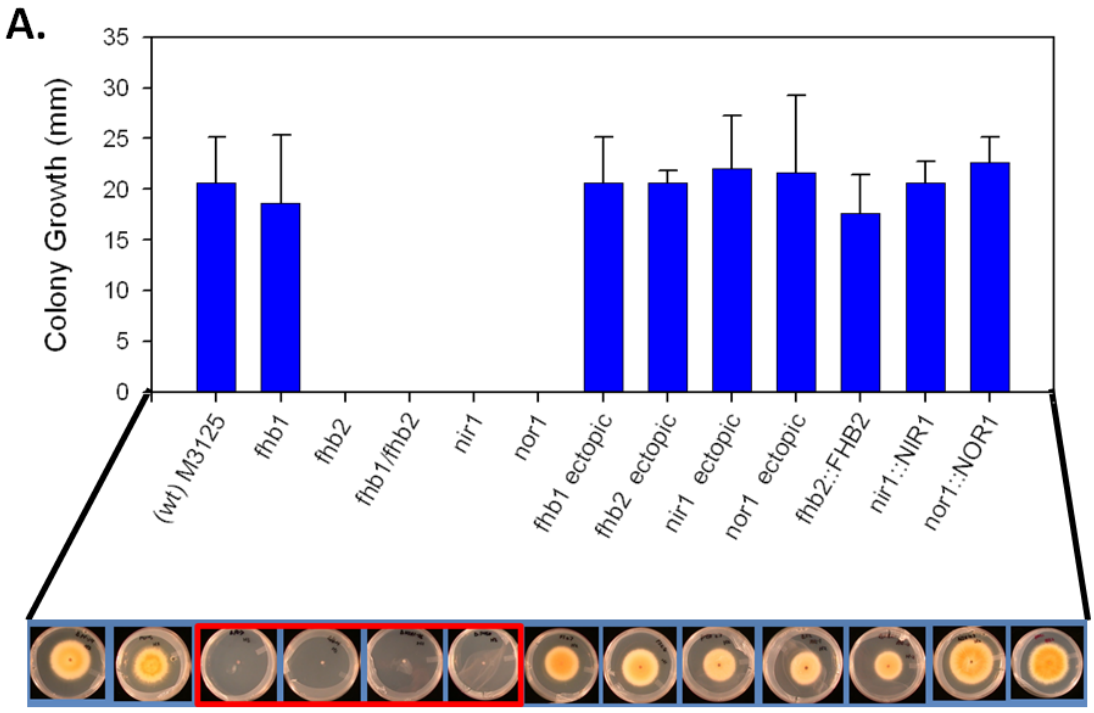
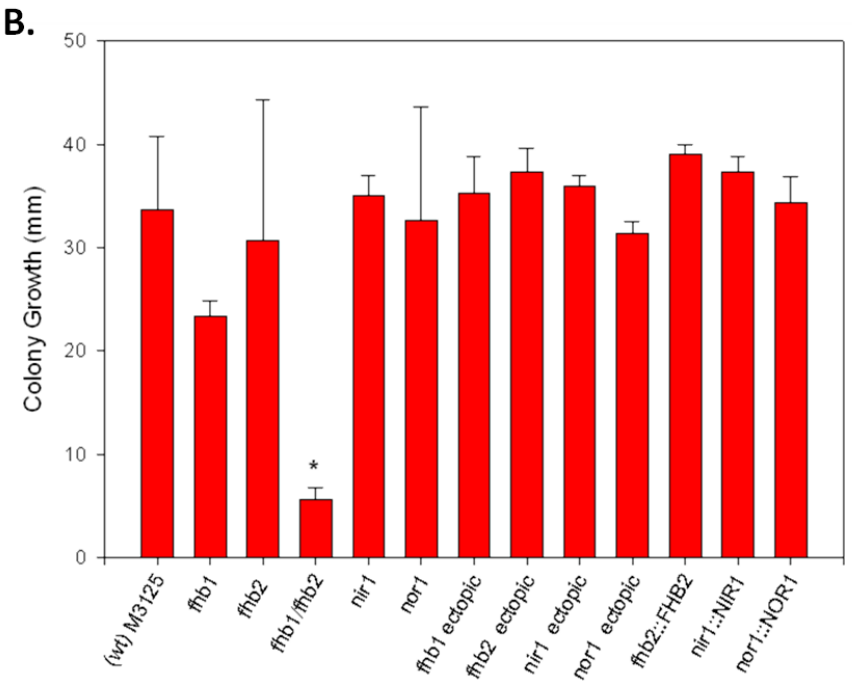

