THE ROLE OF FUMONISINS IN MAIZE SEEDLING DISEASE AND THE ECOLOGICAL INTERACTION BETWEEN *FUSARIUM VERTICILLIOIDES*, SOIL AND PLANTS

by

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(Under the direction of Dr. Mary A. Smith and Dr. Ronald T. Riley)

ABSTRACT

Fumonisins (FB_1, FB_2, and FB_3) are mycotoxins produced by *Fusarium verticillioides* (Sacc.) Nireberg (synonym=*F. moniliforme*; telemorph *Gibberella moniliformis*). They inhibit ceramide synthase, a key enzyme in *de novo* sphingolipid biosynthesis. While fumonisins are not known to cause plant disease under field conditions, they are found in the ear, roots, and stalks of maize and can enter the soil intact where they can be tightly bound and under certain conditions may be released. We hypothesized that fumonisin production in soils, inhibition of ceramide synthase, and disruption of sphingolipid metabolism modulate disease symptoms in *F. verticillioides* infected maize seedling.

To evaluate the ability of *F. verticillioides* to produce fumonisins in synthetic and natural soils, maize seeds were inoculated with a fumonisin producing strain of *F. verticillioides* and planted in potting soil and three different natural soils. The fungus produced fumonisins in each soil type, disrupted sphingolipid metabolism in the roots and induced symptoms of maize seedling disease

To investigate the role of fumonisin-induced inhibition of ceramide synthase in *F. verticillioides* pathogenicity on maize seedlings, maize seeds were inoculated with fumonisin producing strains or fumonisin non-producing strains of *F. verticillioides* and planted in sterile potting soil. Only the seedlings grown from seeds inoculated with the fumonisin producing strains of *F. verticillioides* developed symptoms of *F. verticillioides* maize seedling disease. In addition, the concentration of fumonisin and free sphingoid bases and their 1-phosphate metabolites were correlated with the severity of the pathology.

To further understand the role of fumonisins in maize seedling disease associated with *F. verticillioides,* un-inoculated maize seeds were watered with 1, 5, 10, or 20 µg/ml aqueous fumonisin B_1. There was a dose-dependent reduction in root mass at 5, 10, and 20 µg/ml fumonisin B_1, and there was a dose-dependent elevation in fumonisin B_1, sphingoid bases and sphingoid base 1-phosphates in the root tissues. In addition, there was an increase in leaf lesions and reduced growth. These results show that under laboratory conditions fumonisin B_1 in potting soil can: i) affect root growth and alter seedling performance, ii) be taken up by roots, iii) cause marked dose-dependent elevation in sphingoid bases and their 1-phosphates that are likely to contribute to reduced root growth and other symptoms of seedling disease. These findings support the hypothesis that fumonisins are phytotoxins and pathogenicity factors that contribute to *F. verticillioides* maize seedling disease in both synthetic and natural soils.

INDEX WORDS: Fumonisin, ceramide synthase, maize, *Fusarium verticillioides*, sphingolipids, sphingoid base, sphingoid base 1-phosphate, fumonisin B_1, soil, maize seedling disease

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DEDICATION

Dedicated to my grandmother, Martha Davis.

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CHAPTER 1

INTRODUCTION

Fumonisins are mycotoxins produced by the fungus *Fusarium verticillioides* (Sacc.) Nirenberg (synonym=*F. moniliforme*; telemorph *Gibberella moniliformis*). At present, at least 28 different fumonisins have been reported (Rheeder et al., 2002). Fumonisin B_1 is the most abundant and is believed to be the most toxic of the fumonisins (IARC, 2002). Diseases of maize associated with *F. verticillioides* contamination include: seed rot, seedling blight, root rot, stalk rot, and kernel or ear rot (Cook, 1981; Shurtleff, 1980; Kommedahl and Windels, 1981).

F. verticillioides and the fumonisins have become areas of great concern for maize producers, processors, and consumers. Contamination of maize with fumonisins is worldwide and consumption of contaminated maize can cause disease of farm animals as well as liver and kidney cancer in rodents (WHO, 2002; IARC, 2002). The fumonisins also pose a threat to humans as it has been hypothesized that fumonisins are contributing factors in high incidences of neural tube defects (Marasas et al, 2004) and esophageal cancer (Rheeder et al., 1992) in areas where consumption of low quality maize is high. The Joint Expert Committee on Food Additives recommended to the Codex Committee on Food Additives and Contaminants a provisional maximum tolerable daily intake (PMTDI) for FB_1 of 2µg/kg body weight/day (Bolger et al., 2001). These recommendations are intended to minimize exposure and prevent adverse health effects from consumption of fumonisin contaminated maize-based foods and feeds. However, little is known about the environmental fate of fumonisins and therefore other

routes and sources of exposure could contribute to the total fumonisin intake by humans and animals (Marasas et al., 2000).

Some strains of *F. verticillioides* can be highly infective and associated with maize without producing any sign of disease to the plant (Thomas and Buddenhajen, 1980; Foley, 1962). This aspect of the fungal-plant interaction is referred to as a symptomless association (Bacon and Hinton, 1996). The symptomless association between the fungus and the maize plant is important because the absence of overt signs of infection increases the possibility that fumonisin-contaminated plant residues could enter, undetected, food and feed chains and into the soil environment. Diseased maize parts often containing high levels of *F. verticillioides* (Bullerman, 1996) and possibly the fumonisins, are usually not harvested. *F. verticillioides* has also been detected in soil (Almeida, 2002), therefore it is possible that fumonisin levels in soils could be quite high given that 1) the fumonisins are heat and light stable (Dupuy et al., 1993; Howard et al., 1998; IARC, 1993), 2) the fumonisins can accumulate to high levels in moldy maize parts and plant debris (Bullerman,1996), 3) the majority of fumonisins consumed in contaminated feeds are rapidly excreted by farm animals (Marasas et al., 2000) and 4) *F. verticillioides* is a common contaminant of maize.

There is considerable evidence that fumonisin-induced disruption of sphingolipid metabolism is important in the cascade of events leading to altered cell growth, differentiation and cell injury observed both *in vitro* and *in vivo* (Merrill et al., 2001; Riley et al., 2001). The most important piece of evidence is that fumonisin B_1 and *Alternaria alternata* f.sp. *lycopersici* (AAL) toxin are inhibitors of ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthetic pathway (Wang et al., 1991; Merrill et al., 1993). Fumonisin inhibition of ceramide synthase, as evidenced by elevation in free sphingoid bases, a marker for ceramide synthase

inhibition (Riley et al., 1994), has been shown in several plant models (Abbas et al., 1994; Abbas et al., 1998) including roots of maize seedlings (Riley et al., 1996) and excised maize shoots (Wright et al., 2003). The structurally similar compound, AAL-toxin, has been shown to be a pathogenicity factor for *Alternaria alternata* f.sp. *lycopersici* – induced stem canker disease in tomato (*Lycopersicon esculentum*) (Gilchrist and Grogan, 1976). In susceptible tomato plants, fumonisin B_1 or mycelia and spores of *F. verticillioides* sprayed on aerial parts have been shown to mimic both the symptoms of AAL-toxin and *A. alternata* f.sp. *lycopersici*-induced toxicity (Abbas et al., 1994). The toxicity of AAL-toxin in tomato is closely associated with disruption of sphingolipid metabolism (Abbas et al., 1994), and tomato varieties resistant to *A. alternata* f.sp. *lycopersici* are also resistant to AAL-toxin and fumonisin-induced accumulation of free sphingoid bases (Abbas et al., 1994). In contrast, even though maize tissues (cob, stalks, and kernels) can contain detectable levels of fumonisins, fumonisin-induced disruption of sphingolipid metabolism has not been shown to be a mechanism of any disease in maize. Because fumonisins, produced by *F. verticillioides*, are toxic to plants and animals and their toxicity in both plants and animals is closely linked to inhibition of ceramide synthase, it is critical to develop a sound scientific understanding of the abiotic and biotic processes that regulate the occurrence, biological availability, fate and distribution of fumonisins and *F. verticillioides* in soils.

The hypothesis of this dissertation is that in *F. verticillioides* maize seedling disease, the extent and severity of disease expression is dependent upon fumonisin production and subsequent disruption of sphingolipid metabolism in the infected maize seedlings. The specific objectives of this research are to determine i) if *F. verticillioides* can produce fumonisins in synthetic and complex natural soils when inoculated on maize seeds; ii) if the fumonisins

produced are biologically available to maize roots using an increase in free sphingoid bases and sphingoid base 1-phosphates as markers for fumonisin-induced disruption of sphingolipid metabolism; and iii) the time- and dose- dependent relationship between fumonisins in soil and effects on root development, expressed as root growth, and disruption of sphingolipid metabolism.

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CHAPTER 2

LITERATURE REVIEW

I. The Plant-Fungus Association

The fungus *Fusarium verticillioides* produces a number of toxic chemicals that include the fumonisins (Bacon and Nelson, 1994). *F. verticillioides* is a non-obligate plant pathogen that is commonly associated with maize (Bacon and Hinton, 1996). Most non-obligatory plant pathogens kill their host cells prior to infection and obtain nutrients from nonliving tissue (Bacon and Hinton, 1996). However, *F. verticillioides* does not follow this pattern because a large percentage of the isolates get their nutrition from living cells, they are therefore hemibiotrophic (Bacon and Hinton, 1996). The development of disease symptoms and death of infected maize plants are a result of an interaction between stress induced by the pathogen and stress induced by other factors such as drought and insect damage or the depletion of nutrients (Bacon and Hinton, 1996).

F. verticillioides belongs to the section *Liseola* mating populations A of *Gibberella moniliformis* (Klittich and Leslie, 1992). Fungi of mating population A are primarily associated with maize. *F. proliferatum* (Matsushima) Nirenberg in mating population D, is also commonly found on maize and can produce large amounts of the fumonisins (Munkvold and Desjardin, 1997).

F. verticillioides (as *F. moniliforme*) is not host specific and has been recovered from sorghum, wheat, rice, oats, beans, cotton, peanuts, pecans, bananas, sugar beets, green peppers, flax,

soybean, figs, stone fruits, several forages and sugar cane (Bacon and Nelson, 1994), and many other commercially important crops and plants. Extensive research has been done on the parasitism of maize because this fungus may produce a wide variety of chemically different fungal metabolites on maize (Bacon and Hinton, 1996). Because it is a non-obligate parasite it is able to attack many different plants and plant parts, and may produce and secrete a large number of nonspecific phytotoxins or extracellular enzymes that affect metabolic pathways or cellular functions common to many plants (Bacon and Hinton, 1996). The nature of the interaction between isolates of *F. verticillioides* and maize varieties is diverse (Munkvold and Desjardins, 1997). Some isolates are virulent; however, some non-virulent isolates can be highly infective and associated with maize without producing any sign of disease to the plant (Thomas and Buddenhajen, 1980; Foley, 1962). Infection of maize with *F. verticillioides* without production of disease symptoms is referred to as symptomless association (Bacon and Hinton, 1996).

Diseases of maize associated with *F. verticillioides* contamination include: seed rot, seedling blight, root rot, stalk rot, and kernel or ear rot (Cook, 1981; Shurtleff, 1980; Kommedahl and Windels, 1981). In seed rot the kernels decay before and during germination and very few seeds ever produce coleoptiles and radicals (Bacon and Hinton, 1996). Seedling blight or damping-off, caused by *F. verticillioides*, can be distinguished from diseases caused by other fungi by a white-to-pink mycelium with masses of spores in stem tissues (Bacon and Hinton, 1996). Root rot may be initiated as a part of the seedling blight disease, but persists into the matured maize plant where late in the season symptoms similar to seedling blight appear on roots (Bacon and Hinton, 1996). These symptoms may be exaggerated during plant maturity and seed development. Stalk rot is very destructive and is a major factor responsible for yield reduction. It may be initiated from root rot or initiated directly by conditions favorable to root

rot. The nature of losses due to stalk rot caused by *F. verticillioides* are difficult to define since under field conditions there is a large complex of contributing fungi (Shurtleff, 1980). Symptoms of stalk rot disease caused by *F. verticillioides* include a salmon-colored mycelium in the stem pith, which disintegrates resulting in stalk breakage or lodging. Under warm moist conditions, a cottony-pink growth of mycelium with conidia appears on the leaf sheaths and at the node usually following lodging (Bacon and Hinton, 1996).

II. Chemistry and Natural Occurrence of Fumonisins

At present, at least 28 different fumonisins have been reported and other minor metabolites have been identified, although some of them do not occur naturally (Rheeder et al., 2002). Fumonisin B_1 (FB_1) is the most abundant and toxic of the fumonisins, which occur naturally (Abbas and Shier, 1997; Musser and Plattner, 1997; Plattner, 1995). In maize kernels, FB_1 usually co-occurs with FB_2 and FB_3 (Marasas et al., 2000). FB_1 is the diester of propane-1, 2,3-tricarboxylic acid and 2-amino-12, 16-dimethyl-3, 5,10,14,15-pentahydroxyeicosane and has a molecular mass of 721. The pure substance is a white hygroscopic powder soluble in water, acetonitrile-water or methanol, is stable in acetonitrile-water (1:1), unstable in methanol, and is heat (Dupuy et al., 1993; Howard et al., 1998) and light (IARC, 1993) stable. Fumonisins of the "B" series acquire their polar nature from the presence of the two tricarboxylic acid moieties at carbons 14 and 15 and the primary amino group at carbon 2 (Marasas et al., 2000). Fumonisins are often described as being modified sphingoid bases due to their structural similarity to the sphingoid base sphinganine (Wang et al., 1991). This is most apparent with fumonisins of the "B" series following treatment with dilute bases (eg. NaOH, KOH) which remove the tricarboxylic acid groups at carbons 14 and 15 forming what are called hydrolyzed fumonisins (HFB's). The amphipathic-zwitterionic nature of the fumonisins of the "B" series allows them to

be chemically isolated using clean-up procedures involving the use of strong anion or cation exchange resins and reverse phase C_{18} columns (Marasas et al., 2000). The presence of the primary amino group allows all fumonisins of the "B" series, and their hydrolyzed counterparts, to react with chemicals that covalently bind to primary amino groups or which form Schiff bases such as reducing sugars (Marasas et al., 2000). There is limited data suggesting that fumonisins can be metabolized by some soil microorganisms (Blackwell et al., 1999). However, the microbial flora of the gut in Vervet monkey was shown to be able to remove the tricarboxylic acid side-chains (Shephard and Snijam, 1999) and fumonisin metabolizing fungi and bacteria have been described (Duvick et al., 2001).

The fumonisins were originally isolated in 1988 from laboratory maize cultures of *F. verticillioides* in southern Africa (Bezuidenhout et al., 1988). Since this discovery, the fumonisins have been isolated from maize and maize-based food and feedstuffs naturally contaminated with this fungus in many countries including the United States, Canada, China, Egypt, Argentina, Nepal, Peru and Brazil (Bacon and Nelson, 1994). These locations follow the geographic distribution of both the fungus and maize production, suggesting that fumonisins will be found wherever maize is grown (Bacon and Nelson, 1994). The timing of fumonisin formation and accumulation patterns in the vegetative parts of maize seedlings has been reported (Bacon et al., 2001). Fumonisins have also been shown to appear soon after infection of field maize (1 week after anthesis) and peak within 2 weeks (Payne et al., 2002). A second peak in fumonisin production occurs late in the season and is not associated with an increase in kernel infection (Payne et al., 2002). Fumonisins have been detected in maizecobs; glumes of old maize florets (bee wings), kernels and maize screenings (Bacon and Nelson, 1994). The highest concentrations have been detected in bee wings (Riley et al., 1993). However maize screenings

have higher concentrations than whole maize and have been associated with farm animal diseases in swine and horses (Riley et al., 1993). Fumonisins also are found in maize and maize-based products for human consumption (Chu et al., 1995; Sydenham et al., 1991; Thiel et al., 1991; Richard et al., 1993). Sound maize or "symptom-less" kernels can contain fumonisins, however, visually damaged kernels often contain much higher levels of fumonisin (Desjardin and Plattner, 1998).

F. verticillioides is also saprophytic and normally produces its toxin under field conditions (Bacon and Nelson, 1994). The high levels of fumonisin production under culture conditions suggest that maize kernels, their fragments and dead plant debris can serve as substrates for toxin production under storage conditions or in plant debris in the field (Bacon and Nelson, 1994). However, fumonisin production under normal conditions of maize storage in the United States has not been demonstrated nor has production in natural soils. Laboratory studies have established that the optimum temperature range for growth of isolates of *F. verticillioides* is between 22.5 to 27.5°C, with the maximum between 32 and 37°C and the minimum temperature between 2.5 to 5.0°C (Joffe et al., 1973). Additional studies indicate that the minimum humidity for vegetative growth is at -180 bars water potential, while conidium germination occurs between -140 bars and -150 bars (Joffe et al., 1973). Maize kernels with moisture content between 18.4 and 23% were optimal for growth under storage conditions (Koehler, 1938), whereas growth was inhibited at 28% moisture content. The growth of this fungus in storage is complex since there is an interaction of moisture with levels of oxygen and carbon dioxide. Thus, this specific fungus can grow under storage conditions of 0% oxygen and 60% CO_2 at 26^{o}C, but at similar high levels of CO_2 in the storage atmosphere, growth is reduced at 12^{o}C (Tuite et al., 1967). The growth of this fungus at 0% oxygen and high CO_2 suggests that it can grow anaerobically in storage (Bacon

and Nelson, 1994). Growth reports of this fungus in storage indicate that it has the potential to produce mycotoxins under a wide-range of storage conditions. As a result, it can be concluded that the fumonisins might arise initially from field conditions, but can then increase due to improper storage conditions (Bacon and Nelson, 1994). Nonetheless, there are no studies demonstrating fumonisin-induced farm animal diseases due to consumption of FB_1 that developed due to improper storage of feed.

F. verticillioides has been isolated from maize debris (Bullerman, 1996) and soil (Almeida et al., 2002). But there is only limited data on the fate of fumonisins in soils. In one published report on the fate of FB_1 in soil (Madden and Stahr, 1993), it was found that when FB_1 was mixed with silty clay loam soil, it could not be recovered from the soil. It was concluded that FB_1 was either irreversibly bound or chemically altered in the soil. However, more recent studies using Cecil sandy loam soil indicated that while FB_1 can be tightly bound in the soil, it can also be released intact under acidic conditions (Williams et al., 2003). The possibility that FB_1 from maize debris in field situations could enter the soil is of significant interest since FB_1 could alter the biological activity of soil flora and fauna, and it is also possible that FB_1 from maize debris in field situations could enter the soil environment.

III. Mycotoxin-Animal Association

There are several animal diseases associated with *F. verticillioides* and fumonisins. These include equine leukoencephalomalacia (ELEM), porcine pulmonary edema syndrome (PPE), poultry toxicity, as well as a suggested link between fumonisins and human esophageal cancer (Marasas et al., 2000) and neural tube defects (Marasas et al., 2004). ELEM syndrome is characterized by the presence of liquefactive necrotic lesions in the white matter of the cerebrum, however the gray matter may also be involved (Riley et al., 1993). In 1902, ELEM was

experimentally produced by feeding horses moldy maize obtained from a field in Kansas (Butler, 1902). The disease was known as "moldy maize poisoning," but early attempts to identify the responsible fungus failed. Wilson and Maronpot (1971) isolated *F. verticillioides* as the predominant contaminant of moldy maize that had caused many cases of ELEM and they reproduced ELEM by feeding horses *F. verticillioides* maize culture material. After the discovery of fumonisins, ELEM was also produced in horses by the intravenous administration of FB_1 (Marasas et al., 1988).

In studies with *F. verticillioides* fed to horses, pigs, sheep, rats and baboons, lung edema only occurred in pigs (Kriek et al., 1981). In the late 80's and early 90's, outbreaks of ELEM and PPE were reported in different parts of the United States. In these outbreaks, *F. verticillioides* was found to be the predominant contaminant of the maize (Osweiller et al., 1992). Pure FB_1 was also shown to produce PPE when administered intravenously to pigs (Harrison et al., 1990). Compared to horses and pigs, broiler chickens are considered to be relatively resistant to fumonisin toxicity (Miller et al., 1996). However, reports have been published suggesting that *F. verticillioides* contamination of feed is related to disease of poultry (Bryden et al., 1987). Studies have also confirmed that *F. verticillioides*, *F. proliferatum*, fumonisin B_1 and moniliformin are toxic to broiler chicks (Brown et al., 1992; Dombrink-Kurtzman et al., 1993, Henry and Wyatt, 2000) and chicken embryos (Bacon et al., 1995, Henry and Wyatt, 2001). In studies conducted by Javed et al. (1993a,b) the levels of fumonisins used were relatively high and the co-occurrence of moniliformin posed an additional complication for toxicological interpretation.