A.
Colony Growth (mm)
0
5
10
15
20
25
30
35
B
B
B
A
A
B
B
A,B
B
A,B
B
B
B
B
(wt) M3125
fhb1
fhb2
fhb1/fhb2
nir1
nor1
fhb1/fhb2 :: AB FHB2
fhb2 :: AB FHB2
nir1 :: AB NIR1
nor1 :: AB NOR1
fhb1 ectopic
fhb2 ectopic
nir1 ectopic
nor1 ectopic
B.
Colony Growth (mm)
0
5
10
15
20
25
30
35
(wt) M3125
fhb1
fhb2
fhb1/fhb2
nir1
nor1
fhb1/fhb2 :: AB FHB2
fhb2 :: AB FHB2
nir1 :: AB NIR1
nor1 :: AB NOR1
fhb1 ectopic
fhb2 ectopic
nir1 ectopic
nor1 ectopic

Figure 5.11. Growth of Wild Type and Mutants on Minimal Media Containing Nitrite (40 mM) Under Oxygenic and Hypoxic Environments. (**A**) Oxoid chambers were utilized for generating the hypoxic conditions. (**B**) Normal atmospheric conditions. Radial growth (mm) was measured after 7 days (n=3, $P < 0.05$)


A.
Colony Growth (mm)
35
30
25
20
15
10
5
0
(wt) M3125
fhb1
fhb2
fhb1/fhb2
nir1
nor1
fhb1 ectopic
fhb2 ectopic
nir1 ectopic
nor1 ectopic
fhb2::FHB2
nir1::NIR1
nor1::NOR1


B.
Colony Growth (mm)
50
40
30
20
10
0
*
(wt) M3125
fhb1
fhb2
fhb1/fhb2
nir1
nor1
fhb1 ectopic
fhb2 ectopic
nir1 ectopic
nor1 ectopic
fhb2::FHB2
nir1::NIR1
nor1::NOR1

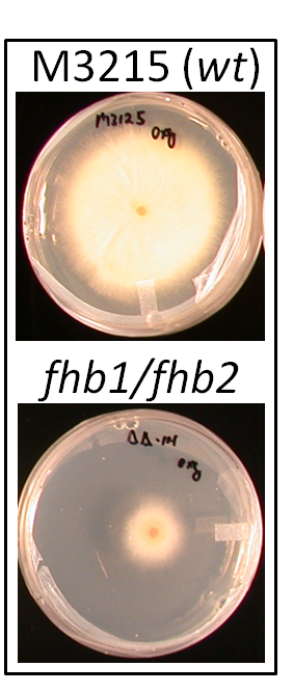

C.
M3215 (wt)
fhb1/fhb2

Figure 5.12. Virulence of WT and Mutants on Susceptible Maize Cultivar Silver Queen (14 Days After Planting). (**A**) Virulence of mutants *fhb1*, *fhb2*, *nir1*, and *nor1* was comparable to wild type. (**B**) Seedling height and (**C**) total biomass of roots and leaves reflects virulence of mutants compared to wild type (n=3, $P < 0.05$). (**D**) Fumonisin B1 detected in soil (n=3, $P < 0.05$).

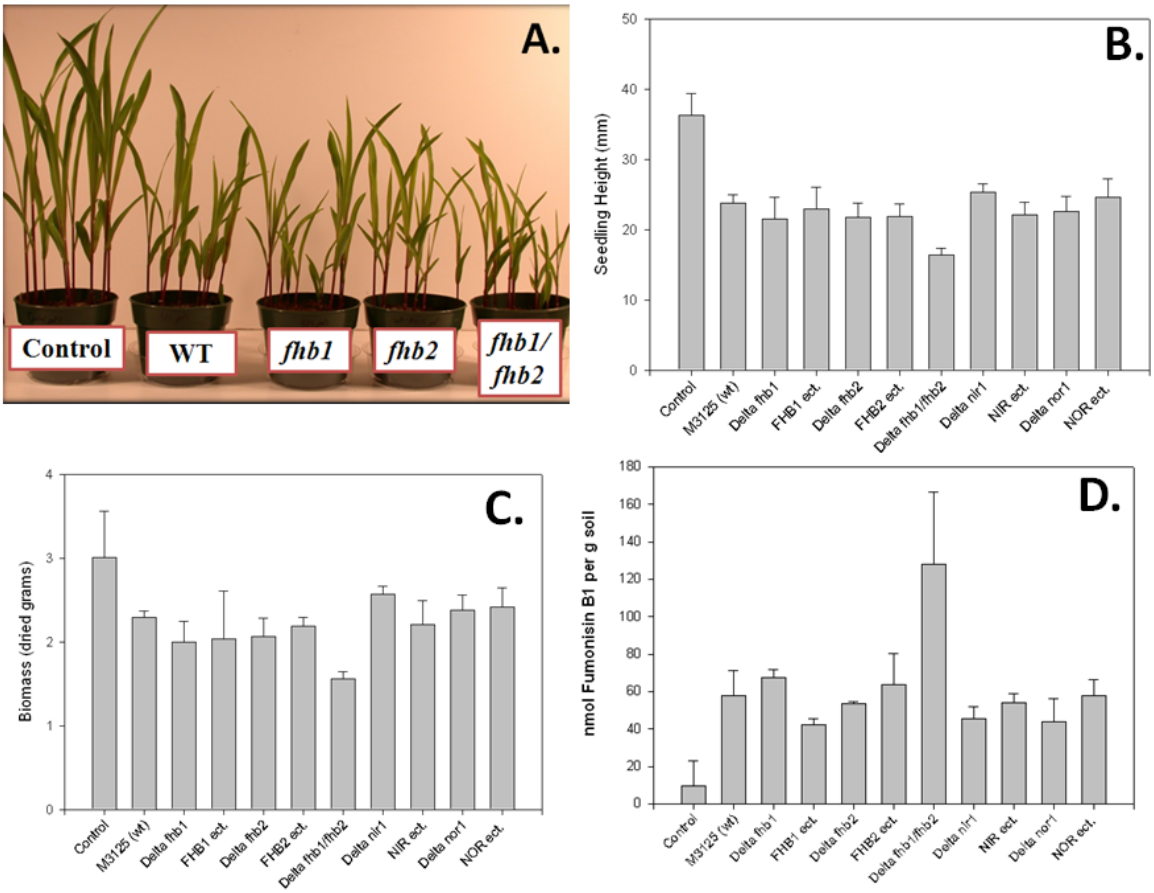

A.
Control
WT
fhb1
fhb2
fhb1/
fhb2
B.
Seedling Height (mm)
0
10
20
30
40
50
Control
M3125 (wt)
Delta fhb1
FHB1 ect.
Delta fhb2
FHB2 ect.
Delta fhb1/fhb2
Delta nir1
NIR ect.
Delta nor1
NOR ect.
C.
Biomass (dried grams)
0
1
2
3
4
Control
M3125 (wt)
Delta fhb1
FHB1 ect.
Delta fhb2
FHB2 ect.
Delta fhb1/fhb2
Delta nir1
NIR ect.
Delta nor1
NOR ect.
D.
nmol Fumonisin B1 per g soil
0
20
40
60
80
100
120
140
160
180
Control
M3125 (wt)
Delta fhb1
FHB1 ect.
Delta fhb2
FHB2 ect.
Delta fhb1/fhb2
Delta nir1
NIR ect.
Delta nor1
NOR ect.

Figure 5.13. Morphology and Detection of NO in M3125 (*WT*) and Deletion Mutants *fhb1*, *fhb2*, and *nir1* Using the NO Probe DAF-FM DA. (**A**) Top left (DIC) and top right (fluorescence) at 200X. Lower panel merged demonstrating detection of endogenous NO production by the fungus.. (B.) Further comparison of *wt* and *fhb1* (100X).