The normal incidence of esophageal cancer is 5 or fewer cases per 100,000 people, however some regions of the world exceed this level. In the Transkei region of South Africa, 50-

200 cases were reported per 100,000 people (van Rensburg, 1985). In this region, the people consume maize as a staple as well as consuming a beer brewed with maize and a non-alcoholic fermented drink made with maize (Rose, 1982; Segal, 1988). The maize was locally grown, stored in open cribs and was often visually moldy, with the moldiest ears hand selected for use in beer brewing (Marasas et al., 1979). *F. verticillioides* was the predominant fungus found and levels of fumonisin contamination were higher in areas with higher incidence of esophageal cancer, as compared to areas with lower rates of esophageal cancer (Rheeder et al., 1992). Whether fumonisins are responsible for the high rate of esophageal cancer in regions of the Transkei cannot be determined. However, the existing evidence suggests that high levels of FB_1 exposure are correlated with high incidence of both esophageal and liver cancer in southern Africa and parts of China (Marasas et al., 2000). Fumonisins are also known to cause neural tube defects in mouse embryos in culture (Marasas et al., 2004) and *in vivo* (Gelineau van Waes et al., 2005), and they have been suggested to contribute to higher incidences of neural tubes defects in humans due to interference with folate utilization (Marasas et al., 2004).

F. verticillioides maize-culture material, maize naturally contaminated with *F. verticillioides*, and pure FB_1 are hepato-carcinogenic in rats (Marasas et al., 2000). Long-term feeding studies have shown that fumonisin B_1 is both a liver and kidney carcinogen in rodents (NTP Technical Report, 2001). Fumonisins are poorly absorbed and metabolized, and are rapidly excreted by animals, with only a small amount of the toxin retained in the liver and kidney (Marasas et al., 2000). Thus, most of the fumonisin consumed by farm animals is rapidly returned to the ground.

IV. Mechanisms of Action

The structure of FB_1 is very similar to the free sphingoid base sphinganine which is why it was hypothesized that the mechanism of action of this mycotoxin might be related to disruption of either sphingolipid metabolism or function (Wang et al., 1991). Wang et al. (1991) demonstrated that FB_1 inhibits the enzyme ceramide synthase, which catalyzes the acylation of sphinganine and sphingosine. This causes an increase in free sphinganine, and a decrease in reacylation of sphingosine derived from complex sphingolipid turnover (Wang et al., 1991). There is also an increase in sphingoid base degradation products and alterations in lipid pools due to the changes in carbon flux resulting from the increase in the sphingoid base degradation products (Riley et al., 1998). These reactions lead to decreased cell growth and increased cell death, in both animals and plants (Riley et al., 1996a).

V. Sphingolipid Metabolism

Sphingolipids are a collection of structurally complex lipids found in all eukaryotic cells as well as some prokaryotic cells (Lynch and Dunn, 2004). The precursors to complex sphingolipids are long chain sphingoid bases. The most common free sphingoid bases in animal cells and plant cells are sphingosine and phytosphingosine, respectively (Hannun et al., 2001), and under normal conditions these sphingoid bases are present in low concentrations in cells. The intracellular maintenance of low levels of sphingoid bases, sphingoid base 1-phosphates, and ceramide is critical because all of these molecules have been shown to be either extremely biologically active or second messengers in signaling systems in mammalian cells (Merrill, 2002) and there is increasing evidence for their importance in plants (Lynch and Dunn, 2004). More complex sphingolipids, which are important structural constituents of cell membranes, also have been shown to be important in regulating many functions in mammalian cells, such as

protein trafficking and sorting, modulation of cell-cell communication, adhesion, and cell morphology as well as the formation and stabilization of axonal branching (Merrill et al., 1997). There is also circumstantial evidence suggesting that glycophosphosphingolipids are involved in cell signaling in plants (Hetherington et al., 1992).

The two most common types of complex sphingolipids in plants are glucosylceramide and inositolphosphorylceramide (Lynch and Dunn, 2004). Glucosylceramide production has been implicated in the induction of pathogenesis-related proteins in rice (Umemura et al., 2000) and the ability to tolerate environmental stress (Lynch and Dunn, 2004) such as drought and freezing. Thus, proper sphingolipid metabolism is critical (Merrill, 2002) because sphingoid bases, sphingoid base metabolites, ceramide and more complex sphingolipids play important roles in cellular processes that modulate growth, differentiation and apoptosis (Merrill et. al., 1997; Brown and London, 2000; Vesper et al., 1999) and fumonisin induced disruption of these processes can be detrimental to cells.

VI. The Role of Fumonisins in Plant Diseases

A precise role for the fumonisins in plant pathogenicity in maize is confusing. Fumonisins (FB_1, FB_2 and FB_3) may contribute to *F. verticillioides* virulence in seedlings grown from inoculated maize seeds, but were suggested to be neither necessary nor sufficient for virulence (Desjardins et al., 1995). The structurally similar compound, Alternaria alternata lycopersici (AAL) toxin, has been shown to be a pathogenicity factor for *Alternaria alternata* f.sp. *lycopersici* – induced stem canker disease in tomato (*Lycopersicon esculentum*) (Gilchrist and Grogan, 1976). Pure fumonisin B_1, mycelia and spores of *F. verticillioides* sprayed on susceptible tomato plants have been shown to mimic both the signs of AAL-toxin and *A. alternata* f.sp. *lycopersici* – induced toxicity (Abbas et al., 1994). The mycelia and spore

suspensions were later shown to contain fumonisins (Abbas and Riley, 1996). Similar experiments involving spraying maize leaves with concentrations of fumonisin B_1 as high as 1,000 µg/ml did not cause any signs of pathology (Abbas and Boyette, 1992). However, Lamprecht et al. (1994) reported significant reductions in growth of maize seedlings grown on water agar containing 10 µM fumonisin B_1.

F. verticillioides and fumonisins are found in maize debris (Bullerman, 1996) and therefore it is reasonable to expect that maize seedlings could become infected with the fungus and also could potentially be exposed to fumonisins in the field. Williams et al. (2003) showed that under laboratory conditions, fumonisins in maize debris can be leached by rainfall and can move through soils intact. However, a large amount is bound in certain soils. In sandy loam soils, fumonisins are bound tightly but can be released under acidic conditions. Thus, fumonisins from maize debris, or produced by *F. verticillioides* in the soil, could under certain environmental conditions become biologically available to plants growing in the soil.

There is considerable supporting evidence that fumonisin-induced disruption of sphingolipid metabolism is important in the cascade of events leading to altered cell growth, differentiation and cell injury observed both *in vitro* and *in vivo* in animals (Riley et al., 2001; Merrill et al., 2001). The most important piece of evidence is that fumonisin B_1 and AAL toxin are inhibitors of ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthetic pathway (Wang et al., 1991; Merrill et al., 1993). Fumonisin induced disruption of sphingolipid metabolism, as evidenced by elevation in free sphingoid bases has been shown in several plant models (Abbas et al., 1994; Abbas et al., 1998) including roots of maize seedlings (Riley et al., 1996) and excised maize shoots (Wright et al., 2003). The toxicity of AAL toxin in tomato is closely associated with disruption of sphingolipid metabolism (Abbas et al., 1994), and tomato

varieties resistant to *A. alternata* f.sp. *lycopersici* are also resistant to AAL toxin and fumonisin-induced accumulation of free sphingoid bases (Abbas et al., 1994). In contrast, even though maize tissues (cob, stalks, and kernels) can contain detectable levels of fumonisins, fumonisin-induced disruption of sphingolipid metabolism has not been shown to be a mechanism of any disease in maize (Nelson et al., 1993).

The biochemical consequences of ceramide synthase inhibition have been closely correlated with fumonisin induced diseases. Inhibition of ceramide synthase leads to an increase in free sphingoid bases in animals (Riley et al., 1996b) and plants (Abbas et al., 1994). It has also been shown that inhibition of ceramide synthase will cause an increase in sphingoid base 1-phosphates in animals (Piva et al., 2005; Riley et al., 2005), but this has not been experimentally demonstrated in plants. Elevation by exogenously adding these intermediates to animal cells induced increased cell death (sphingosine and sphinganine) or cell proliferation (sphinganine 1-phosphate and sphingosine 1-phosphate) (Merrill et al., 1997). Elevation of exogenously added free sphingoid bases have also been shown to influence reactions which cause morphological changes and promote plant necrosis (Tanaka et al., 1993) and increased apoptosis (Wang et al., 1996). Fumonisin inhibition of ceramide biosynthesis also leads to a decrease in the biosynthesis of more complex sphingolipids which also play important roles in regulating numerous cell functions in both animals and plants and in particular lipid raft function (Merrill et al., 2001).

In plant systems, there has only been limited research done on sphingolipid metabolism and the role of sphingolipids in cellular regulation and disease. While many of the enzymatic steps and intermediates involved in plant sphingolipid biosynthesis have been demonstrated directly or indirectly (Lynch, 2000; Wright et al., 2003; Lynch and Dunn, 2004), most of the

enzymes involved have not been purified and characterized. Much of what is known about sphingolipid metabolism in animals has, therefore, been inferred in plants.

The hypothesis of this research project is that fumonisin production, inhibition of ceramide synthase and disruption of sphingolipid metabolism modulates pathogenicity in *F. verticillioides* maize seedling disease. The objectives are to determine i) if *F. verticillioides* can produce fumonisins in synthetic and complex natural soils when inoculated on maize seeds, ii) if the fumonisins produced are biological available to maize roots using an increase in free sphingoid bases and sphingoid base 1-phosphates as markers for fumonisin-induced disruption of sphingolipid metabolism, and iii) the time- and dose- dependent relationship between fumonisins in soil and effects on root development, expressed as growth, and disruption of sphingolipid metabolism.

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CHAPTER 3

FUMONISIN PRODUCTION AND BIOAVAILABILITY TO MAIZE SEEDLINGS GROWN FROM SEEDS INOCULATED WITH FUSARIUM VERTICILLIOIDES AND GROWN IN NATURAL SOILS[1]

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Abstract

The fungus *Fusarium verticillioides* infects maize and produces fumonisins. The purpose of this study was to determine the ability of *F. verticillioides* to produce fumonisins in synthetic and natural soils and their biological availability to maize roots. Maize seeds were inoculated with a pathogenic strain of *F. verticillioides* (MRC826) and planted in synthetic and three different natural soils. Statistically significant reductions were evident in stalk weight and root mass along with increased leaf lesions in the MRC826 treated seedlings in all soil types. Fumonisins were detected in all the soils of seedlings grown from MRC826 inoculated seeds. The fumonisin produced in the soils was biologically available to seedlings based on statistically significant elevation of free sphingoid bases and sphingoid base 1-phosphates in their roots. These results indicate that *F. verticillioides* produced fumonisins in the autoclaved synthetic and natural soils and that the fumonisin produced is biologically available based on evidence of inhibition of ceramide synthase.

KEYWORDS: Fumonisin, *Fusarium verticillioides*, maize, seedling disease

INTRODUCTION

The fungus *Fusarium verticillioides* is a non-obligate, genetically diverse plant pathogen that is commonly associated with maize (*Zea mays*) (1). *F. verticillioides* is not host specific and has been recovered from many commercially important crops and plants (2). Diseases of maize associated with *F. verticillioides* include seed rot, root rot, stalk rot, kernel or ear rot and seedling blight (3,4). *F. verticillioides* produces a number of fungal metabolites, most notably, the fumonisins (2). Fumonisins are water soluble mycotoxins that cause diseases in farm animals are contributing factors to plant diseases (5), and possible human carcinogens (6,7). At present, at least 28 different fumonisins have been reported (8). Fumonisin B_1 (FB_1) is the most abundant and is believed to be the most toxic of the fumonisins (6).

Although suspected to contribute to *F. verticillioides* virulence in seedlings grown from inoculated maize seeds, the role that fumonisins play in host-pathogen interaction with maize has not been fully investigated. Desjardins et al (9) compared the ability of fungal strains to produce fumonisin and their virulence on maize seedlings and concluded that, while fumonisin played a role in virulence, it was not necessary nor sufficient for seedling disease development. Seedlings from two maize cultivars sprayed with concentrations of FB_1 as high as 1,000 µg/mL showed no symptoms of disease (10). However, significant reductions in growth of maize seedlings grown on water agar containing fumonisin B_1 (11) and maize callus (12) have been reported. FB_1 inhibits ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthetic pathway (13,14). Fumonisin inhibition of ceramide synthase, as evidenced by elevation in free sphingoid bases (15), has been shown in several plant models (16,17) including roots of maize seedlings (18) and excised maize shoots (19).

F. verticillioides has been isolated from maize debris (20) and soil (21). There is only limited data on the fate of fumonisins in soils (22, 23). In the first published report on the fate of FB_1 in soil, it was found that when FB_1 was mixed with silty clay loam soil, it could not be recovered from the soil (22). It was concluded that FB_1 was either irreversibly bound or chemically altered in the soil. However, more recent studies using Cecil sandy loam soil indicated that while FB_1 can be tightly bound in the soil, it can also be released intact under acidic conditions (23). The possibility that FB_1 from maize debris in field situations could enter the soil environment is of significant interest since FB_1 could alter the biological activity of soil flora and fauna. Also, the more complex the soil, the more likely that FB_1 will be retained in the soil matrix (23), however, this does not preclude that it is not biologically available.

The purpose of this study was to determine i) if *F. verticillioides* can produce fumonisins in synthetic and complex natural soils when inoculated onto maize seeds, and if so, ii) whether the fumonisins produced are biological available to maize roots. Increases in free sphingoid bases and sphingoid base 1-phosphates were used as markers for fumonisin-induced disruption of sphingolipid metabolism. The results of this study show that fumonisin can bind tightly to some soils and that binding is correlated with the cation exchange capacity of the soil, that *F. verticillioides* can produce fumonisins in synthetic and natural soils, and that the fumonisin produced is biologically available based on evidence of inhibition of ceramide synthase.

MATERIALS AND METHODS

Soil Types. The soil types used in this study were washed fine sand, commercial potting mix (45% sphagnum peat, Conrad Fafard Inc., Agawam, MA, USA), Cecil sandy loam (clayey, kaolinitic, thermic typic kanhapludult, collected in Watkinsville, GA, USA) Downer sandy loam (coarse-loamy, siliceous, semiactive, mesic typic hapludults, collected in East New Market, MD, USA), and Roper muck sandy loam (fine-silty, mixed, semiactive, acid, thermic, histic humaquepts, collected in Elizabeth City, NC, USA). All of the complex natural soils came from fields where maize was grown as a commercial crop. Samples of each soil type were analyzed by the University of Georgia Soil Testing and Plant Analysis Laboratory (Athens, GA, USA) and characteristics are given in Table 3.1. The washed fine sand, Cecil sandy loam, Downer sandy loam and Roper muck sandy loam were texturally distinguishable from each other by their percentage of sand, silt and clay. The commercial potting mix was distinguished from the others by its high content of organic material.

Soil Binding Assay. To determine how tightly FB_1 binds to the test soils, a modification of a previously described acid displacement procedure (23) was utilized using twice autoclaved commercial potting mix, Cecil sandy loam, Downer sandy loam, and Roper Muck sandy loam. Briefly, the procedure consisted of mixing 2 g of each soil in 50 ml culture tubes with 25 ml of water containing 3.2 μg FB_1/ml. The tubes were shaken for 12 h and then centrifuged at 240 rcf, supernatants removed, and aliquots analyzed by liquid chromatography mass spectrometry (LCMS) for unbound FB_1. Acetonitrile (ACN) and H_2O were added to each tube so as to attain a 1:1 mixture based on the calculated void volumes. The tubes were shaken again for 12 h, centrifuged and aliquots of the supernatants analyzed by LCMS for FB_1 defined as loosely bound. Samples were then extracted with ACN:H_2O containing 5% formic acid, and the

supernatants were analyzed for acid displaced FB_1. The difference between the total amount of FB_1 added to the soils and the sum of FB_1 recovered (unbound plus loosely bound plus acid displaced) was defined as irreversibly bound to the soils. In order to facilitate comparison of the binding capacity of the various soil types, the sum of the unbound and loosely bound FB_1 was defined as "not tightly bound" and the sum of acid displaced and irreversibly bound FB_1 was defined as "tightly bound". It was assumed that microbial degradation of FB_1 during the initial 12 h shaking period was negligible since the soils were twice autoclaved and the FB_1 water solution was filter sterilized using a 0.2 μm Nalgene filter (Nalge Nunc International, Rochester, NY, USA).

Virulence Assay. *F. verticillioides* strain MRC826 (Medical Research Council, Tygerberg, South Africa) is known to be highly virulent to some maize cultivars (24, 25) and also produces several fumonisins including fumonisins B_1, B_2 and B_3 (8). The conidia were frozen at -80 in 15% glycerol until inoculated on potato dextrose agar and incubated at 27°C in the dark to initiate experimental cultures. The conidia for seed inoculation were obtained by flooding the agar surface with 10 ml sterile water and diluting this suspension to 10^6 conidia/ml.

Untreated maize seed ('Silver Queen', Gurney's Seed & Nursery Co., Yankton, SD, USA) 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 seed were then heat shocked by placing them in a 60°C water bath for 5 min for internal sterilization (26). Inoculations were performed by placing 40 sterilized seeds in a Petri dish (100 mm) and flooding the seed with 10 ml of the conidial suspension. Sterile water was used for the control group. The seed were incubated overnight at 27°C. Five 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 mix or twice-autoclaved Cecil sandy loam, Downer sandy loam or Roper muck sandy loam. Pots were watered as needed throughout the duration of the assay. Assays were performed under aseptic conditions in a plant growth chamber at 26^{o}C under a 14 h light (cool-white, high-output fluorescent tubes at an average of 254 µmol m^{-2} s^{-1}) and a 10 h dark regime at 22^{o}C.

Disease symptoms were visually assessed for indications of seedling blight (24) from 7 to 21 days after planting. The exposure time was chosen based on other studies with the sweet maize hybrids 'Polar Vee' (24) and 'Silver Queen' (27). These earlier studies found that seedlings grown in potting mix from seeds inoculated with MRC826 showed signs of stunting, necrotic leaf lesions and abnormal leaf development as compared to seedlings grown from surface sterilized control seeds, which showed no signs of fungal infection or disease.

Preparation, Extraction and Fumonisin Analysis of Soils. After harvest, the soils from each replicate in the virulence assay were carefully separated from the roots and allowed to air-dry in a fume hood and then stored at -20^{o}C. The soils were carefully inspected to remove all visible root materials. Fumonisins in the soil were extracted as described in Williams et al (23). Briefly, two grams of the dried soil from each replicate were placed into 50 ml conical tubes and 25 ml of 1:1 ACN:5% formic acid in water was added to each tube. The tubes were placed on a rotary shaker for 16 h, after which, samples were centrifuged for 20 min at 1100 rcf, and 1 ml samples were placed into polypropylene tubes and centrifuged at 16,000 rcf for 10 min. Samples of the extracts (1 ml) were transferred to polypropylene tubes containing 0.67 ml of H_2O and mixed to make a final concentration of 30:70 ACN:1.5% formic acid in water. The samples were allowed to stand approximately 1 h then centrifuged at 16,000 rcf for 5 min and 1 ml was transferred to a sample vial that contained 10 µl of a 10 ng/µl phytosphingosine internal standard

(added to monitor instrument performance (28)) to make a final concentration of 0.1 ng phytosphingosine/µl of sample. Samples containing the internal standard were analyzed by high performance liquid chromatography (HPLC) tandem mass spectrometry (LCMS) (described below), with values expressed as µg FB_1 per gram soil (FB_2 and FB_3 were measured but only FB_1 levels are reported).