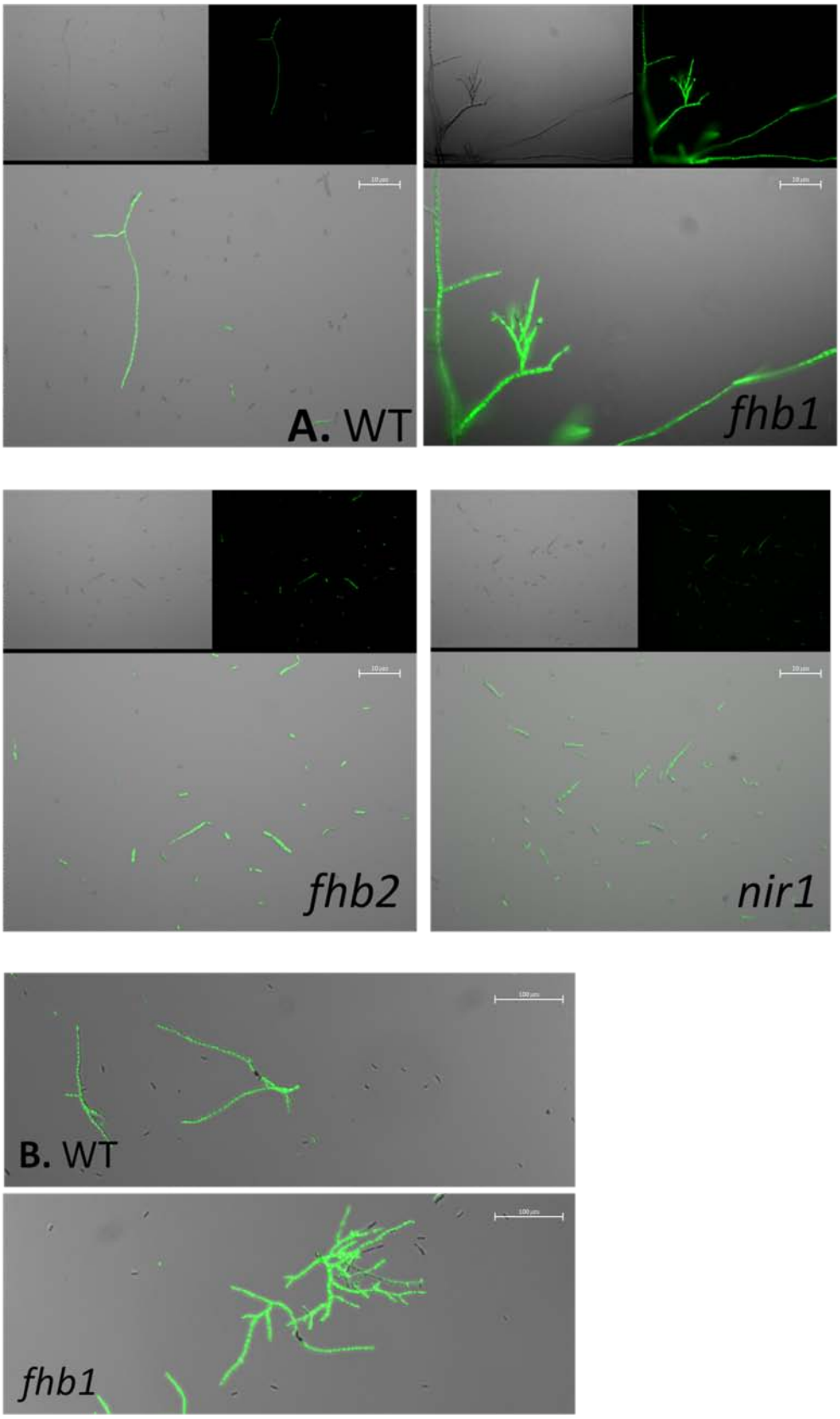
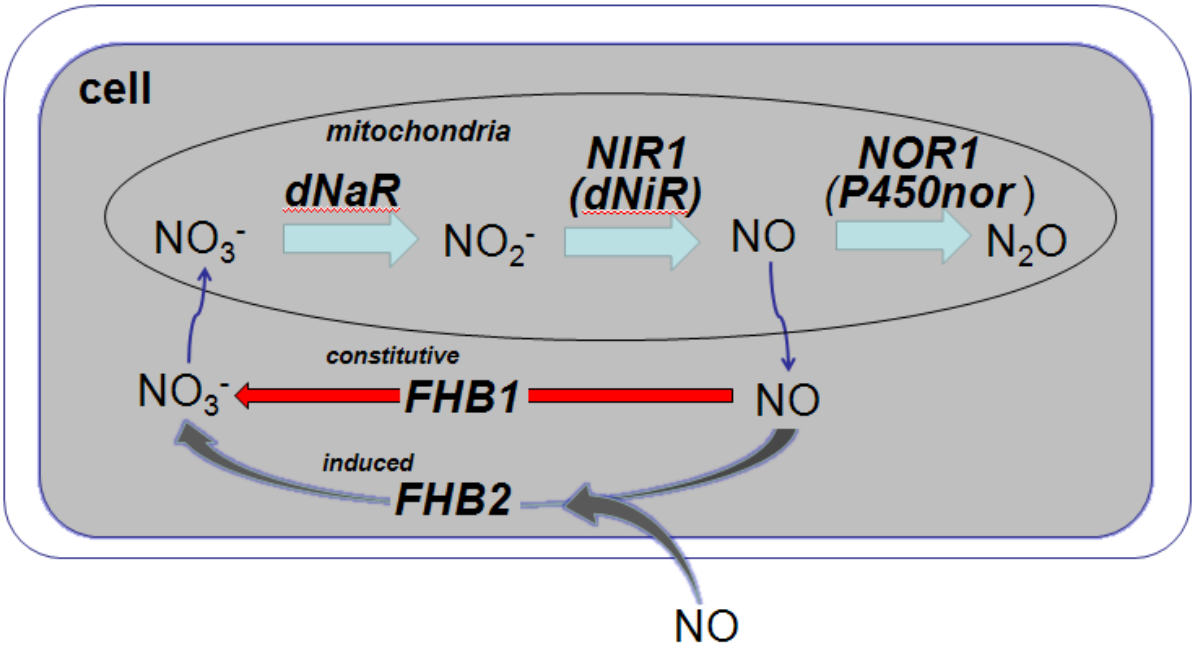