Extraction of Free Sphingoid Bases and Sphingoid Base 1-Phosphates. The intact roots from each pot and treatment were immersed and rinsed in an ice water-bath to remove any remaining soil. The washed roots were blotted dry and placed in a -80^{o}C freezer overnight. The shoots and roots were then freeze-dried, and the roots were separated from the shoots and placed into labeled zip-lock bags and stored at -20^{o}C. Prior to extraction, the freeze-dried root tissues were carefully inspected, to remove any remaining soil and were weighed to determine the effects on root growth. The root tissues were then placed in Pyrex 9825 (15 x 123 cm, 18 ml) round bottom centrifuge tubes and pulverized with a glass rod to a fine powder under liquid nitrogen and stored in a vacuum desiccator with anhydrous calcium sulfate. The ground root tissues were extracted and analyzed for free sphingoid bases (sphinganine and phytosphingosine) and their 1-phosphate metabolites (sphinganine 1-phosphate and phytosphingosine 1-phosphate). The extraction method was a modification of Sullards et al (29). Briefly, samples consisting of 20 mg of ground root tissue from each replicate were transferred into Pyrex round bottom centrifuge tubes, then 100 µl cold phosphate buffer was added and the samples were homogenized on ice at 4^{o}C for 5 minutes. Then 0.6 ml methanol and 0.3 ml chloroform plus 10 µl internal standard (10 ng/µl C_{17} sphinganine 1-phosphate and 50 ng/µl C_{20} dihydrosphingosine each dissolved in ethanol, Avanti Polar Lipids, Inc., Alabaster, AL, USA) was added to each sample. Samples were sonicated 1 min at room temperature, capped tightly and incubated for 16 h at 48^{o}C in a

heating block. Samples were then allowed to cool, after which, 75 µl of 1 M methanolic KOH was added and the samples were sonicated 30 s and incubated 2 h at 37°C. The samples were then centrifuged at 1100 rcf for 10 min and the supernatants were transferred to 13 mm x 100 mm (Pyrex 9826) glass tubes. The samples were neutralized by adding two drops of 1N HCl, and then evaporated to dryness (without heat) in a vacuum centrifuge (1.5 – 2 h) and stored under N_2 at -20°C. The samples were then reconstituted in 500 µl of (49.5:49.5:1) ACN:water:formic acid containing 5 mM ammonium formate and clarified by filter centrifugation (4500 rcf for 10 min) using a 0.45 Nylon Microspin filter (Lida Manufacturing Corp., Kenosha, Wisconsin, USA). The 500 µl samples were then analyzed by LCMS.

LCMS Methods. Fumonisin was separated on a Thermal Separations HPLC (Riviera Beach, FL, USA) consisting of a model P2000 solvent delivery system and an AS3000 autosampler. Separations were done using an Intersil 5µ ODS-3 column (150 x 3 mm, Metachem Technologies, Inc, Torrance, CA, USA). The flow was 0.2 ml/min and the mobile phase was a 28 min programmed gradient starting at 30% of 97% ACN:2% water:1% formic acid (solvent "A") and 70% of 2% ACN:97% water: 1% formic acid (solvent "B") and after 15 min the proportions of "A" and "B" were 60% and 40% respectively, and at 20 min the proportions of "A" and "B" were 90% and 10% respectively followed by an 8 min gradient returning to 30% "A" and 70% "B". The total run time was 28 min and there was a 5 min equilibration between each injection. The column effluent was directly coupled to a ThermoFinnigan LCQ Duo ion trap mass spectrometer (MS) (Woodstock, GA, USA). The MS was operated in the electrospray ionization (ESI) positive ion mode with an inlet capillary temperature of 200°C and the sheath gas was nitrogen. For MS/MS of fumonisin B_1, fumonisin B_2 and fumonisin B_3 the collision energy was 32% and the parent m/z were 722.3 and 706.3, 706.3, respectively, and mass

fragments were scanned from 195 to 800 m/z and compared to authentic standards.

Sphingoid bases and sphingoid base 1-phosphates were chromatographically separated on the same LCMS system as fumonisin. The gradient started at 50% solvent A and at 15 min was 70% solvent A and at 20 min it was 100% solvent A which was held until 25 min at which time the column was re-equilibrated with 50% A for 15 min before the next injection (10 μl). The total run time was 40 min. The MS was operated in the ESI positive ion mode with an inlet capillary temperature of 170^{o}C and the sheath gas was nitrogen. For MS/MS the collision energy was 30% and the parent m/z for MS/MS were 318.2, 302.2, 398.5 and 382.5 for phytosphingosine, sphinganine, phytosphingosine 1-phosphate and sphinganine 1-phosphate, respectively. The m/z for the internal standards were 366.5 and 330.2 for C_{17} sphingosine 1-phosphate and C_{20} dihydrosphingosine, respectively. MS/MS mass fragments were scanned from 195 to 400 m/z and compared to authentic standards.

Statistical Analysis. Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). Where many groups were compared, one-way analysis of variance was used, followed by post hoc multiple comparisons. Where only two groups were compared, a Student t-test or Mann Whitney Rank Sum test was used. The Pearson Product Moment Correlation was used to measure the strength of the association between pairs of variables. All data were expressed as mean ± standard deviation, and differences among or between means were considered significant if the probability (p) was ≤ 0.05.

RESULTS AND DISCUSSION

In a previous study (23) it was shown that FB_1 did not bind appreciably (< 10% bound) to washed sand but was bound by Cecil sandy loam soil. In the present study both the potting soil and all of the complex natural soils bound appreciable amounts of FB_1 (Figure 3.1). However, the Roper muck sandy loam bound more FB_1 than the Cecil sandy loam, Downer sandy loam, or potting mix (Figure 3.1) based on the amount of FB_1 recovered in the water after 12 h and in the ACN:water extracts. The best predictors of FB_1 binding to soils were the calculated effective cation exchange capacity (CEC_e) and the calcium content of the soil (Table 3.2). The correlation between tightly bound FB_1, CEC_e and calcium content were statistically significant ($p< 0.05$, n=5), whereas, the correlation between FB_1 not tightly bound, CEC_e and calcium were ≥ 0.87 and the "p" values were both < 0.06 (Table 3.2). The fact that 5% formic acid is necessary to extract tightly bound fumonisins from complex natural soils is consistent with the hypothesis that the tight binding is due to ionic interactions with soil constituents and that soils with a high cation exchange capacity and calcium content will bind fumonisins tightly.

Maize seedlings grew much better in the synthetic potting mix than in any of the natural complex soils. This was most evident when comparing the weight of aerial plant tissues. For example, the stalk weight (Table 3.3) of the control maize seedlings grown in potting mix were significantly greater ($p< 0.001$, $F=48.1$, degrees of freedom (df) = 19) compared to control plants grown in the complex natural soils. A similar statistically significant reduction in root weight was evident in control seedlings grown in complex natural soils compared to seedlings grown in potting soil ($p< 0.001$, $F=9.6$, df = 19). Nonetheless, there were no necrotic leaf lesions or signs of abnormal leaf development in seedlings grown from un-inoculated seeds planted in either synthetic or natural soil. Symptoms of *F. verticillioides*-induced seedling disease were evident

in seedlings grown from seeds inoculated with MRC826 and planted in either synthetic or natural soils. For each soil type, symptoms of disease included necrotic leaf lesions and abnormal leaf development, stunting and reduced root development compared to the respective un-inoculated control group (Table 3.3).

All soil types were analyzed prior to planting the seeds and no fumonisins were detected. At planting, the MRC826 inoculated seeds did not contain detectable fumonisins (< 0.004 μg/g). After 21 days in the virulence assay all the soils from the MRC826 pots contained fumonisins and fumonisin was also detected in only 1 of the control pots of the synthetic potting soil group (Table 3.3). The FB_1 content of the potting soil, Cecil sandy loam and Roper muck sandy loam soils from the MRC826 treatment groups all had similar levels of FB_1 and while the level of FB_1 in the Downer sandy loam was much less than in the other soils, the difference was not statistically significant ($p = 0.084$, $F=2.6$, $df=19$). However, when comparing just the complex natural soils the Downer sandy loam contained significantly ($p< 0.05$) less FB_1 compared to the Cecil sandy loam (Table 3.3).

Within the complex natural soils from the MRC826 treatment, there was a statistically significant correlation between leaf lesions and root weights, between leaf lesions and root weights normalized to the total weight of the seedling (stalk weight plus root weight), and between leaf lesions and FB_1 in the soils (Table 3.4). The correlation between root weights and FB_1 in the soils was not statistically significant ($p=0.069$), however, the FB_1 in the soil was significantly correlated with the root weight when normalized to the total weight (stalks plus roots) of the seedling ($p=0.027$). In another study, the fumonisins had no effect on percent seed germination, but it inhibited radicle elongation by 75% (30). These results offer additional

support for the hypothesis that FB_1 available in the rhizosphere is a virulence factor in *F. verticillioides* seedling disease.

In order to determine if the fumonisins present in the soil were biologically available (able to enter the intracellular space), root tissues were analyzed for elevation in free sphingoid bases, (phytosphingosine (Pso) and sphinganine (Sa)) and their sphingoid base 1-phosphates (Sa-1-P and Pso-1-P); elevation in free sphingoid bases and sphingoid base 1-phosphates is a consequence of inhibition of ceramide synthase, an enzyme localized primarily in the endoplasmic reticulum (14). There was a statistically significantly elevation of Sa and Pso in the roots of seedlings grown from seeds inoculated with MRC826 and planted in potting mix, Cecil sandy loam, and Downer sandy loam, as compared to the seedlings grown from un-inoculated controls (Figure 3.2). There was also an increase in Sa and Pso in the roots of seedling grown from seeds inoculated with MRC826 and planted in Roper muck sandy loam soil, however the increase was not statistically significant when compared to roots of seedlings grown from un-inoculated seeds planted in Roper muck sandy loam (Figure 3.2). The sphingoid base 1-phosphates (Sa-1-P and Pso-1-P) were significantly increased in the roots of seedlings grown from seeds inoculated with MRC826 with the exception of the Sa-1-P in the MRC826 group grown in Cecil sandy loam; nonetheless the mean Sa-1-P concentration was over 500% of the control (Figure 3.3A). The fact that free sphingoid bases and sphingoid base 1-phosphates are elevated in root tissues is consistent with the conclusion that the fumonisin produced in the rhizosphere can enter the intracellular space of the growing maize seedling.

F. verticillioides is frequently found in maize seed and soils and is a frequent cause of disease in maize, an important agricultural commodity worldwide. Thus, it is important to understand the interactions between the plant, the soil, and this toxin. In this study, maize seeds

were grown in synthetic soil (potting mix) and three complex natural soils (Cecil sandy loam, Downer sandy loam and Roper Muck sandy loam) from different geographic regions to determine if *F. verticillioides* can produce fumonisins in soils representative of those in which maize is grown commercially. In addition, because earlier studies (22, 23) showed that fumonisins are tightly bound in some soils, it was important to determine if the fumonisin produced in complex natural soils is biologically available to the plant. *F.* verticillioides, MRC826, was able to produce fumonisins in each of the complex soils and induce maize seedling disease. Compared to controls, the root mass was reduced in the seedlings inoculated with MRC826 and the roots had increased levels of free sphingoid bases and sphingoid base 1-phosphates. The degree of reduction in root mass, amount of fumonisins detected in the soils, as well as the increase in free sphingoid bases and their 1-phosphate metabolites varied in the different soil types. However, regardless of the soil type *F. verticillioides* MRC826 was able to produce fumonisins in 4 soil types, disrupt sphingolipid metabolism in the roots, and induce maize seedling disease. In addition, there was a statistically significant correlation between FB_1 levels in the soil and both the number of leaf lesions and decreased root weight normalized to total seedling weights.

These findings are important because they show that *F. verticillioides* and FB_1 can alter maize seedling performance and viability in natural soil. Nonetheless, the extent of crop damage would depend on maize genotype and soil biotic and abiotic factors, none of which were examined in this study. These factors are equally important in assessing risk management of the complex mechanisms indicated in this study of the expression of maize seedling disease induced by *F. verticillioides*.

ABREVIATIONS USED

FB_1, fumonisin B_1; Sa, sphinganine; Pso, phytosphingosine; Sa-1-P, sphinganine 1-phosphate; Pso-1-P, phytosphingosine 1-phosphate; ACN, acetonitrile; LCMS, liquid chromatography mass spectrometry; HPLC, high performance liquid chromatography; ESI, electrospray ionization; CEC_e, effective cation ion exchange capacity.

SAFETY

Fumonisin B_1 is a known liver and kidney carcinogen in rodents; therefore it should be handled using proper precautionary measures.

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Table 3.1. Comparison of the Chemical and Physical Analysis of Sand, Potting Mix, Cecil Sandy Loam, Downer Sandy Loam, and Roper Muck Sandy Loam[a]

sample	sand (n = 2)	potting mix (n = 3)	Cecil sandy loam (n = 7)	Downer sandy loam (n = 6)	Roper muck sandy loam (n = 6)
CEC (meq/100g)	0.21	ND	5.96	6.98	17.1
$CEC_{effective}$ (meq/100g)	0.07	0.61	3.20	4.52	8.76
pH	5.7	6.3	5.1	6.6	5.4
Organic Matter (%)	0.0	63.0	3.2	1.9	13.0
Ca (meq)	0.05	0.15	2.30	3.10	6.30
K (meq)	0.01	0.16	0.30	0.32	0.26
Mg (meq)	0.01	0.30	0.60	1.10	2.20
% Sand	98	ND	72	72	62
% Silt	0.0	ND	13.0	19.0	25.0
% Clay	2.0	ND	15.0	9.0	13.0

[a]Samples of sand, potting mix, Cecil sandy loam, Downer sandy loam, and Roper muck sandy loam were analyzed by the University of Georgia soil testing and plant analysis laboratory (Athens, Ga, USA). $CEC_{effective}$ is an estimate of the cation exchange capacity (CEC) at the current soil pH. It is based on soil test extractable calcium (Ca), potassium (K), and magnesium (Mg) (31). ND, indicates that these measurements can not be done with synthetic soils (potting mix).

Table 3.2. Correlation Between the Amount of FB_1 Not Tightly Bound[a], FB_1 Tightly Bound[b], Effective Cation Exchange Capacity[c] (CEC_e) and Calcium Content of the Soils (Ca^{2+}) (n=5 soil types described in Table 3.1).

	tight	CEC_e	Ca^{2+}
Not Tightly Bound			
correlation coefficient	-0.998	-0.877	-0.870
p value	<0.001	0.0510	0.0552
Tightly Bound			
correlation coefficient		0.890	0.885
p value		0.0429	0.0457
CEC_e			
correlation coefficient			0.997
p value			<0.001

[a]FB_1 not bound (water) + FB_1 loosely bound (extracted from soil with ACN:H_2O) n=3/soil type from Figure 3.1.

[b]FB_1 acid displaced (extracted with ACN:H_2O containing 5% formic acid) + FB_1 irreversibly bound (not recovered from soils) n=3/soil type from Figure 3.1.

Table 3.3. Summary of Phytotoxic Effects and FB_1 in Roots and Soil from the Virulence Assay with Fumonisin Producing (MRC826) and Control-Treated Maize Seedlings Grown in Commercial Potting Mix (Potting), Cecil Sandy Loam (Cecil), Downer Sandy Loam (Downer), or Roper Muck Sandy Loam (Roper) at 21 Days

soil and treatment	survival incidence[a]	leaf lesion[b]	stalk weights (g)[c]	root weight (g)[d]	FB_1 in soil (μg/g)[e]
Potting					
control	49/50	0/49	2.9 ± 0.3	1.11 ± 0.15	0.04 ± 0.01
MRC826	49/50	35/49	1.5 ± 0.3[f]	0.33 ± 0.09[f]	1.74 ± 1.43[f]
Cecil					
control	48/50	0/48	1.3 ± 0.3	0.66 ± 0.13	0.00 ± 0.00
MRC826	49/50	43/49	1.0 ± 0.2[f]	0.41 ± 0.11[f]	2.21 ± 1.47[f]
Downer					
control	50/50	0/50	1.4 ± 0.1	0.82 ± 0.15	0.00 ± 0.00
MRC826	50/50	15/50	1.1 ± 0.1[f]	0.68 ± 0.08[f]	0.37 ± 0.19[f]
Roper					
control	50/50	0/50	1.6 ± 0.2	0.81 ± 0.09	0.00 ± 0.00
MRC 826	49/50	26/49	1.1 ± 0.1[f]	0.68 ± 0.06[f]	1.76 ± 0.66[f]

[a]Indicates the total number of seedlings harvested on day 21 over the number of seeds planted (10/pot).

[b]Indicates the total number of seedlings exhibiting leaf lesions over the total number of surviving seedlings. All MRC826 treated pots (n=5/soil type) had at least 1 seedling that exhibited leaf lesions.

[c]Values are the mean ± standard deviation (n=5) of the average stalk weight of all plants harvested on day 21 from each treatment group.

[d]Values are the mean ± standard deviation (n=5) of the average root weight of all plants harvested on day 21 from each treatment group. Differing superscripts indicates value is significantly ($p \leq 0.05$) different from the control group.

[e]Values are the mean ± standard deviation (n=5) of the total fumonisin extracted from soil. Differing superscripts indicates value is significantly ($p \leq 0.05$) different from the control group.

[f]Value is significantly ($p \leq 0.05$) different from the control corresponding group.

Table 3.4. Correlation between FB_1 in the Natural Soils, Leaf Lesions, Stalk Weight and the Ratio of Root Weight to Plant Weight of the Seedlings Grown from Seeds Inoculated with MRC826 [a] (n = 15 pots total).

	stalk weight	root weight	Ratio[b] (root weight/plant weight)	FB_1 in soil
leaf lesion correlation coefficient	-0.024	-0.711	-0.708	0.545
p value	0.932	0.002	0.003	0.026
stalk weight correlation coefficient		0.291	-0.112	0.273
p value		0.293	0.691	0.310
root weight correlation coefficient			0.915	-0.470
p value			<0.001	0.069
ratio[a] correlation coefficient				-0.568
p value				0.022

[a]Cecil Sandy Loam, Downer Sandy Loam, and Roper Muck Sandy Loam.

[b]Ratio is the root weights normalized to the total plant weight (root weight + stalk weight).

FIGURE CAPTIONS

Figure 3.1. The ability of potting soil (A), Cecil sandy loam (B), Downer sandy loam (C), and Roper Muck sandy loam (D) to bind FB_1. "Unbound" is defined as the amount of FB_1 in the water solution after mixing with the soil for 12 h. "Loosely bound" is defined as the FB_1 subsequently extracted from the soil with ACN:water (1:1, v/v) after shaking for an additional 12 h. "Acid displaced" is defined as the FB_1 subsequently extracted from the soil with ACN:H_2O with 5% formic acid (1:1, v/v) after shaking for an additional 12 h. "Irreversibly bound" is the FB_1 that was not recovered calculated as the difference between the FB_1 in the original solution minus the total FB_1 recovered from the three other fractions. Values are means±SD (n=3).

Figure 3.2. (A) Free sphinganine (Sa) and (B) free phytosphingosine (Pso) in roots from maize seedlings grown from sterilized seeds (control) or sterilized seeds inoculated with the pathogenic strain (MRC826) of *F. verticillioides* and planted in potting mix, Cecil sandy loam, Downer sandy loam or Roper Muck sand loam and harvested 21 days after planting. Values for free sphingoid bases are expressed as nmol/g root tissue (mean ± SD, n=5 pots containing 10 seedlings). Differing superscripts (a,b) indicate significant differences ($p \leq 0.05$) in Sa or Pso between treatments.

Figure 3.3. (A) Free sphinganine 1-phosphate (Sa-1-P) and (B) phytosphingosine 1-phosphate (Pso-1-P) in roots from maize seedlings grown as described in Figure 3.2. Values for free sphingoid bases and sphingoid base 1-phosphates are expressed as nmol/g root tissue (mean ± SD, n=5 pots containing 10 seedlings). Differing superscripts (a,b) indicate significant differences ($p \leq 0.05$) in Sa-1-P or Pso-1-P between treatments; "ND" = not detected.

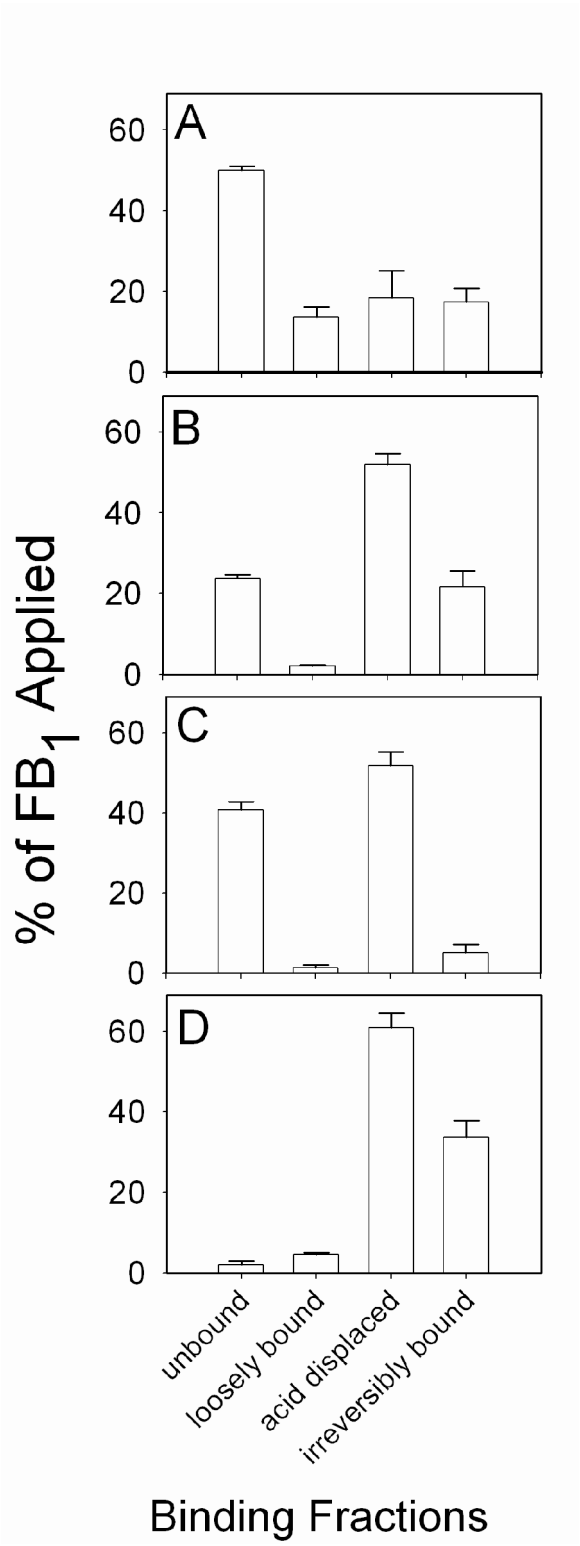

Fig. 3.1 Williams et al.

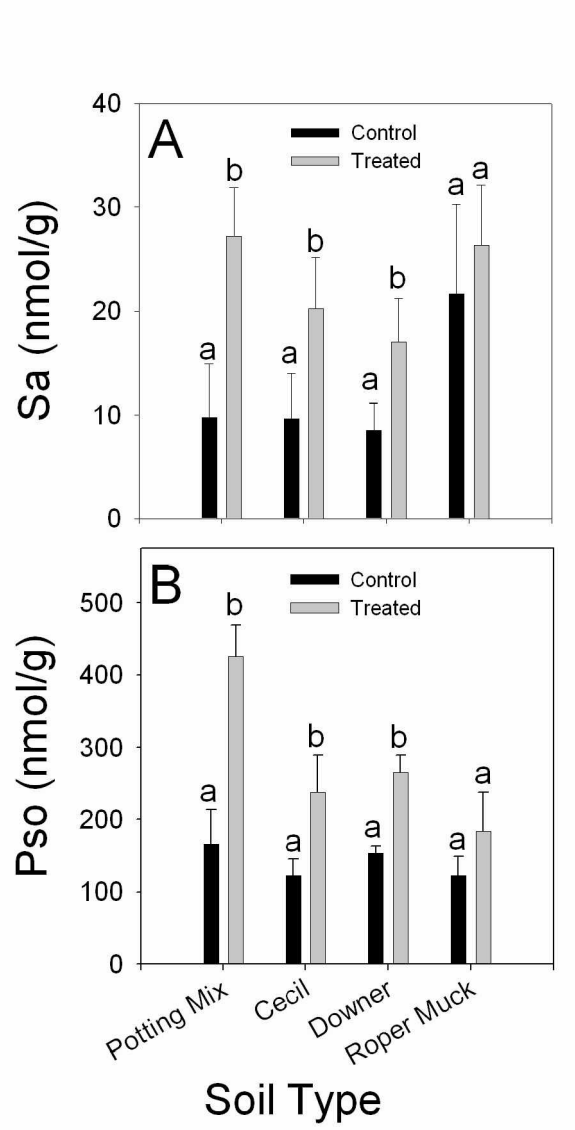

Fig. 3.2 Williams et al.

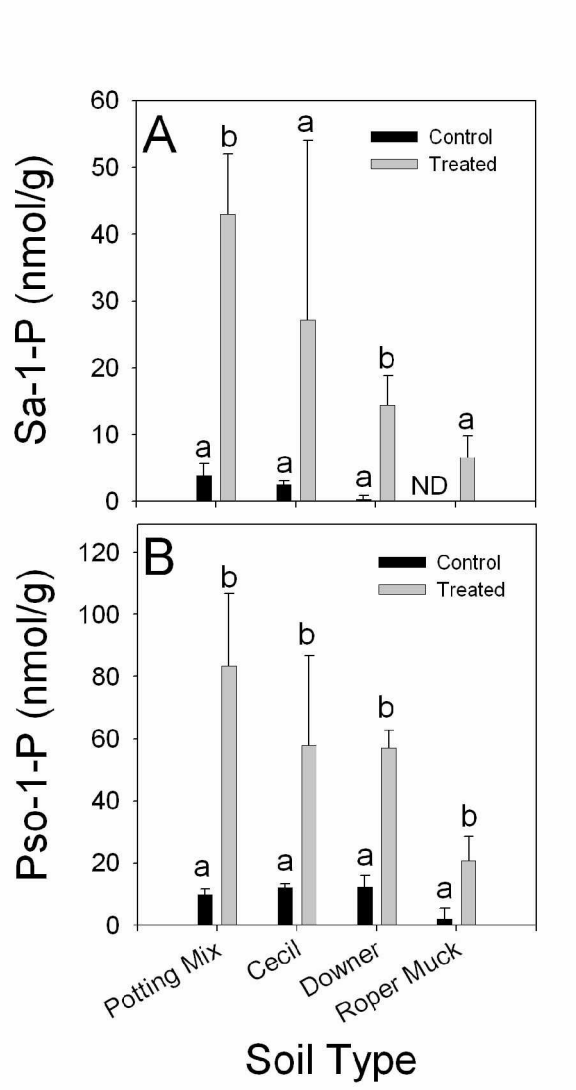

Fig. 3.3 Williams et al.

CHAPTER 4

DISRUPTION OF CERAMIDE BIOSYNTHESIS AND ACCUMULATION OF SPHINGOID BASES AND SPHINGOID BASE 1-PHOSPHATES: A MECHANISM FOR *FUSARIUM VERTICILLIOIDES* EFFECTS ON ROOT DEVELOPMENT IN MAIZE-SEEDLING DISEASE[2]

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Abstract

The fungus *Fusarium verticillioides* infects maize (*Zea mays*) and produces fumonisins, inhibitors of acyl coenzyme A dependent ceramide synthase. To determine the role of fumonisins on maize root development, seeds were inoculated with pathogenic or non-pathogenic strains of *F. verticillioide*s. Roots were analyzed for sphingoid bases, sphingoid base 1-phosphates, and fumonisins. Potting soils also were analyzed for fumonisins. Seedlings grown from seeds inoculated with the pathogenic strain had detectable fumonisins in roots and soils, and elevated levels of sphingoid bases and sphingoid base 1-phosphates in roots. In a subsequent study, un-inoculated seeds were grown in soil watered with fumonisin B_1. In addition to aboveground symptoms indicative of *F. verticillioides*-induced seedling disease, there was a dose-dependent reduction in root mass that was inversely correlated with accumulated fumonisin B_1, sphingoid bases and sphingoid base 1-phosphates in roots. The correlation between fungal pathogenicity, fumonisin production, ceramide synthase inhibition, the production of qualitatively similar pathology by both pathogenic strains of *F. verticillioides* and direct exposure to fumonisin B_1, and the known biological activity of sphingolipids as regulators of cell function in plants strongly support the hypothesis that fumonisin is necessary and sufficient to produce *F. verticillioides* maize seedling disease and that the proximate cause of effects on root development is disruption of sphingolipid metabolism. The data offer additional evidence for the importance of sphingolipids in the physiological well being of plants and indicate the need for developing a better understanding of the role of sphingolipids in physiological and disease processes in plants.

INTRODUCTION

Fumonisins, mycotoxins produced by the fungus *Fusarium verticillioides*, are the cause of animal diseases and may be contributing factors to plant diseases (Minorsky, 2002). The fungus *F. verticillioides* is a non-obligate plant pathogen that is commonly associated with maize (*Zea mays*) (Bacon and Hinton, 1996). *F. verticillioides* is not host specific and has been recovered from many commercially important crops and plants (Bacon and Nelson, 1994). Extensive research has been done on the parasitism of maize (Bacon and Hinton, 1996). Diseases of maize associated with *F. verticillioides* include seed rot, root rot, stalk rot, kernel or ear rot and seedling blight (Cook, 1981; Kommedahl and Windels, 1981). *F. verticillioides* produces a number of fungal metabolites that include the fumonisins (Bacon and Nelson, 1994), which are water soluble mycotoxins that cause diseases in farm animals and are possible human carcinogens (IARC, 2002; WHO, 2002). At present, at least 28 different fumonisins have been reported (Rheeder et. al., 2002). Fumonisin B_1 is the most abundant and is believed to be the most toxic of the fumonisins (IARC, 2002).

The role that fumonisins play in pathogenicity in maize is not understood. Although suspected to contribute to *F. verticillioides* virulence in seedlings grown from inoculated maize seeds, the most definitive study to date concluded that fumonisins are neither necessary nor sufficient for virulence (Desjardins et al., 1995). The structurally similar compound, Alternaria alternata lycopersici (AAL) toxin, has been shown to be a pathogenicity factor for *Alternaria alternata* f.sp. *lycopersici* – induced stem canker disease in tomato (*Lycopersicon esculentum*) (Gilchrist and Grogan, 1976). In tomato protoplasts and leaflets the mechanism of cell death induced by AAL-toxin and fumonisin B_1 is apoptotic-like (Wang et al., 1996). Studies in protoplasts and infiltrated leaflets from *Arabidopsis thaliana* indicate that fumonisin-induced

programmed cell death is light dependent and requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways (Asai et al., 2000). Interestingly, *Arabidopsis* seedlings grown on agar media containing fumonisin, developed similar lesions on leaves but root growth was largely unaffected (Stone et al., 2000). In susceptible tomato plants, fumonisin B_1 or mycelia and spores of *F. verticillioides* sprayed on aerial parts have been shown to mimic both the symptoms of AAL-toxin and *A. alternata* f.sp. *lycopersici*-induced toxicity (Abbas et al., 1994). Similar experiments involving spraying maize plants with concentrations of fumonisin B_1 as high as 1,000 μg mL^{-1} did not produce any symptoms of disease (Abbas and Boyette, 1992). However, significant reductions in growth of maize seedlings grown on water agar containing fumonisin B_1 (Lamprecht et al., 1994) and maize callus (Van Asch et al., 1992) have been reported.

F. verticillioides is found in maize debris (Bullerman, 1996) and soil (Almeida et al., 2002). Therefore it is reasonable to expect that fungus-infected debris and soil could serve as a source of fungal inoculum, and possibly fumonisins, in the soil and rhizosphere, which is the soil environment in close proximity to the developing root system (Walker et al., 2003). In sandy loam soils, fumonisins are bound tightly but can be released under acidic conditions (Williams et al., 2003). Thus, fumonisins from maize debris, or produced by *F. verticillioides* in the rhizosphere, could become biologically available to plants growing in the soil.

The structure of fumonisin B_1 is very similar to the free sphingoid base sphinganine which is why it was originally hypothesized that the mechanism of action of this mycotoxin might be related to disruption of either sphingolipid metabolism or function (Wang et al., 1991). Briefly, sphingolipids are a collection of structurally complex lipids found in all eukaryotic cells as well as some prokaryotic cells (Lynch and Dunn, 2004). In plant systems, there has only been

limited research done on sphingolipid metabolism and the role of sphingolipids in cellular regulation and disease (Worrall et al., 2003). While many of the enzymatic steps and intermediates involved in plant sphingolipid biosynthesis have been demonstrated directly or indirectly (Lynch, 2000; Wright et al., 2003; Lynch and Dunn, 2004; Imai and Nishiura, 2005; Coursol et al., 2005), many of the enzymes involved have not been purified and characterized. Much of what is known about sphingolipid metabolism in animals and yeast (*Saccharomyces cerevisiae*) has, therefore, been inferred to also be true in plants. There is increasing evidence for the importance of sphingolipids as signaling molecules in plants (Liang et al., 2003; Worrall et al., 2003; Lynch and Dunn, 2004; Coursol et al., 2005).

Today, there is considerable evidence that fumonisin-induced disruption of sphingolipid metabolism is important in the cascade of events leading to altered cell growth, differentiation and cell injury observed both *in vitro* and *in vivo* (Merrill et al., 2001; Riley et al., 2001). The most important piece of evidence is that fumonisin B_1 and AAL-toxin are inhibitors of ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthetic pathway (Figure 4.1) (Wang et al., 1991; Merrill et al., 1993). Fumonisin inhibition of ceramide synthase, as evidenced by elevation in free sphingoid bases, a marker for ceramide synthase inhibition (Riley et al., 1994), has been shown in several plant models (Abbas et al., 1994; Abbas et al., 1998) including roots of maize seedlings (Riley et al., 1996) and excised maize shoots (Wright et al., 2003). The toxicity of AAL-toxin in tomato is closely associated with disruption of sphingolipid metabolism (Abbas et al., 1994), and tomato varieties resistant to *A. alternata* f.sp. *lycopersici* are also resistant to AAL-toxin and fumonisin-induced accumulation of free sphingoid bases (Abbas et al., 1994). In contrast, even though maize tissues (cob, stalks, and kernels) can contain

detectable levels of fumonisins, fumonisin-induced disruption of sphingolipid metabolism has not been shown to be a mechanism of any disease in maize.

The biochemical consequences of ceramide synthase inhibition have been closely correlated with fumonisin induced diseases. Inhibition of ceramide synthase leads to an increase in free sphingoid bases in animals (Riley et al., 1996) and plants (Abbas et al., 1994). It has also been shown that inhibition of ceramide synthase will cause an increase in sphingoid base 1-phosphates in animals (Piva et al., 2005; Riley et al., 2005), but this has not been experimentally demonstrated in plants. Elevation of free sphingoid bases in animal cells has been shown to be the cause of increased cell death and inhibition of cell proliferation, whereas, fumonisin inhibition of ceramide biosynthesis has been shown to protect cells from cell death mediated by increased intracellular ceramide (Riley et al., 2001). Conversely, sphingoid base 1-phosphates in animal cells typically induce increased cell survival (Merrill et al., 2001; Riley et al., 2001) and phosphorylated ceramides appear to be protective in both animals and plants (Sugiura et al., 2002; Liang et al., 2003). In plants, elevated free sphingoid bases have been shown to influence reactions which cause morphological changes and promote plant necrosis (Tanaka et al., 1993; Spassieva et al., 2002). Inhibition of ceramide biosynthesis also leads to a decrease in the biosynthesis of more complex sphingolipids.

More complex sphingolipids, which are important structural constituents of cell membranes, also have been shown to be important in regulating many functions in mammalian cells, such as protein trafficking and sorting, modulation of cell-cell communication, adhesion, and cell morphology (Merrill et al., 1997). The two most common types of complex sphingolipids in plants are glucosylceramide and inositolphosphorylceramide (Lynch and Dunn, 2004). Glucosylceramide production has been implicated in the induction of pathogenesis-

related proteins in rice (Umemura et al., 2003) and the ability to tolerate environmental stress (Lynch and Dunn, 2004) such as drought and freezing. There is also circumstantial evidence suggesting that glycophosphosphingolipids (Worrall et al., 2003) and glycosylphosphatidylinositol (GPI)-anchored proteins (Fischer et al., 2004), which are found in lipid rafts whose function require sphingolipids and is disrupted by fumonisins (Merrill et al., 2001), are involved in cell signaling in plants (Borner et al., 2005). Thus, proper sphingolipid metabolism is critical (Merrill, 2002) because sphingoid bases, sphingoid base metabolites, ceramide and more complex sphingolipids are known to play important roles in cellular processes that modulate growth, differentiation and apoptosis (Merrill et. al., 1997; Vesper et al., 1999; Brown and London, 2000) and fumonisin induced disruption of these processes can be detrimental to cells.

The purpose of this study was to determine the role of fumonisin inhibition of ceramide synthase in *F. verticillioides'* effect on root development in maize seedling disease using changes in free sphingoid bases and sphingoid base 1-phosphates as markers for fumonisin induced disruption of sphingolipid metabolism. The specific objectives were to determine: i) if *F. verticillioides* can produce fumonisins in the rhizosphere, ii) if the fumonisins that are produced in the rhizosphere are biologically available, iii) if soil bound fumonisins are toxic to maize seedlings, and iv) the time- and dose- dependent relationship between fumonisins in soil and effects on root development, expressed as growth, and disruption of sphingolipid metabolism.

RESULTS

Fumonisin Producing *F. verticillioides* Strain is Pathogenic in Maize Seedling Virulence Assay and Disrupts Sphingolipid Metabolism

Pathogenic and non-pathogenic strains of *F. verticillioides* were tested for their ability to produce fumonisins on maize. The non-pathogenic strains NRRL25059 and JFL-A04516 of *F. verticillioides* did not produce significant amounts of fumonisins when grown on maize culture for 14 days. Analysis of maize kernels from 14 day cultures of pathogenic strains MRC826, AEG 3-A3-5, AEG 3-A3-6 and JFL-A00999 produced 319, 98, 102, and 1000 μg fumonisin B_1 g^{-1}, respectively. After 18 h, the MRC826 inoculum (10^7 total colony forming units) used to treat the 'Silver Queen' seeds prior to planting contained low but detectable levels of fumonisins (7 ng total), however, when planted, the MRC826 inoculated seeds contained no detectable fumonisins (< 0.004 μg g^{-1}).

In the virulence assay, similar to what has been reported previously (Glenn et al., 2002 and 2004), there were no visual symptoms of disease in the developing seedlings grown from control seeds or those inoculated with the non-pathogenic *F. verticillioides* strain NRRL25059. Conversely, clear evidence of seedling disease was apparent as early as 7 days and persisted for at least 21 days (Figure 4.2) after planting seeds inoculated with the pathogenic *F. verticillioides* strain MRC826. Symptoms of disease included stunting, increased necrotic leaf lesions, increased abnormal leaf development and reduced root development (Figure 4.2) compared to the control group and the group inoculated with NRRL25059. Fumonisin B_1 was detected in extracts of the potting soil in which maize-seedlings were grown for 9 days from seeds inoculated with MRC826 (Figure 4.3A); fumonisin B_2 and fumonisin B_3 were also detected (data not shown). There was no detectable fumonisin B_1 (< 0.004 μg g^{-1} dry soil) in the soils from

control seeds or the seeds inoculated with NRRL25059 (Figure 4.3A) after 9 days. At 9 days, the only group showing elevated levels of free sphingoid bases was the MRC826 group (Figure 4.3B). There was a statistically significant increase in free sphinganine in the MRC826-treated group as compared to the control or NRRL25059-treated groups. There was also a significant increase in the level of free phytosphingosine in the MRC826-treated group as compared to the NRRL25059-treated group (Figure 4.3B); however, the increase seen in the MRC826-treated group was not statistically significant compared to the control group ($p>0.05$). Analysis of sphingoid base 1-phosphates and fumonisins in roots were not done in the 9 day assay.

In a follow-up experiment, conducted similar to that described above, seedlings were grown from MRC826 inoculated seeds for 21 days and then the soil was analyzed for fumonisin B_1 content and the roots were analyzed for fumonisin B_1, free sphingoid bases and sphingoid base 1-phosphates. Once again symptoms of disease included stunting, necrotic leaf lesions, abnormal leaf development and reduced root development (Figure 4.2). The levels of fumonisin B_1 in the soil at 21 days (Figure 4.4A) were similar to those at 9 days (Figure 4.3A) and although the average fumonisin B_1 level in the soil was higher in the 9 day study compared to the 21 day study, the difference was not statistically significant ($p>0.05$).

The root tissues of the plants harvested at 21 days were analyzed for "loosely bound" and "tightly bound" fumonisin B_1. Tightly bound was defined as that fumonisin B_1 which was not extracted from the water-washed freeze dried roots with distilled water but was extracted in acetonitrile:5% formic acid in water (1:1, v/v) (see methods for additional details). Tightly bound fumonisins (B_1, B_2 and B_3) were detected in the roots of seedlings grown from MRC826 inoculated seeds (Figure 4.4B).

Free sphinganine and free phytosphingosine were significantly elevated, compared to controls, in the roots from seedlings grown for 21 days from seeds inoculated with MRC826 (Figure 4.5A). The increase in free sphinganine at 9 and 21 days (Figure 4.3B vs. 4.5A) in the roots of the MRC826-treated groups was not significantly different ($p > 0.05$) but the increase in free phytosphingosine at 21 days was significantly greater compared to the increase after 9 days (425 nmol g^{-1} vs 146 nmol g^{-1}, $p \leq 0.05$). Sphinganine 1-phosphate and phytosphingosine 1-phosphate were significantly elevated in the roots of the MRC826 treatment group compared to the control group at 21 days (Figure 4.5B). The percent increase in sphinganine 1-phosphate and phytosphingosine 1-phosphate compared to controls was similar (900 to 1100%) and was much greater than the percent increase in free sphinganine and free phytosphingosine (250 to 300%) (Figure 4.5A and 4.5B). While the roots of two of the five control-treated replicates at 21 days were contaminated with low levels of fumonisin B_1, there was no statistical difference ($p>0.05$) between the levels of free sphingoid bases or sphingoid base 1-phosphates in the roots from the control pots that had low levels of fumonisin B_1 and those in which fumonisin B_1 was not detected.