A. WT
fhb1
fhb2
nir1
B. WT
fhb1

Figure 5.14. Proposed NO Detoxification and Denitrification Pathway in *F. verticillioides*. NO is detoxified to nitrate by either FHB1 or FHB2. FHB1 is proposed to have a primary role in detoxifying endogenous NO, whereas FHB2 is most active during denitrification and in response to exogenous NO. *FHB1* (red) is constitutively expressed at high levels, compared to induction of *FHB2* (grey) by NO. dNaR, dNiR (*NIR1*), and P450nor (*NOR1*) are predicted to be mitochondrial. FHB1 and FHB2 are predicted to be cytosolic. Predictions made using TargetP, MitoProt II, and WoLF PSORT.


cell
mitochondria
dNaR
NIR1
(dNiR)
NOR1
(P450nor)
NO3-
NO2-
NO
N2O
constitutive
NO3-
FHB1
NO
induced
FHB2
NO

CHAPTER 6

CONCLUSION

The ascomycetous fungus *Fusarium verticillioides* (tel. *Gibberella moniliformis*) of the order Hypocreales is a non-obligate plant pathogen of maize causing a number of economically significant diseases including root rot, kernel rot, seed rot, stalk rot, and seedling blight (Kommedahl and Windels, 1981; Kuldau and Yates, 2000). In particular, seedling blight disease results in fumonisin mycotoxin accumulation, which is necessary for pathogenicity. Seedling blight and fumonisin exposure results in a decrease in seedling root mass, stunted aerial growth, and manifestation of various leaf abnormalities (Glenn et al., 2008; Williams et al., 2006; Williams et al., 2007). The mechanism of fumonisin toxicity involves inhibition of ceramide synthase and disruption of sphingolipid metabolism resulting in accumulation of toxic levels of sphingoid base metabolites and depletion of complex sphingolipids (Wang et al., 1991).