Fumonisin B_1 Produces Qualitatively Similar Pathology in Watering Assay

In the watering assay, germination and seedling survival was > 93% for all treatment groups harvested at 8 days or 21 days (Table 4.1). Stunting and leaf lesions were not seen in the 8 day study but were seen in the plants harvested on day 21 (Table 4.1) and developed between days 13 and 21. Leaf lesions were not observed in the control or 1 μg mL^{-1} fumonisin B_1 treatment groups on day 21 but were seen in all other groups (Table 4.1) and the total incidence of necrotic leaf lesions in the 5, 10 and 20 μg mL^{-1} fumonisin B_1 treatment groups were 3/28, 4/28 and 10/30 (number of plants exhibiting lesions over total plants harvested), respectively.

Abnormal leaf development was only observed in the 20 μg mL^{-1} fumonisin B_1 group harvested on day 21 and was much less severe compared to that seen in seedlings from the MRC826 treatment group in the virulence assay. The mean height of the seedlings on day 21 (8-10 plants /pot; n=3 pots) was decreased in the 5, 10 and 20 μg mL^{-1} fumonisin B_1 groups compared to the control group but was only statistically significant in the 20 μg mL^{-1} fumonisin B_1 group ($p \leq 0.05$). A statistically significant decrease in root mass was observed after 8 days in the 20 μg mL^{-1} fumonisin B_1 treatment group (Figure 4.6B). At 21 days there was a clear dose-dependent decrease in root mass (4.6A). The decrease was statistically significant in the 5, 10 and 20 μg mL^{-1} fumonisin B_1 treatment groups compared to the control group (Figure 4.6C). Nonetheless, total root mass was significantly ($p \leq 0.05$) increased in all groups at 21 days compared to 8 days (Figure 4.6B and 4.6C) indicating that although inhibited, root growth continued after 8 days albeit at a reduced rate. In order to better visualize the effects of fumonisin B_1 on root development, sterilized 'Silver Queen' seed were germinated and grown on culture media in the presence of 0 or 10 μM (14 μg mL^{-1}) fumonisin B_1 for 14 days. Compared to the control, the root system of seedlings germinated and grown on medium containing 10 μM fumonisin B_1 exhibited reduced growth of the primary, seminal and lateral roots (Figure 4.7). While the initiation of lateral roots was apparent, lateral root elongation was impaired and there was a complete lack of root hair development in all roots that were in contact with the medium containing fumonisin. In addition to the effects on the development of the root system, there was reduced above-ground growth although it was much less marked compared to the reduced growth of the root system.

Low Levels of Fumonisin B_1 are Tightly Associated with Seedling Roots

After harvest, the soils from each treatment were collected and analyzed for the presence of fumonisins. Fumonisin B_1 was detected in each treatment and at both time points at similar levels and there was a dose-dependent increase in the levels detected (Figure 4.8A and 4.8B). With the exception of the soils watered with the 1 μg fumonisin B_1 mL^{-1} solution, the levels of fumonisin B_1 detected in the soils at 8 days and 21 days were not significantly different ($p > 0.05$). No fumonisin B_1 was detected in the control soils at either time point and neither fumonisin B_2 nor fumonisin B_3 was detected in any of the soils watered with fumonisin B_1. The roots of the plants harvested at both time points were analyzed for "loosely bound" and "tightly bound" fumonisin B_1 (see methods for details). Fumonisin B_1 was detected in the acetonitrile: 5% formic acid in water extracts of treated roots at both time points, and there was a dose-dependent increase in the levels detected (Figure 4.9A and 4.9B). The levels of fumonisin B_1 tightly associated with the roots at 21 days were significantly elevated, relative to the 8 day values, in the plants watered with 5, 10 and 20 μg mL^{-1} fumonisin B_1 ($p \leq 0.05$).

Decrease in Root Mass and Degree of Disruption of Sphingolipid Metabolism are Correlated

Watering with pure fumonisin B_1 (96%) resulted in inhibition of ceramide synthase as indicated by the accumulation of sphingoid bases in the roots. There was a qualitatively similar increase in the levels of both total phytosphingosine (Figure 4.10) and total sphinganine (Figure 4.11) in roots on both days 8 and 21. The absolute amount of total accumulated phytosphingosine was greater than total sphinganine (Figures. 4.10 and 4.11) and the levels of total accumulated phytosphingosine and total sphinganine were clearly dose-dependent on day 21 (Figures 4.10B and 4.11B) but the dose dependence was less clear in the roots sampled on

day 8 (Figures 10A and 11A), similarly, the dose dependent effect of fumonisin B_1 on root weights was most evident at 21 days (Figure 4.6C). Statistical comparison of the change in root weight with the other variables at 21 days showed that the decrease in root mass was significantly inversely correlated with the increase in fumonisin B_1 content, free sphingoid bases and sphingoid base 1-phosphates (Table 4.2). At 8 days the correlation was not statistically significant for the free sphingoid bases but was significant for all other variables (Table 4.2). The correlation was much better when comparing the 21 day root weights with the other variables at 8 days (Table 4.2), indicating that there was a lag-time between disruption of sphingolipid metabolism and decreased root growth at the lower doses.

With the exception of free phytosphingosine in the 20 µg fumonisin B_1 mL^{-1} group (Figure 4.12A), the differences in the levels of free phytosphingosine and free sphinganine on days 8 and 21 in the roots from the fumonisin B_1 watered seedlings were not statistically significant (Figures 4.12A and 4.13A). However, there was a statistically significant reduction in the levels of both phytosphingosine 1-phosphate and sphinganine 1-phosphate on day 21 compare to the values at day 8 (Figures 4.12B and 4.13B). Neither phytosphingosine 1-phosphate nor sphinganine 1-phosphate was detected in the roots of the control seedlings on days 8 or 21 (Figures 4.12B and 4.13B).

In order to determine if long chain bases and long chain base 1-phosphates other than phytosphingosine (t18:0), sphinganine (d18:0), phytosphingosine 1-phosphate and sphinganine 1-phosphate were elevated by fumonisin B_1–induced ceramide synthase inhibition, root extracts were analyzed using data-dependent scanning of the two most intense ions (150-600 m/z). There was no evidence that any other long chain base or their 1-phosphates were present in either the

extracts of fumonisin-treated or control-treated seedling roots. In addition, the precursor for sphinganine, 3-keto-sphinganine, was not detected (Figure 4.14).

DISCUSSION

In 1992 there was little evidence supporting a role of fumonisin in *F. verticillioides* induced diseases in maize (Gilchrist et al., 1992). In 1994 fumonisins were shown to cause dose-dependent reduction in root and shoot mass in maize seedlings grown on water agar (Lamprecht et al., 1994). Desjardins et al. (1995) compared the ability of fungal strains to produce fumonisin and virulence and concluded that fumonisin played a role in virulence but was not necessary or sufficient for virulence on maize seedlings. Glenn et al. (2002) used a seedling blight virulence assay in the sweet maize cultivar 'Polar Vee' and found that the fumonisin producing strain MRC826 caused reduced survival and stunting. However, there was no effect on mean survival or mean height of seedlings grown from seeds inoculated with the *F. verticillioides* strain NRRL25059, which is fully capable of establishing systemic infection (Glenn et al., 2002), and we now know that the NRRL25059 strain does not produce fumonisins either in maize culture or soil. In the present study, maize seed of the sweet maize cultivar 'Silver Queen' were inoculated with a pathogenic or non-pathogenic strain of *F. verticillioides* and grown in sterile potting mix. Disease symptoms were not seen in seedlings grown from seeds inoculated with the non-pathogenic strain NRRL25059 or JFL-A04516 nor were fumonisins detected in the soil. Seedlings developed disease symptoms only when grown from seeds inoculated with the pathogenic strain (MRC826) and at 9 days fumonisin was only detected in the soil from the

seedlings grown from seeds inoculated with the MRC826 strain. Other strains (AEG3-A3-5, AEG3-A3-6 and JFL-A00999) that were pathogenic in the virulence assay also were fumonisin producers. In the 21 day virulence assay neither inoculated seeds nor soil contained any detectable fumonisins at the time of planting but at 21 days the soil and roots from the MRC826 pots contained a total of 348 μg of fumonisin B_1; indicating that fumonisin is produced in the soil environment during the process of seedling development and growth.

Fumonisin induced disruption of sphingolipid metabolism has been suggested to contribute to *F. verticillioides* effects on maize seedlings (Lamprecht et al., 1994; Desjardins et al., 1995; Riley et al., 1996). In the virulence assay, accumulation of free sphingoid bases and sphingoid base 1-phosphates in roots indicate that the fumonisins produced by the pathogenic strain MRC826 entered, or was produced in, the cells of the roots in sufficient quantities to inhibit the acyl CoA dependent ceramide synthase. These findings show that the ability of the fungus to produce fumonisin is required for development of *F. verticillioides* maize seedling disease symptoms, and are consistent with the hypothesis that fumonisin is a pathogenicity factor necessary for *F. verticillioides* maize seedling disease. However, the virulence assay alone did not determine definitively if the disease symptoms observed were caused by fumonisin, or by an interaction between the fungus, fumonisin and possibly other unidentified phytotoxins within the rhizosphere where the fungal-root-soil interaction is occurring.

To further examine the role of fumonisin as a pathogenicity factor in maize seedling disease caused by *F. verticillioides*, sterilized maize seeds were planted and watered with fumonisin B_1 (96% pure), applied as an aqueous solution. The seedlings developed pathology that was qualitatively similar to that seen in seedlings grown from seeds inoculated with the pathogenic, fumonisin producing *F. verticillioides* strain MRC826. The extent and severity of

stunting and leaf lesions in the watering assay were less marked as compared to those observed in the virulence assay. For example, symptoms of disease (stunting and leaf lesions) were first observed on day 7 in the virulence assay, whereas, in the watering assay symptoms of disease developed between days 13 and 21. However, in both the virulence assay and the watering assay there was clear evidence of reduced root development and disruption of sphingolipid metabolism and in the watering assay there was a close correlation between the elevation in sphingoid bases and sphingoid base 1-phosphates and effects on root growth.

In the virulence assay, the mean fumonisin B_1 concentration in the soil at 21 days (Figure 4.4A) was 0.6 $\mu g\ g^{-1}$ soil and was clearly associated with adverse effects in the seedlings (Figure 4.2). Fumonisin B_1 defined as tightly bound in roots in the virulence assay at 21 days (Figure 4.4B) was 0.45 $\mu g\ g^{-1}$ root tissue and in the group watered with 5 $\mu g\ mL^{-1}$ and 10 $\mu g\ mL^{-1}$, respectively (Figure 4.9B), it was 0.36 $\mu g\ g^{-1}$ and 0.60 $\mu g\ g^{-1}$ roots. Thus, the level of fumonisin B_1 in root tissue that caused adverse effects on root development was comparable in both the virulence assay and the watering assay. The no observable adverse effect (reduced root mass on day 21 compared to controls) level of tightly bound fumonisin B_1 was 0.08 $\mu g\ g^{-1}$ root tissue (Figure 4.9); however, these levels did cause clear evidence of disruption of sphingolipid biosynthesis (Figures 4.10 and 4.11) suggesting that there is a threshold for adverse effects induced by disrupted sphingolipid metabolism.

Genetic susceptibility to stem-canker disease in tomato is closely correlated with sensitivity to disruption of sphingolipid metabolism (Abbas et al., 1994). In tomato plants resistant (Asc/Asc) to the toxic effects of fumonisin B_1 and AAL-toxin, free sphinganine and phytosphingosine did not accumulate to high levels compared to the susceptible (asc/asc) variety (Abbas et al., 1994). The resistance in tomato plants was attributed to the *Asc-1* gene (Clouse

and Gilchrist, 1986) that is now known to be homologous to the yeast longevity assurance genes (*LAG1* and *LAC1*) (Brandwagt et al., 2000 and 2002) which confer resistance to fumonisin inhibition of the CoA-dependent ceramide synthase in yeast (Lynch and Dunn, 2004); the precise mechanism is unclear. Recently, it has been shown that the endoplasmic reticulum proteins Lag1p, Lac1p and Lip1p are subunits of the acyl-CoA dependent ceramide synthase in yeast (Vallee and Riezman, 2005) which are less sensitive to the toxic effects of ceramide synthase inhibitors when compared to plants (Abbas et al., 1994; Wu et al., 1995). The K_i for fumonisin B_1 determined with the purified yeast ceramide synthase was 400 μM (Vallee and Reizman, 2005). In maize roots, marked elevation in free sphingoid bases and sphingoid base 1-phosphates were seen at 1 μg fumonisin B_1 mL^{-1} in the watering assay. The no observable effect level for fumonisin-induced accumulation of sphingoid base 1-phosphates is clearly less than 0.05 ± 0.02 μg tightly bound fumonisin B_1 g^{-1} roots since significant elevation in sphingoid bases and sphingoid base 1-phosphates were seen at 8 days in roots from plants watered with the 1 μg fumonisin B_1 mL^{-1} solutions. Assuming the water content of roots is 80%, the calculated intracellular molar concentration of fumonisin B_1 necessary to disrupt sphingolipid metabolism (significant elevation in free sphingoid bases) in roots is less than 0.014 μM. This is reasonably close to the IC_{50} values reported in tomato microsomes (Gilchrist et al., 1994) consistent with the hypothesis that the ceramide synthase in intact maize roots is of similar sensitivity to that in the susceptible tomato (asc/asc).

As reported previously (Riley et al., 1996) and shown in this study, the levels of free phytosphingosine in control and fumonisin-exposed maize roots were always much higher than free sphinganine (Figures 4.3B, 4.5A, 4.12A and 4.13A). Wright et al. (2003) showed unequivocally in maize shoots, free phytosphingosine is formed by the hydroxylation of free

sphinganine (Figure 4.1). In both maize roots and shoots free phytosphingosine levels were always greater than free sphinganine after fumonisin exposure which is not the case in tomato leaf discs, fronds and roots of duckweed, or leaf discs of black nightshade (Abbas et al., 1994 and 1998), suggesting that the kinetics of metabolism of free sphingoid bases can vary widely between species and possibly tissues.

The ability to reverse the biochemical consequences of ceramide synthase inhibition in roots of 'Silver Queen' maize seedlings is clearly minimal since the level of free sphingoid bases remained elevated over the entire 21 days of exposure. Nonetheless, the fact that on day 21 both sphinganine 1-phosphate and phytosphingosine 1-phosphate were both reduced by approximately 50% to 75% at every dose level compared to their levels on day 8 (while free sphinganine and free phytosphingosine were not) (Figures 4.12 and 4.13) suggests that prolonged fumonisin exposure resulted in a change in the relative activity of the enzymes responsible for metabolizing free sphingoid bases. The fact that sphingoid base 1-phosphates accumulate in roots suggests that the activity of serine palmitoyltransferase and sphingoid base kinase exceed the ability of sphingoid base 1-phosphate lyase and phosphohydrolases to degrade the sphingoid base 1-phosphates. The reduction in accumulated sphingoid base 1-phosphates on day 21 could indicate that the sphingoid base 1-phosphate lyase(s) or phosphohydrolase(s) is (are) induced after prolonged exposure and that this results in more efficient degradation of the products of the sphingoid base kinase(s). The fact that both phytosphingosine 1-phosphate and sphinganine 1-phosphate are both reduced in a similar proportion (50%-75%); suggests that either the same enzymes degraded both compounds equally effectively, or their expression is tightly coupled.

The data presented in this study are consistent with the hypothesis that the proximate cause of the adverse effects seen in the virulence and watering assays is the inhibition of

ceramide synthase. The potential downstream cellular consequences of inhibition are too numerous to describe in detail but *in vitro* and *in vivo* studies in animals indicate that the most likely biochemical changes that lead to altered biological responses are i) increased apoptosis and inhibited cell growth induced by elevation in free sphingoid bases (Riley et al., 2001), ii) increased proliferation and disrupted function of S1P receptor (also known as G-protein coupled receptors and EDG receptors) mediated responses induced by decreased levels of ceramide and increased levels of sphingoid base 1-phosphates (Merrill et al., 2001), and iii) altered function of glycosylphosphatidylinositol-anchored proteins via altered lipid raft function (Stevens and Tang, 1997; Gelineau van Waes et al., 2005). It is quite likely that all of the downstream biochemical changes, collectively referred to as disruption of sphingolipid metabolism, occur concurrently at high dosages of fumonisin. In the following paragraphs we will summarize the current evidence supporting the role of the three most likely biochemical changes listed above as contributing factors in the observed adverse effects of fumonisins in maize seedlings.

Free sphingoid bases have been shown to modulate the activity of numerous enzymes that are key components of signaling pathways regulating cell growth, death and differentiation (Merrill et al., 2001; Riley et al., 2001). Very little is known about the role of sphingoid bases in regulating physiological processes in plants, however, there is some evidence that the biological activity of sphingoid bases seen in animal cells is also seen in plants. Free sphingoid bases added to cultures of duckweed has been shown to cause electrolyte leakage and chlorophyll loss (Tanaka et al., 1993) and myriocin, an inhibitor of serine palmitoyltransferase, the first and rate limiting step in *de novo* sphingolipid biosynthesis, has been shown to reduce both the accumulation of free sphingoid bases and the necrotic lesions observed in tomato leaf discs treated with fumonisin B_1 (Spassieva et al., 2002). Free sphingoid bases in animal cells have

been shown to modulate key transport ATPases (Oishi et al., 1990) and phospholipid lipid signaling pathways that are known to modulate cell growth. In plants, fumonisin B_1 and free sphingoid bases can inhibit the plasma membrane H^+-ATPase in vesicles prepared from maize embryos (Gutierrez-Najera et al., 2005) and lipid pathways shown to be modulated by free sphingoid bases in animal cells are also known to modulate cell growth in plants. For example phosphatidic acid phosphatase and phospholipase D which have been shown to play important roles in polar root hair initiation and polar tip growth (Fischer et al., 2004).

When ceramide biosynthesis is blocked or serine palmitoyltransferase activity increases rapidly, elimination of sphingoid bases is critical for maintaining intracellular concentrations of sphingoid bases and ceramide at non-toxic levels. The most likely path for elimination of sphingoid bases is via sphingosine kinase with the production of sphingoid base 1-phosphates. The activity of sphingosine kinase in maize shoot microsomes has been characterized and shown to phosphorylate sphinganine and sphingosine but not phytosphingosine (Crowther et al., 1997). Clearly, in this study, the maize roots are capable of phosphorylating phytosphingosine. The sphingosine kinase from *Arabidopsis thaliana* has recently been cloned and characterized (Coursol et al., 2005; Imai and Nishiura, 2005) and has been shown to be primarily membrane associated (Coursol et al., 2005) and to phosphorylate sphingosine, 4, 8-sphingadienine (d18:2), phytosphingosine and sphinganine. The maize root sphingosine kinase would appear to be more like the sphingosine kinase isolated from *Arabidopsis* since it can presumably utilize both sphinganine and phytosphingosine.

In mammalian cells, yeast and the slime mold (*Dictyostelium discoideum*) the balance between intracellular ceramide and sphingosine 1-phosphate (the sphingolipid rheostat) determines whether a cell dies or survives (Spiegel and Milstein, 2002; Min et al., 2005).

Ceramide, which is a cell death mediator, can be generated either *de novo* by increased activity of serine palmitoyltransferase or by breakdown of membrane sphingolipids (Speigel and Milstein, 2002). Sphingosine 1-phosphate, but not sphinganine 1-phosphate, has been hypothesized to be an intracellular second messenger in pathways regulating calcium homeostasis and activation of pathways involved in promoting cell survival (Spiegel and Milstien, 2000; Hla, 2003). Ceramide-induced apoptotic-like cell death has been demonstrated in *Arabidopsis* during pathogen attack and the ability to produce ceramide 1-phosphate is protective (Liang et al., 2003). The source of the increased ceramide during pathogen attack is unknown; however, Birch et al. (1999) showed that in potato (*Solanum tuberosum*) pathogen infection induced the serine palmitoyltransferase gene (Birch et al., 1999). If pathogen infection in maize root had a similar effect, then fumonisin would inhibit ceramide biosynthesis and stimulate biosynthesis of sphingoid base 1-phosphates which would be protective. Conversely, if ceramide was produced via hydrolysis of complex sphingolipids fumonisin would not be protective.