Subsequent investigation of the aerial tissues by analyzing the first, second, and third leaves determined the highest fumonisin accumulation occurred in the first leaf of maize seedlings grown from seed inoculated with *F. verticillioides* (Zitomer et al., 2008). Accumulation of fumonisins in these tissues is noted as containing a significantly higher ratio of FB_1 to FB_2 and FB_3 compared to the observed accumulations in the roots (Zitomer et al., 2008), suggesting a mechanism of either greater accumulation of FB_1 over the other analogs by selective transport or by selective production of FB_1 by the fungus colonizing the leaf. Further questions arose about the mechanism of fumonisin accumulation when uninoculated seedlings were watered with FB_1 and FB_2 without detectable accumulation in the leaf tissues (Zitomer et

al., 2010), thus suggesting the fungus is essential for accumulation of FB_1 in the leaves. This study investigates mobility and accumulation of FB_1 with systemic infection by inoculating seedlings with fumonisin-producing and fumonisin non-producing strains of *F. verticillioides.* To separate systemic infection and fumonisin mobility, strains lacking the ability to produce conidia were also examined. FB_1 was detected in the first and second leaves with no detected colonization of the aerial tissues of seedlings grown from seed inoculated with the fumonisin-producing aconidial strain. In comparison, wild-type *F. verticillioides* colonized the leaves and accumulated FB_1. Therefore, root colonization by *F. verticillioides* is necessary for discriminant accumulation of FB_1 in leaves but not by direct fungal colonization of the leaf.

Accumulation of FB_1 also results in the accumulation of sphingoid bases (phytosphingosine and sphinganine) and their 1-phosphates in the roots and leaves of maize seedlings. Others have demonstrated that in *Arabidopsis thaliana* sphingosine-1-phosphate (S1P) and phytosphingosine-1-phosphate (Pso-1P) regulate stomatal behavior. Our objectives were to evaluate physiological effects of *F. verticillioides* on maize seedlings, including whether FB_1 alters maize transpiration and stomatal aperture behavior. This interaction could play a dramatic role in *Fusarium* pathogenesis as a consequence of the elevated levels of sphingoid base 1-phosphates in leaf tissues during pathogenesis by effecting plant physiological processes, such as transpiration. Our research shows a direct effect on transpiration by either inoculation of the fungus or by watering with FB_1. Our epidermal peel experiments showed a significant decrease in stomatal aperture in the 10 μM ABA treatment ($t=2.910$, $P<0.05$) (Figure 4.4) and demonstrates the capability for further investigation into possible molecular effects of FB_1, the sphingoid bases, or the sphingoid base 1-phosphates on stomatal aperture.

Cross-talk between signaling pathways in pathogenic interactions is common. Recently, sphingoid bases have been shown to induce an early defense and signaling event in plants known as the nitric oxide (NO) burst (Guillas et al., 2013). Also, given the prominence of NO in other pathogenic interactions we investigated the possible role of NO in the biology and virulence of *F. verticillioides* using molecular methods. Specifically this study addressed detoxification of the NO by the flavohemoglobins *FHB1* and *FHB2* in *F. verticillioides*. Microarray analysis revealed a significant induction of *FHB2* (17-fold) when the fungus was exposed to exogenous NO (1.5 mM) and a 2-fold increase of *FHB1*. In comparison the highest induction at 246-fold was a dissimilatory nitrite reductase (*NIR1*) along with high induction of other genes from the denitrification pathway, including a P450 nitric oxide reductase (*NOR1*). Deletion mutants (*fhb1*, *fhb2*, *nor1* and *nir1*) were generated and challenged on NO, nitrate, and nitrite media. The *fhb1*, *fhb2*, and *nor1* mutants were restricted in growth and the *fhb1/fhb2* double deletion mutant was unable to grow when challenged with NO. All mutants were able to grow on nitrate and nitrite media. However, mutants *fhb2*, *nir1*, and *nor1* were unable to grow on nitrite under hypoxic conditions. Also, *fhb1/fhb2* had restricted growth on nitrite under normal atmospheric conditions. Combined, data suggests the importance of the flavohemoglobins in nitrite metabolism. Seedling blight assays using Silver Queen maize seedlings revealed the *fhb1/fhb2* double deletion mutant to be more virulent than wild type and had double the production of FB_1. Our data demonstrate a significant role of the denitrification pathway in NO detoxification and links such detoxification to virulence and FB_1 production.

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