In addition to their possible role as intracellular second messengers, in mammals and yeast sphingosine 1-phosphate and sphinganine 1-phosphate are also known to act as ligands for a family of extracellular G protein coupled receptors known as S1P receptors (Spiegel and Milstien, 2000). There is increasing evidence for sphingoid base 1-phosphates as signaling molecules in plants (Worrall et al., 2003). For example, in *Arabidopsis*, sphingosine 1-phosphate and phytosphingosine 1-phosphate (but not sphinganine 1-phosphate) have been shown to regulate stomatal aperture by causing an increase in intracellular calcium and a decrease in potassium, resulting in a decrease in guard cell turgor pressure (Ng et al., 2001; Coursal et al., 2005). The fact that sphinganine 1-phosphate was not active indicates that the C4-double bond

or hydroxyl group in sphingosine 1-phosphate and phytosphingosine 1-phosphate, respectively, are critical for their activity. Regulation of stomatal aperture by phytosphingosine 1-phosphate is impaired in *Arabidopsis* plants where the sole putative S1P-like receptor, GPA1, is non-functional (Coursol et al., 2005), a finding that is consistent with the hypothesis that S1P receptors are important in plants. Additional support for the possibility that phytosphingosine 1-phosphate could play a role in G-protein mediated processes in plants (Coursol et al., 2005) is that the mammalian $S1P_4$ receptor has been shown to have high affinity for phytosphingosine 1-phosphate (Candelore et al., 2002).

There is no evidence for S1P receptors in maize roots; however, if they are present then the persistent elevation in sphingoid base 1-phosphates would most likely have an adverse effect on their normal physiological function. There is no data available as to the cytotoxicity and biological activity of sphinganine 1-phosphate or the effects of sustained elevation of phytosphingosine 1-phosphate, in either plants or animals; however accumulation of phosphorylated long chain bases in yeast causes growth inhibition (Kim et al., 2000). Thus, fumonisin, used in conjunction with inhibitors of other enzymes in the sphingolipid pathways, could become an important research tool for revealing the possible roles of these sphingoid base 1-phosphates in regulating physiological and disease processes in maize roots.

One of the consequences of fumonisin disruption of sphingolipid metabolism *in vitro* and *in vivo* is inhibition of transport processes and other functions mediated by GPI-anchored proteins in cell membranes (Stevens and Tang, 1997; Gelineau van Waes et al., 2005). GPI-anchored proteins in the plasma membrane reside in membrane micro-domains commonly referred to as lipid rafts. Lipid rafts are enriched in cholesterol and sphingolipids and can affect the function of proteins found in them (Brown and London, 2000). AAL-toxin has been shown

to inhibit the biosynthesis of complex sphingolipids (Spassieva et al., 2002) and exogenous ceramide counteracts the cell death induced by AAL-toxin (Brandwagt et al., 2000) in sensitive tomato (asc/asc) leaf discs. Thus, it is reasonable to expect that sphingolipid-dependent lipid raft functions would be inhibited in plants sensitive to fumonisin inhibition of ceramide synthase (Brandwagt et al., 2000 and 2002). Recent studies show that detergent-resistant membranes in plants, consistent with known lipid rafts in other eukaryotes, are enriched in sphingolipids and sterols (Borner et al., 2005). There is growing evidence that lipid raft function and GPI-anchored proteins play important roles in plant cell polarity essential for the proper directional expansion of root epidermis (Fischer et al., 2004). In addition, there is evidence that bulk sterol composition in plants contributes to cell and tissue polarity and is essential for lipid raft dependent processes (Fischer et al., 2004). Increased levels of sphingoid base 1-phosphate degradation products have been shown to inhibit sterol biosynthesis when membrane phosphatidylethanolamine levels are high (Dobrosotskaya et al., 2002). Not surprisingly, another consequence of fumonisin inhibition of ceramide synthase is the increased biosynthesis of phosphatidylethanolamine (Merrill et al., 2001) utilizing the increased ethanolamine phosphate that is a product of the sphingoid base 1-phosphate lyase (Figure 4.1). Whether this occurs in plants is unknown.

It is highly likely that the elevation in free sphingoid bases, sphingoid base 1-phosphates, depletion of more complex sphingolipids and altered phospholipid biosynthesis could have independent but additive adverse effects on root growth and development. In addition the metabolism of sphinganine to phytosphingosine consumes NADPH/NADH and the phosphorylation to create the 1-phosphates requires ATP. Thus, at the very least, persistent fumonisin inhibition of ceramide biosynthesis results in generation of toxic free sphingoid bases,

elevation in sphingoid base 1-phosphates, depletion of critical membrane glycosphingolipids, altered function of GPI anchored proteins and lipid rafts, modulation of phospholipid signaling pathways and is an energy drain for affected cells.

The results we have described in 'Silver Queen' are also seen in other maize varieties. In preliminary studies (data not shown) using field maize cultivars (Pioneer 3167 and 3156) we have found that seed inoculated with MRC826 result in assays qualitatively similar effects on root growth, fumonisin production, accumulation in roots, and disruption of sphingolipid metabolism. However, the extent and severity of the effects are less and the field maize cultivars did not develop leaf lesions nor did they show any symptoms of abnormal leaf development. These results suggest that the effects of fumonisin inhibition of ceramide biosynthesis seen in sweet maize varieties are also seen in field maize cultivars, however, with differences in susceptibility to the toxic effects. This supports the findings of a recent study showing that sensitivity of maize varieties to fumonisin B_1 is likely to be an ancestral trait in *Z. mays* and that insensitivity is a rare but inheritable trait in maize (Desjardins et al., 2005).

Conclusion

In conclusion, the correlation between fungal pathogenicity, fumonisin B_1 production, ceramide synthase inhibition, the production of qualitatively similar pathology by both pathogenic strains of *F. verticillioides* and direct exposure to fumonisin B_1, and the known biological activity of sphingolipids as regulators of cell function in animals, yeast and plants strongly support the hypothesis that fumonisin is necessary and sufficient to produce *F. verticillioides* maize seedling disease and that the effects on root development are a consequence of fumonisin-induced disruption of sphingolipid metabolism. In addition, the data offer additional evidence for the importance of sphingolipids in the physiological well being of plants

and indicate the need for developing a better understanding of the role of sphingolipids and their metabolism in physiological processes and disease resistance in plants. Future studies will determine whether or not fumonisins are translocated in maize seedlings and if disruption of sphingolipid metabolism occurs in affected aerial tissues.

MATERIALS AND METHODS

Virulence Assay

Two strains of *F. verticillioides*, designated NRRL25059 (Northern Regional Research Laboratory (=NCAUR), USDA ARS, Peoria, IL) and MRC826 (Medical Research Council, Tygerberg, South Africa) were used in this study. NRRL25059 lacked virulence in a maize seedling blight assay (Glenn et al., 2002), whereas, MRC826 was highly virulent (Glenn et al., 2002). MRC826 produces several fumonisins including fumonisin B_1, fumonisin B_2 and fumonisin B_3 (Rheeder et al., 2002). The conidia were frozen at -80 in 15% glycerol until inoculated on potato dextrose agar and incubated at 27^{o}C in the dark to initiate experimental cultures. The inoculum (conidia and mycelia) was obtained by flooding the agar surface with 10 mL sterile water and diluting the concentrated inoculum to 10^6 colony forming units mL^{-1}. In order to determine the ability of NRRL25059, MRC826 and other strains to produce fumonisins on maize kernels, twice autoclaved cracked maize (5 g hydrated with 45% water in a 20 mL glass vial) was inoculated with the conidial suspension (~2.5 x 10^7 conidia). Two vials of maize were inoculated for each strain. After 14 days incubation at 25ºC in the dark, 10 mL of acetonitrile:water (1:1) were added to each vial, which was shaken and allowed to stand and then analyzed for fumonisin as described below. Strains of *F. verticillioides* other than MRC826 and

NRRL25059 were also tested (AEG3-A3-5, AEG3-A3-6, JFL-A00999 and JFL A04516). AEG = Anthony E. Glenn, Russell Research Center, USDA, ARS, Athens, GA, USA; JFL = John F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan, KS, USA (Glenn et al, 2001 and 2002). The MRC826 inoculum used in the virulence assay were also analyzed for fumonisins.

Maize seed ('Silver Queen', Gurney's Seed & Nursery Co., Yankton, SD, USA) were surface-disinfected for 10 min in 100% bleach (5.25% hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water. The seed were then heat shocked by placing them in a 60^{o}C water bath for 5 min for internal sterilization (Bacon et al., 1994). Inoculations were performed by placing sterilized seeds in a Petri dish (100 mm) and flooding the seed with 10 mL of the conidial suspension. Sterile water was used for the control group. The seed were incubated overnight at 27^{o}C. Samples of the seeds before and after inoculation were analyzed for fumonisins. Three or five replicates of 10 seeds each were planted in sterile 4 inch plastic azalea pots (Hummert International, Earth City, MO, USA) containing twice-autoclaved commercial potting mix (45% sphagnum peat, Conrad Fafard Inc., Agawam, MA, USA). Pots were watered from below for the first two days and then watered as needed from above throughout the duration of the assay. Assays were performed under aseptic conditions in a plant growth chamber at 26^{o}C under a 14 h light (cool-white, high-output fluorescent tubes at an average of 254 µmol m^{-2} s^{-1}) and a 10 h dark regime at 22^{o}C.

Disease symptoms were visually assessed for indications of seedling blight (Glenn et al., 2002) from 7 to 21 days after planting. The exposure time was chosen based on other studies with the sweet maize cultivars 'Polar Vee' (Glenn et al., 2002) and 'Silver Queen' (Glenn et al., 2004). These earlier studies found that seedlings grown from seeds inoculated with MRC826

showed symptoms of stunting compared to seedlings grown from seeds inoculated with NRRL25059 or control-treated seeds, and MRC826 treatment also resulted in necrotic leaf lesions and abnormal leaf development which first appear about 7 days after planting and become progressively worse for up to 21 days after planting (the longest time over which inoculated seedlings were observed). The earlier studies (Glenn et al., 2002) also showed that seedlings grown from seeds inoculated with MRC826 or NRRL25059 had endophytic infection with the inoculated strains in all tested aerial tissues (leaf, whorl and node). Control tissues from surface sterilized kernels showed no infection with fungi. In the present study, two separate virulence assays were conducted, the first lasted a total of 9 days and the second lasted 21 days.

Fumonisin Preparation and Fumonisin Watering Assays

The fumonisin B_1 test material and analytical standard were prepared by the method of Meredith et al., (1996) and the purity (>96%) was determined by the procedure of Plattner and Branham, (1994). Impurities were primarily other fumonisins and fumonisin dimers. Fumonisin B_1 test material was dissolved in 1L of sterile distilled water to make concentrations of 1 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1} and 20 μg mL^{-1} (1.4, 6.9, 13.9 and 27.7 μM, respectively), to be used for the watering assay described below.

Three replicates (=pots) of 10 sterilized seeds each were planted in twice-autoclaved commercial potting mix containing no detectable fumonisins. Replicates were watered with 100 mL of each designated concentration of fumonisin B_1 on days 2, 4, and 6 after planting. The plants were then watered with sterile water as needed until the plants were harvested 8 and 21 days after planting. Control groups were similarly treated but with sterile distilled water. Assays were performed in a plant growth chamber cycling as previously described. After harvest, disease symptoms were assessed. The number of surviving plants, height of surviving plants,

dry weight of roots, number of plants with leaf lesions, and number of plants with leaf developmental abnormalities was recorded.

In order to more easily observe effects on the young root system, sterilized 'Silver Queen' seed (n=3/treatment) were grown on Murashige and Skoog (1962) medium with 8g L^{-1} Caisson plant culture gelling agent in the presence of 0 or 10 μM (14 μg mL^{-1}) fumonisin B_1 at room temperature on the bench-top under fluorescent lights. The developing root system was observed daily and after 14 days, seedlings were removed from the tubes and photographed.

Extraction of Fumonisins from the Soils and Roots

After harvest, the soils from each replicate in both the virulence (days 9 and 21) and the fumonisin B_1 watering (days 8 and 21) assays were carefully separated from the root mass and collected and allowed to air-dry in a fume hood and then stored at -20°C. The intact roots from each pot and treatment were immersed in a water-bath to remove any remaining soil. The washed soil-free roots were then allowed to drain, and were blotted dry and placed in a -80°C freezer overnight. Because fumonisins are water soluble compounds (> 20 g L^{-1}), most if not all, fumonisins external to the roots should have been removed by the water washing. The plants were then freeze-dried, and the roots from each plant were separated from the leaves and stalks and placed into labeled zip-lock bags. Soils were also carefully inspected to remove all visible root materials. Fumonisins in the soil were extracted as described in Williams et al. (2003). Briefly, two grams of soil from each replicate was placed into 50 mL conical tubes and 25 mL of 1:1 (v/v) acetonitrile:5% formic acid in water was added to each tube. The tubes were placed on a rotary shaker over night, after which, samples were centrifuged for 20 min at 1100 rcf, and 1 mL samples were placed into polypropylene tubes and centrifuged at 16,000 rcf for 10 min. Samples of the extracts (1 mL) were transferred to polypropylene tubes containing 0.666 mL of

water and mixed to make a final concentration of 30:70 acetonitrile:3% formic acid in water. The samples were allowed to stand approximately 1 h then centrifuged at 16,000 rcf for 5 min and 1 mL was transferred to a sample vial that contained 10 µL of a 10 ng μL^{-1} phytosphingosine internal standard to make a final concentration of 0.1 ng μL^{-1} of sample. Samples containing the internal standard were analyzed by high performance liquid chromatography (HPLC) tandem mass spectrometry (LCMS) (described below), with values expressed as µg fumonisin B_1 $gram^{-1}$ soil (fumonisin B_2 and fumonisin B_3 were measured but only fumonisin B_1 levels are reported). The purpose of the phytosphingosine was to monitor instrument performance; it was not used for quantitation.

Prior to fumonisin extraction, the water washed, freeze-dried root tissues were carefully inspected, to remove any remaining soil. Clean root tissues were weighed to determine the effects of fumonisin on root growth. The root tissues were then placed in round bottom tubes and pulverized with a glass rod to a fine powder under liquid nitrogen and stored in a vacuum desiccator with anhydrous calcium sulfate. To measure the amount of fumonisins in the roots, samples consisting of 10 mg ground, homogenized root tissue from each replicate were weighed and placed into 2 mL polypropylene tubes. Distilled water (1mL) was added to each tube and the tubes were shaken for 2 hours. The tubes were then centrifuged for 10 min at 16,000 rcf and the supernatants were collected to be analyzed for water extractable fumonisin that was defined as “loosely” associated with the roots. More “tightly” bound fumonisin was extracted by adding distilled water, formic acid, and acetonitrile to the pelleted roots to make a final volume of 1 mL of acetonitrile:5% formic acid in water (1:1, v/v). This approach was developed for extracting fumonisins from sandy loam soils (Williams et al., 2003) where it was shown that after soils were sequentially extracted with water and then acetonitrile and water (1:1 v/v) the soils still

retained significant amounts of fumonisin which could only be released by extraction with acidic solutions (5% formic acid or acetonitrile:5% formic acid in water (1:1, v/v). The tubes were shaken for 2 hours and centrifuged for 10 min at 16,000 rcf and the supernatants were collected and placed into new polypropylene tubes. Distilled H_2O was added to the supernatant to make a final proportion of 30:70 acetonitrile:3% formic acid in water. Samples were analyzed for fumonisin by LCMS, with values expressed as µg fumonisin $gram^{-1}$ root tissue.

Extraction of Free Sphingoid Bases and Sphingoid Base 1-Phosphates

To determine the effect of treatments on sphingolipid biosynthesis, the ground root tissues were analyzed for free sphingoid bases, sphinganine and phytosphingosine, and their 1-phosphate metabolites, sphinganine 1-phosphate and phytosphingosine 1-phosphate. The extraction method was a modification of that of Sullard and Merrill (2001). Briefly, samples consisting of 20 mg of ground root tissue from each replicate were transferred into round bottom tubes, then 100 µL cold phosphate buffer was added and the samples were homogenized on ice for 5 minutes. Then 0.6 mL MeOH and 0.3 mL $CHCl_3$ plus 10 µl internal standard (10 ng/µL C_{17} sphinganine 1-phosphate and 50 ng/µL C_{20} dihydrosphingosine, Avanti Polar Lipids, Inc., Alabaster, AL, USA) in ethanol was added to each sample. Samples were sonicated 1 min at room temperature, capped tightly and incubated overnight at 48°C in a heating block. Samples were then allowed to cool, after which, 75 µL of 1 M methanolic KOH was added and the samples were sonicated 30 s and incubated 2 h at 37°C. The samples were then centrifuged at 1100 rcf for 10 min and the supernatants were transferred to glass tubes. The samples were neutralized by adding two drops of 1N HCL, and then evaporated to dryness in a vacuum centrifuge and stored under N_2 at -20°C. The samples were then reconstitute in 500 µL of (49.5:49.5:1) acetonitrile:water:formic acid containing 5 mM ammonium formate and clarified

by filter centrifugation (4500 rcf for 10 min) using a 0.45 µ Nylon Microspin filter (Lida Manufacturing Corp., Kenosha, Wisconsin, USA). The 500 µL samples were then analyzed by LCMS.

LCMS Methods

Fumonisin was separated on a Thermal Separations HPLC (Riviera Beach, FL, USA) consisting of a model P2000 solvent delivery system and an AS3000 autosampler. Separations were done using an Intersil 5µ ODS-3 column (150 x 3 mm, Metachem Technologies, Inc, Torrance, CA, USA). The flow was 0.2 mL min^{-1} and the mobile phase was a 28 min programmed gradient starting at 30% of 97% acetonitrile:2% water:1% formic acid (solvent "A") and 70% of 2% acetonitrile:97% water: 1% formic acid (solvent "B") and after 15 min the proportions of "A" and "B" were 60% and 40% respectively, and at 20 min the proportions of "A" and "B" were 90% and 10%, respectively, followed by an 8 min gradient returning to 30% "A" and 70% "B". The total run time was 28 min and there was a 5 min equilibration between each injection. The column effluent was directly coupled to a ThermoFinnigan LCQ Duo ion trap mass spectrometer (MS) (Woodstock, GA, USA). The MS was operated in the electrospray ionization (ESI) positive ion mode with an inlet capillary temperature of 200^{o}C and the sheath gas was nitrogen. For MS/MS of fumonisin $B_{1,}$ fumonisin B_2 and fumonisin B_3 the collision energy was 32% and the parent m/z (molecular weight plus 1) were 722.3 and 706.3, 706.3, respectively, and mass fragments were scanned from 195 to 800 m/z and compared to authentic standards.

Sphingoid bases and sphingoid base 1-phosphates were chromatographically separated on the same LCMS system as fumonisin, however, the gradient started at 50% solvent A and at 15 min was 70% solvent A and at 20 min it was 100% solvent A which was held until 25 min at which time the column was re-equilibrated with 50% A for 15 min before the next injection (10

μL). The total run time was 40 min. The MS was operated in the ESI positive ion mode with an inlet capillary temperature of 170^{o}C and the sheath gas was nitrogen. For MS/MS the collision energy was 30% and the parent m/z for MS/MS were 318.2, 302.2, 398.5 and 382.5 for phytosphingosine, sphinganine, phytosphingosine 1-phosphate and sphinganine 1-phosphate, respectively. The m/z for the internal standards was 366.5 and 330.2 for C_{17} sphingosine 1-phosphate and C_{20} dihydrosphingosine, respectively. MSMS mass fragments were scanned from 195 to 400 m/z and compared to authentic standards. Some samples were also analyzed using data-dependent scanning of the two most intense ions (150-600 m/z).

Statistical Analysis

Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). Where many groups were compared one-way analysis of variance (ANOVA) was used, followed by post hoc multiple comparisons. Where only two groups were compared a Student t-test or Mann Whitney Rank Sum test was used. A Chi-square test was performed to determine the significance of leaf lesion frequency data. The Pearson Product Moment Correlation was used to measure the strength of the association between pairs of variables. All data were expressed as mean ± standard deviation, and differences among or between means were considered significant if the probability (p) was ≤ 0.05.

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FIGURE LEGENDS

Figure 4.1. Proposed *de novo* sphingolipid biosynthesis and turnover pathway in plants showing point of disruption by fumonisin B_1 ("**X**") (coenzyme A [CoA] dependent ceramide synthase). Bold arrows indicate known effect of fumonisin B_1 inhibition of ceramide synthase on accumulation of free sphinganine (d18:0; dihydrosphingosine), free phytosphingosine (t18:0; 4-hydroxysphinganine), sphinganine 1-phosphate, and phytosphingosine 1-phosphate in roots from maize seedlings (Riley et al., 1996 and this manuscript). Key enzymes or enzymatic steps in the pathway that will be referred to in the text are indicated in italics. The term sphingoid base refers to all long chain bases including sphinganine and phytosphingosine. The key steps in the pathway are derived from the review of Lynch and Dunn (2004) with modifications to include the findings reported in this manuscript. Possible free long chain bases other than phytosphingosine and sphinganine include sphingosine (d18:1; 4-sphingenine), 8-sphingenine (d18:1), 4, 8-sphingadienine (d18:2) and 4-hydroxy-8-sphingenine (t18:1). These unsaturated sphingoid bases would most likely be derived from sphinganine with both hydroxylation and reduction most likely occurring subsequent to ceramide formation (Lynch and Dunn, 2004). Free phytosphingosine is formed from sphinganine prior to N-acylation and is not a substrate for ceramide synthase in plants (Wright et al., 2003). Plant tissues have been shown to have both an acyl CoA dependent ceramide synthase and a ceramide synthase activity that utilizes fatty acids

in the absence of CoA (Lynch, 2000). Only the CoA dependent ceramide synthase is inhibited by fumonisin B_1 and the fumonisin insensitive ceramide synthase activity (possibly a reverse ceramidase) contributes < 5% of total ceramide synthase activity in the same tissue (Lynch and Dunn, 2004).

Figure 4.2. Example of stunting, necrotic leaf lesion, abnormal leaf development, and reduced root development in 'Silver Queen' seedlings grown for 21 days from seeds inoculated with the pathogenic fumonisin producing *F. verticillioides* strain MRC826. A pot of control seedlings grown for 21 days from un-inoculated seeds are shown for comparison. Similar symptoms of seedling disease were seen beginning at 7 days after planting the MRC826-treated seeds. Not shown are seedlings grown from seeds inoculated with the non-pathogenic strain NRRL25059 which were visually the same as the un-inoculated control seedlings. These results are similar to those reported previously using a seedling blight virulence assay with the same fungal strains (MRC826 and NRRL25059) and the sweet maize cultivar 'Polar Vee' (Glenn et al., 2002). Fumonisin producing strains AEG3-A3-5, AEG3-A3-6 and JFL-A00999 were also pathogenic (data not shown), whereas, the non-producing strain JFL-A04516 was not pathogenic (data not shown).

Figure 4.3. (A) Total fumonisin B_1 (FB_1) extracted from potting soil in which maize seedlings of the sweet maize cultivar 'Silver Queen' were grown from sterilized seeds (control) or sterilized seeds inoculated with a non-pathogenic (NRRL25059) or pathogenic strain (MRC826) of *F. verticillioides* and harvested 9 days after planting. Values for fumonisins B_1 are expressed as μg fumonisin B_1 g^{-1} of potting soil. "ND" = fumonisin B_1 not detected. (B) Free sphinganine

(Sa) and phytosphingosine (Pso) in roots from maize seedlings grown in the potting soil and treated as described in (A). Values for free sphingoid bases are expressed as nmol g^{-1} root tissue (mean ± SD, n=3 pots containing 10 seedlings and 100 g of soil). Differing superscripts (a,b) indicate significant differences ($p \leq 0.05$) in Sa or Pso among treatments.

Figure 4.4. (A) Total fumonisin B_1 extracted from potting soil in which maize seedlings of the sweet maize cultivar 'Silver Queen' were grown from sterilized seeds (control) or sterilized seeds inoculated with the pathogenic strain (MRC826) of *F. verticillioides*, and harvested 21 days after planting. One difference between the soils from the 9 and 21 day experiments was that there were low but detectable levels of fumonisins (B_1, B_2 and B_3) in soil from 2 of the 5 control pots at 21 days. The mean fumonisin B_1 concentration detected in the control soils was only 3.1% of that in the soil of the MRC826 inoculated group. (B) Fumonisin B_1 tightly associated with root tissues from maize seedlings grown as described in (A). The roots from 2 of the 5 control pots also had low but detectable levels of fumonisins suggesting that the fungus in the inoculated plants had contaminated the pots that were planted with un-inoculated seeds. However, the mean fumonisin B_1 concentration detected in the control roots was only 0.06% of that in the roots of the MRC826 inoculated group. Values for fumonisin B_1 are expressed as μg fumonisin B_1 g^{-1} of potting soil or root tissue. Differing superscripts (a,b) indicate significant differences ($p \leq 0.05$) in fumonisin B_1 between treatments (Control and MRC826).

Figure 4.5. (A) Free sphinganine (Sa) and free phytosphingosine (Pso) and (B) sphinganine 1-phosphate (Sa-1-P) and phytosphingosine 1-phosphate (Pso-1-P) in roots from maize seedlings grown in potting soil and treated as described in Figure 4.4. Values for free sphingoid bases and sphingoid base 1-phosphates are expressed as nmol g^{-1} root tissue (mean ± SD, n=5 pots

containing 10 seedlings and 100 g of soil). Differing superscripts (a,b) indicate significant differences ($p \leq 0.05$) in Sa or Pso and Sa-1-P or Pso-1-P between treatments.

Figure 4.6. Effect of fumonisin B_1 on root development. (A) Photograph representative of root masses from each dose level on day 21. Effects of fumonisin B_1 on root weights from seedlings harvested on day 8 (B) and day 21 (C) from control and treated maize seedlings watered on days 2, 4, and 6 with fumonisin B_1 dissolved in water at 0, 1, 5, 10 and 20 $\mu g\ mL^{-1}$ (total fumonisin B_1 exposure at each dose level per pot was 0.3 mg, 1.5 mg, 3 mg and 6 mg, respectively). Values for root weight are total mg dry root tissue (mean ± SD, n=3 pots/treatment containing 10 seedlings). Differing superscripts (a,b,c) indicate significant differences ($p \leq 0.05$) among treatments on days 8 or 21.

Figure 4.7. Example of the root system of 14 day old seedlings grown on Murashige and Skoog (1962) medium with $8g\ L^{-1}$ Caisson plant culture gelling agent in the presence of 0 and 10 μM ($14\ \mu g\ mL^{-1}$) fumonisin B_1.

Figure 4.8. Fumonisin B_1 in potting soil collected on day 8 (A) and day 21 (B) after watering with fumonisin B_1 as described in Figure 4.6. Values for fumonisin B_1 (mean ± SD, n=3 pots/treatment containing 100 g of soil) are expressed as μg fumonisin $B_1\ g^{-1}$ of potting soil and "ND" = fumonisin B_1 not detected. Differing superscripts (a,b,c and d) indicate a significant difference ($p \leq 0.05$) in fumonisin B_1 among treatments on days 8 or 21.

Figure 4.9. Fumonisin B_1 tightly associated with root tissue after watering with fumonisin B_1 as described in Figure 4.6 and harvested on day 8 (A) and day 21 (B). Values for fumonisin B_1 (mean ± SD, n=3 pots /treatment containing 10 seedlings) are expressed as μg fumonisin B_1 g^{-1} of potting soil and "ND" = fumonisin B_1 not detected. Differing superscripts (a,b,c and d) indicate a significant difference ($p \leq 0.05$) in fumonisin B_1 among treatments on days 8 or 21. A small amount of fumonisin B_1 (but not B_2 or B_3) was detected in 2 of the 3 pots of control plants on day 21 (0.03 ± 0.03 μg g^{-1}) even though fumonisin B_1 was not detected in any of the soils collected from control seedlings.

Figure 4.10. Total phytosphingosine (Pso) (sum of free Pso and Pso in Pso 1-phosphate), at 8 days (A) and at 21 days (B) in root tissues from maize seedlings after being watered with fumonisin B_1 as described in Figure 4.6. Values are expressed as nmol g^{-1} root tissue (mean ± SD, n=3 pots/treatment containing 8-10 seedlings. Differing superscripts (a,b and c) indicate a significant difference ($p \leq 0.05$) in total Pso among treatments on days 8 or 21.

Figure 4.11. Total sphinganine (Sa) (sum of free Sa and Sa in Sa 1-phosphate), at 8 days (A) and at 21 days (B) in root tissues from maize seedlings after being watered with fumonisin B_1 as described in Figure 4.6. Values are expressed as nmol g^{-1} root tissue (mean ± SD, n=3 pots/treatment containing 8-10 seedlings). Differing superscripts (a,b,c and d) indicate a significant difference ($p \leq 0.05$) in total Sa among treatments on days 8 or 21.

Figure 4.12. Free phytosphingosine (Pso) (A) and phytosphingosine 1-phosphate (Pso-1-P) (B) at 8 days and at 21 days in root tissues from maize seedlings after being watered with fumonisin

B_1 as described in Figure 4.6. Values are expressed as nmol g^{-1} root tissue (mean ± SD, n=3 pots/treatment containing 8-10 seedlings). Differing superscripts (a,b) indicate a significant difference ($p \leq 0.05$) in Pso or Pso-1-P among treatments on day 8 or 21; "ND" = not detected.

Figure 4.13. Free sphinganine (Sa) (A) and sphinganine 1-phosphate (Sa-1-P) (B) at 8 days and at 21 days in root tissues from maize seedlings after being watered with fumonisin B_1 as described in Figure 4.6. Values are expressed as nmol g^{-1}root tissue (mean ± SD, n=3 pots/treatment containing 8-10 seedlings). Differing superscripts (a,b) indicate a significant difference ($p \leq 0.05$) in Sa or Sa-1-P among treatments on day 8 or 21; "ND" = not detected.

Figure 4.14. Example of LC electrospray ionization data dependent MS and MSMS two dimensional ion maps showing the relative intensities at each m/z and retention time in extracts from roots of maize seedlings grown in potting soil and watered with water (A) or water containing 20 μg fumonisin B_1 mL^{-1} (B). The areas representing the parent ion for phytosphingosine (t18:0; Pso), sphinganine (Sa; d18:0), phytosphingosine 1-phosphate (Pso-1-P), sphinganine 1-phosphate (Sa-1-P), and the internal standards (C17-So-1-P and C20 Sa) are indicated. There was no evidence for the presence of other intermediates or common plant free long chain bases or their 1-phosphates, (i.e., 3-keto-sphinganine (d18:0), 4- or 8-sphingenine (d18:1), 4-hydroxyl-8-sphingenine (t18:1), or 4, 8-sphingadienine (d18:2)). The relative intensities are represented with white and green being high intensity and red is low intensity. While free sphinganine was detected in the control root it is not easily seen in the ion map. The scan was performed from 150 to 600 m/z but only the portion from 250 to 400 m/z is shown here since the areas below 250m/z and above 400 m/z showed no elevated ions in the fumonisin B_1

treatment group compared to the control. There is an elevated ion detected at approximately 15 min with an m/z of 383; the fragmentation pattern provided no evidence that the compound contained a sphingoid-base backbone.

Table 4.1. Summary of phytotoxic effects from watering maize seedlings with fumonisin B_1.

Fumonisin B_1 ($\mu g\ mL^{-1}$)	Survival incidence[a]	Leaf lesion[b]	Mean height (cm)[c]
0	29/30	0/3 (0/29)	26.7
1	30/30	0/3 (0/30)	28.2
5	28/30	2/3 (3/28)	25.7
10	28/30	2/3 (4/28)	23.8
20	30/30	3/3 (10/30)	20.7[d]

[a] Indicates the total number of seedlings harvested on day 21 over the number of seeds planted (10/pot).

[b] Indicates the number of pots that had at least 1 seedling exhibiting leaf lesions over the total number of pots per treatment (n=3). In parentheses is the number of plants exhibiting leaf lesions/total plants. Based on Chi-Square analysis, there was a statistically significant increase in the incidence of leaf lesions with dose (Chi-square = 71.5, degrees of freedom = 4, $p < 0.0001$).

[c] Value is the mean ± standard deviation (n=3) of the average height of all plants harvested on day 21 from each treatment group.

[d] Value is significantly ($p \leq 0.05$) different from the control group (0 ppm).

Table 4.2. Correlation between change in root weight in each pot (n=15 per day) from the watering assay and the associated values for fumonisin B_1 (FB) tightly associated with roots, free phytosphingosine (Pso), free sphinganine (Sa), phytosphingosine 1-phosphate (Pso-1-P), sphinganine 1-phosphate (Sa-1-P) and total sphingoid bases in roots on days 8 and 21.[a]

	FB_1	Pso	Sa	Pso-1-P	Sa-1-P	Totals
Day 8						
Correlation Coefficient	-0.609	-0.465	-0.434	-0.624	-0.543	-0.530
P value	0.015	0.078	0.101	0.012	0.035	0.041
Day 21						
Correlation Coefficient	-0.871	-0.771	-0.873	-0.867	-0.914	-0.889
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Day 21 Weights vs Day 8 for other variables						
Correlation Coefficient	-0.853	-0.780	-0.717	-0.648	-0.668	-0.728
P value	<0.001	<0.001	0.002	0.009	0.006	0.002

[a]Totals=Pso+Sa+Pso 1-P+Sa 1-P

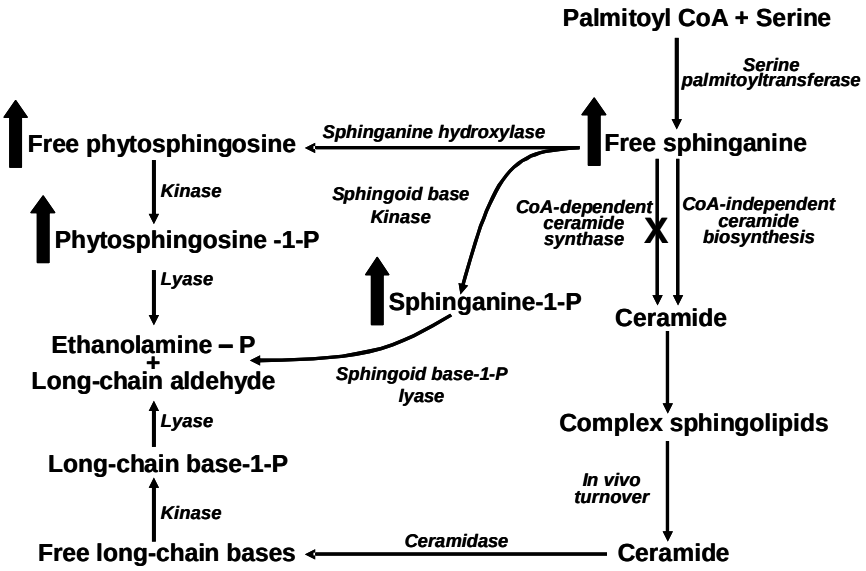


Figure 4.1 Williams et al.

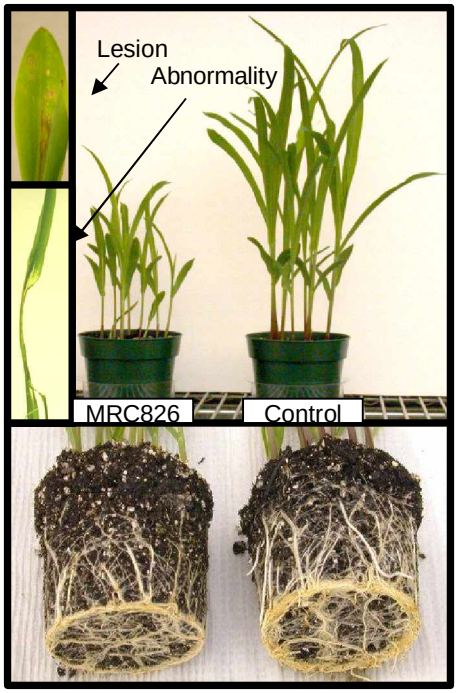


Figure 4.2 Williams et al.

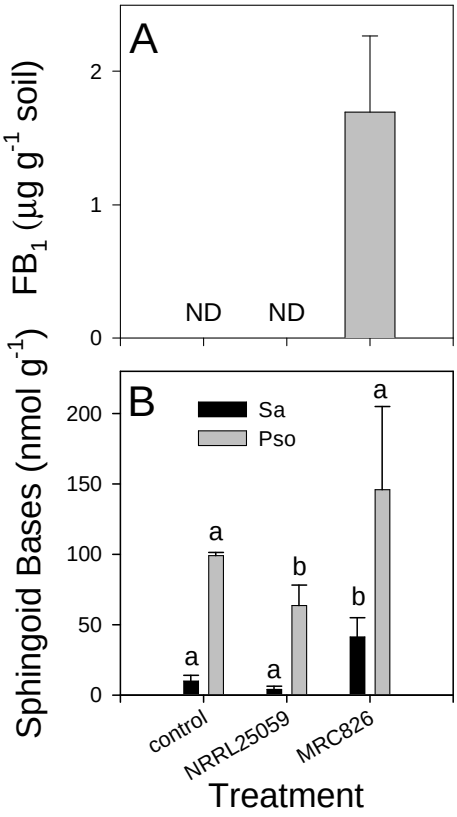


Figure 4.3 Williams et al.

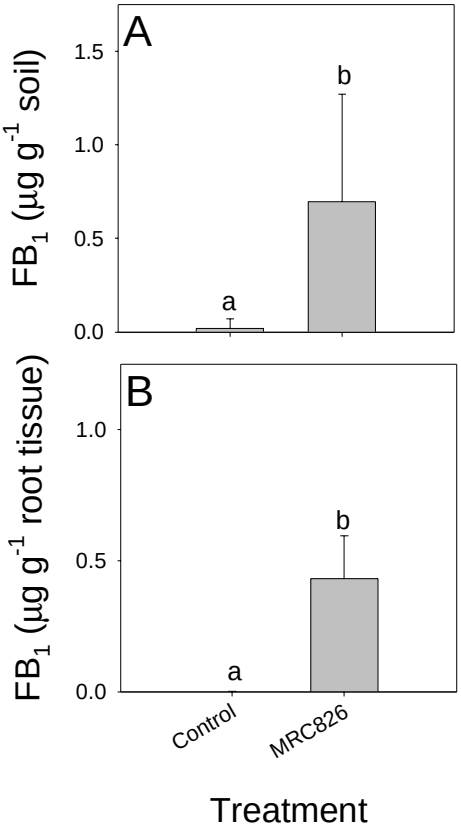


Figure 4.4 Williams et al.

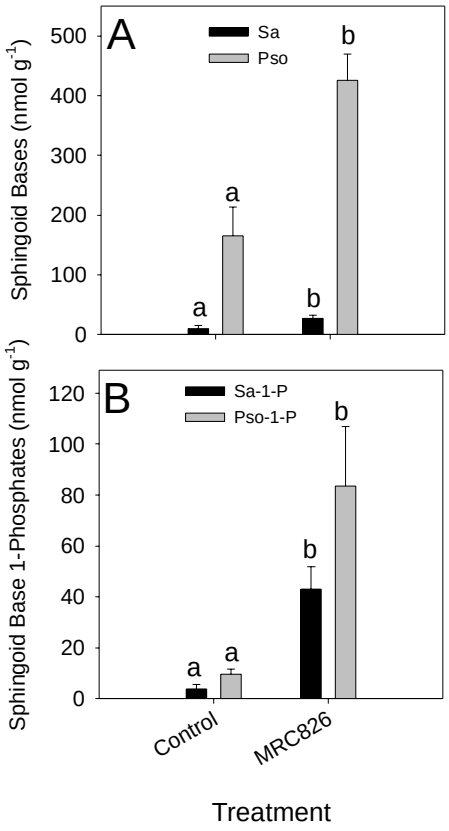


Figure 4.5 Williams et al.

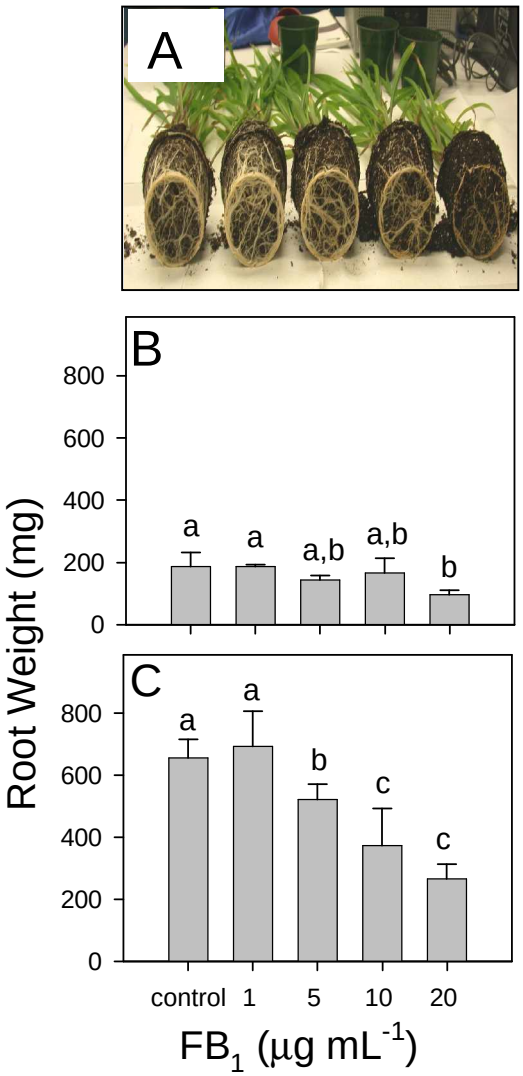


Figure 4.6 Williams et al.

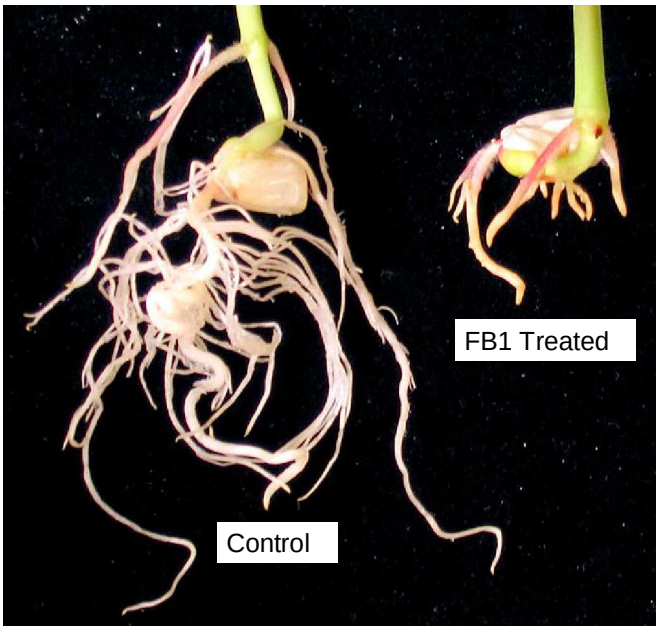


Figure 4.7 Williams et al.

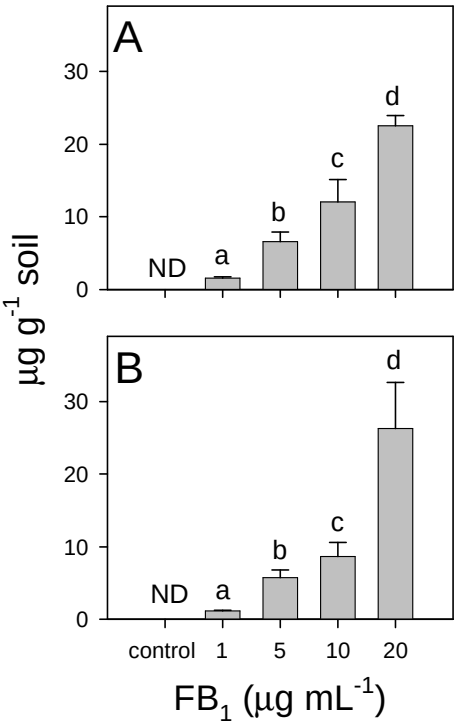


Figure 4.8 Williams et al.

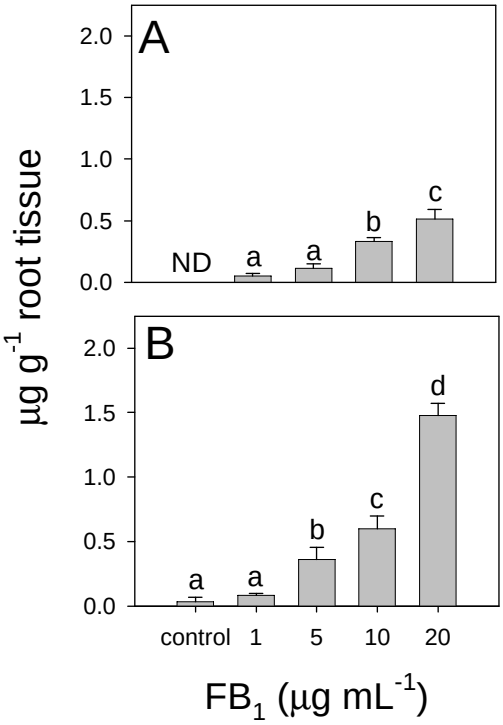


Figure 4.9 Williams et al.

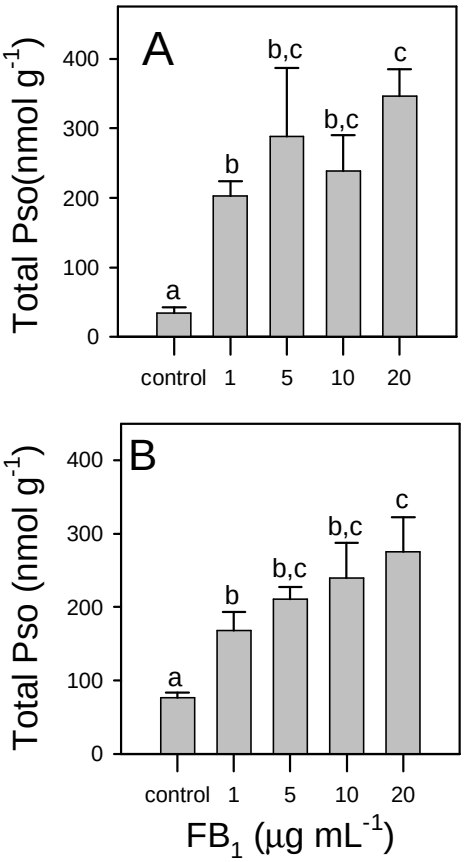


Figure 4.10 Williams et al.

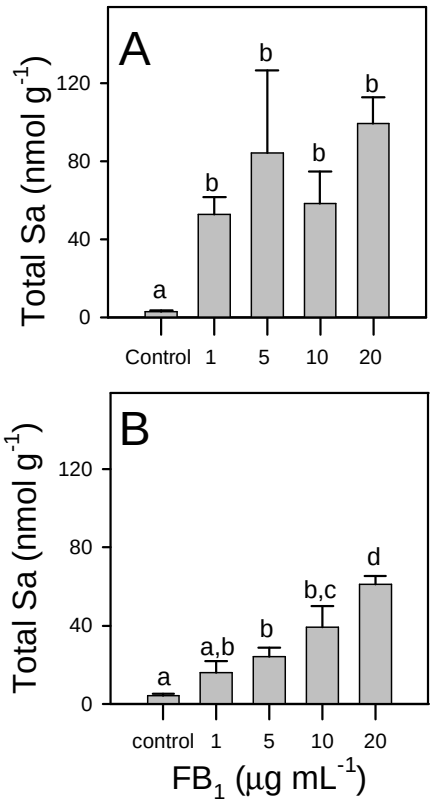


Figure 4.11 Williams et al.

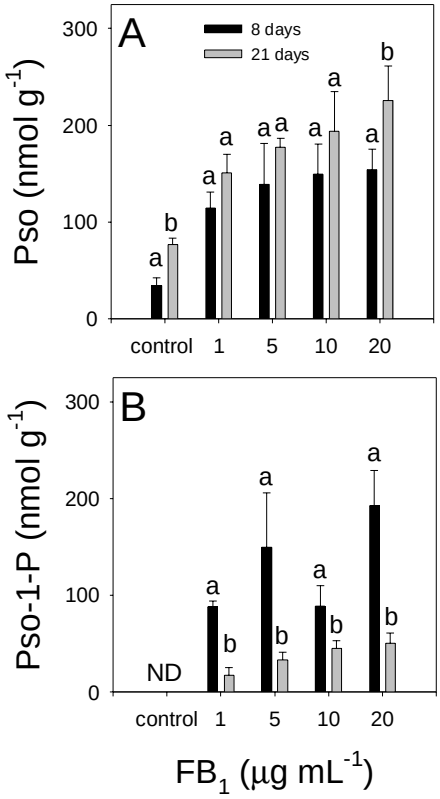


Figure 4.12 Williams et al.

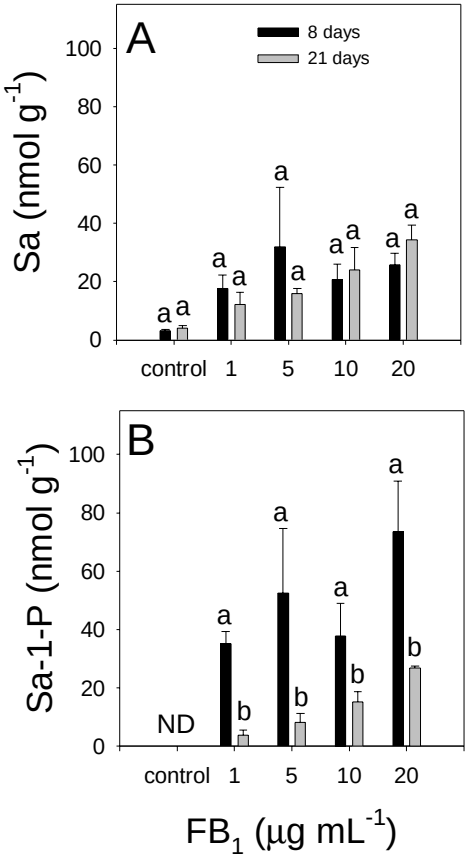


Figure 4.13 Williams et al.

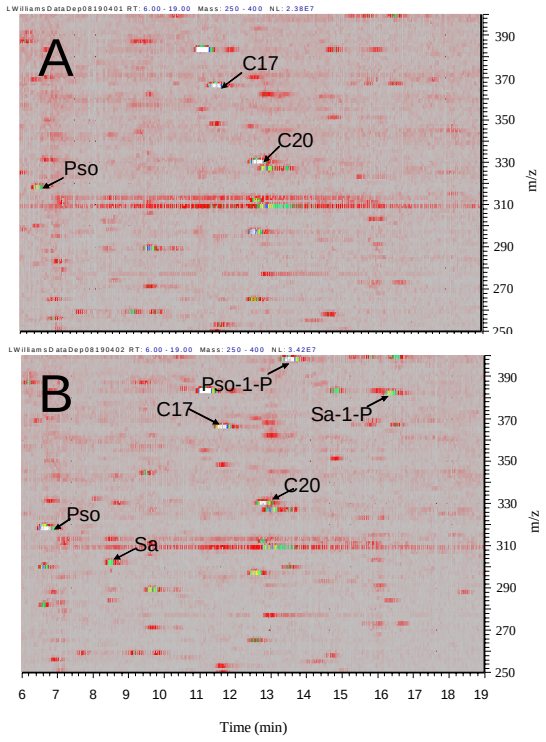


Figure 4.14 Williams et al.

CHAPTER 5

SUMMARY AND CONCLUSION

Fumonisins (FB_1, FB_2 and FB_3), produced by *Fusarium verticillioides*, are carcinogenic mycotoxins that are parasitic to maize plants. They are prevalent contaminants of maize and their mechanism of toxicity is the inhibition of ceramide synthase; a key enzyme in *de novo* sphingolipid biosynthesis. Fumonisin induced inhibition of ceramide synthase leads to disruption of sphingolipid metabolism, which has been shown to be the most likely cause of toxicity of the structurally similar compound, AAL-toxin in tomato plants. In contrast, even though maize tissues (cob, stalks, and kernels) can contain detectable levels of fumonisins, fumonisin-induced disruption of sphingolipid metabolism has not been shown to be a mechanism of any disease in maize. Fumonisins can be present at high levels in maize plant debris and can enter the soil intact where they can be tightly bound and under certain conditions may be released. The objectives of the present study were to i) investigate the ability of *F. verticillioides* to produce fumonisins in synthetic and complex natural soils when inoculated on maize seeds, ii) evaluate if the fumonisins produced are biological available to maize roots using an increase in free sphingoid bases and sphingoid base 1-phosphates as markers for fumonisin-induced disruption of sphingolipid metabolism, and iii) determine the time- and dose- dependent relationship between fumonisins in soil and seedling growth, leaf lesions and effects on root development (expressed as growth) and disruption of sphingolipid metabolism in maize roots.

In a previous study it was shown that FB_1 binding in a simple soil matrix (washed sand) was minimal (> 90% unbound) but was bound appreciably by more complex soil (Cecil sandy

loam soil). In the present study both the potting mix and three complex natural soils bound appreciable amounts of FB_1 (Figure 3.1). However, the Roper muck sandy loam bound more FB_1 than the Cecil sandy loam, Downer sandy loam, or potting mix (Figure 3.1) based on the amount of FB_1 recovered in the water after 12 h and in the ACN:water extracts. Some of the tightly bound (not recovered in the ACN:water extracts) fumonisins were extracted from the soils with ACN:H_2O containing 5% formic acid after shaking for an additional 12 h (Figure 3.1). The best predictors of FB_1 binding to soils were the calculated effective cation exchange capacity (CEC_e) and the calcium content of the soil (Table 3.2). The correlation between tightly bound FB_1, CEC_e and calcium content were statistically significant ($p< 0.05$, $n=5$), whereas, the correlation between FB_1 not tightly bound, CEC_e and calcium were ≥ 0.87 and the "p" values were both < 0.06 (Table 3.2). The fact that 5% formic acid is necessary to extract tightly bound fumonisins from complex natural soils is consistent with the hypothesis that the tight binding is due to ionic interactions with soil constituents and that soils with a high cation exchange capacity and calcium content will bind fumonisins tightly.

F. verticillioides MRC826 produced fumonisins in potting mix as well as in the three complex natural soils (Cecil sandy loam, Downer sandy loam and Roper muck sandy loam) when inoculated on maize seeds ('Silver Queen'), and the seedlings developed all the symptoms of *F. verticillioides* maize seedling disease (leaf lesions, stunted aerial growth, abnormal leaf development and reduced root development). Fumonisins were detected in the roots and the soil of maize seedling grown from MRC826 inoculated seeds and there was a statistically significant reduction in root mass and elevated levels of free sphingoid bases and sphingoid base 1-phosphates in the roots. These findings show that *F. verticillioides* MRC826 can produce

fumonisins in synthetic and complex natural soils and that the fumonisins produced in soils are biologically available to maize plants growing in the soil.

In subsequent studies, the role of fumonisins in diseases of maize seedlings associated with *F. verticillioides* was investigated. Maize seeds ('Silver Queen') were inoculated with fumonisin producing (AEG3-A3-6 and MRC826) or non-producing strains (NRRL25059, JFLA04516 and AEG1-1-57) of *F. verticillioides*. Fumonisins were only detected in the soil used to grow seed inoculated with the fumonisin producing strains, and only the seedlings grown from seeds inoculated with the fumonisin producing strain developed all the symptoms of *F. verticillioides* maize seedling disease. However, the seedlings grown from seeds inoculated with the non-producing strains, JFLA04516 and AEG1-1-57, had a statistically significant reduction in root mass and the seedlings grown from seeds inoculated with JFLA04516 had a statistically significant reduction in mean stalk height when compared to the un-inoculated control seedlings. Nonetheless, the most severe effects on root and stalk growth were seen in the seedlings grown from seeds inoculated with the fumonisin producing strains, and the stalks of the seedlings grown from seeds inoculated with the non-producing strains did not have necrotic leaf lesions or symptoms of abnormal leaf development. These findings suggested that although some non-producing strains of *F. verticillioides* are capable of producing less marked effects on root and stalk growth, fumonisin production is necessary for expression of all aspects of *F. verticillioides*-induced maize seedling disease.

To further investigate the role of fumonisin in *F. verticillioides* maize seedling disease, sterilized maize seeds were planted in potting soil and watered with aqueous fumonisin B_1. When watered onto sterilized maize seeds, pure aqueous fumonisin B_1, was able to induce symptoms of *F. verticillioides* maize seedling disease in the absence of the fungus. However, the

extent and severity of the disease symptoms observed in the seedlings grown from fumonisin B_1 watered seeds were less marked as compared to those observed in the seedlings grown from seeds inoculated with fumonisin producing strains of *F. verticillioides*. Fumonisin B_1 was detected in the soil as well as in the roots and the seedling had reduced root mass when compared to the un-treated control seedlings. The roots of the fumonisin B_1 watered seedling also had increased levels of sphingoid bases and sphingoid base 1-phosphates.

Taken together, these findings show that *F. verticillioides* can produce fumonisins in a variety of soil types which are capable of entering maize roots and inhibiting ceramide synthase. They also suggest that the ability of the *F. verticillioides* to produce fumonisin is essential for the development of all of the aspects of *F. verticillioides* maize seedling disease symptoms, and that fumonisins alone are sufficient for production of all the symptoms of *F. verticillioides* maize seedling disease. However, some non-producing strains (i.e. JFLA04516 and AEG1-1-57) are capable of causing reduced root and stalk growth compared to controls, but the effects are less marked, compared to the effects seen in the seedlings grown from seedlings inoculated with the fumonisin producing *F. verticillioides* strains. The most likely proximate cause of the adverse effects seen in maize seedlings grown from seeds inoculated with the fumonisin producing strains of *F. verticillioides* and in seedlings watered with fumonisin B_1, is the inhibition of ceramide synthase.

Chapter 6

SUPPLEMENTAL DATA

In chapter 3, it was shown that *Fusarium verticillioides* can produce fumonisins that are biologically available to the roots of sweet maize seedlings grown in synthetic and natural soils. Chapter 4 demonstrated that the fumonisin producing strain of *F. verticillioides*, MRC826, as well as pure fumonisin B_1 could induce all the characteristic symptoms of *F. verticillioides* seedling disease on sweet maize, whereas, the non-producing strain, NRRL25059, did not produce any symptoms of seedling disease. In both chapters, the sweet maize cultivar 'Silver Queen' was used due its high level of sensitivity to fumonisins. In addition, only one fumonisin producing (MRC826) and one non-producing strain (NRRL25059) were used in the virulence assay. However, in this country, a variety of field maize cultivars are grown with varying levels of sensitivity to fumonisins and there are numerous strains of *F. verticillioides*.

To compare the ability of *F. verticillioides* to induce symptoms of maize seedling disease in sweet maize and field maize cultivars, sterile maize seed from the sweet maize cultivar 'Silver Queen' and the field maize cultivars 'Pioneer-3167' and 'Pioneer-3156' were inoculated with the fumonisin producing strain of *F. verticillioides*, MRC826. Five replicates of 10 seeds from each cultivar were planted in sterile pots containing sterile commercial potting mix and harvested 21 days after planting. The roots from each cultivar were analyzed for sphingoid bases, sphingoid base 1-phosphates, and fumonisins (FB_1, FB_2 and FB_3); the potting soils used to grow seedling of each cultivar were analyzed for fumonisins. There was a statistically significant reduction in

root mass of seedling grown from seeds inoculated with MRC826 when compared to the un-inoculated control seedlings in all cultivars (Figure 6.1). There was detectable fumonisins in the potting soils used to grow seedlings from seeds inoculated with MRC826 in all cultivars and fumonisin was also detected in the soil of 1 of the 5 control pots (Figure 6.2A). Fumonisins were also detected in the roots of seedlings grown from MRC826 inoculated seeds from each cultivar and fumonisin was also detected in the roots of the seedlings grown in the 1 control pot which had detectable fumonisin in the soil (Figure 6.2B). There were also elevated levels of the free sphingoid bases sphinganine (Sa) and phytosphingosine (Pso) in the roots of seedlings grown from MRC826-inoculated Pioneer-3167 and Silver Queen seeds but not in the Pioneer-3156 (Figure 6.3A-B), however, the sphingoid base 1-phosphates (Sa-1-P and Pso-1-P) were significantly elevated in all the varieties but the effects was most marked in the Silver Queen (Figure 6.4A-B).

The degree of reduction in root mass, amount of fumonisins detected in the roots, as well as the increase in free sphingoid bases and their 1-phosphate metabolites varied in the different maize cultivars. However, regardless of the cultivar, MRC826 was able to produce fumonisins and disrupt sphingolipid metabolism in the roots. This is important because it shows that the ability of *F. verticillioides* to produce fumonisins in soil, and for fumonisins to accumulate in maize roots and disrupt sphingolipid metabolism in sweet maize cultivars is similar in field maize cultivars. However, the extent and severity of the effects on maize roots are less in the field maize cultivars and the field maize cultivars did not develop leaf lesions nor did they show any symptoms of abnormal leaf development.

In chapter 4, it was also shown that *F. verticillioides* strain NRRL25059 did not produce fumonisins and did not induce any symptoms of *F. verticillioides* maize seedling disease. To

further examine the relationship between fumonisin (FB_1, FB_2 and FB_3) production and *F. verticillioides* pathogenicity on maize, other fumonisin producing and non-producing strains of *F. verticillioides* were tested for their ability to produce fumonisins on maize kernels. Samples consisting of five grams of sterile maize seeds ('Silver Queen') were inoculated with a fumonisin producing or a non-producing strain of *F. verticillioides* (10^6 total colony forming units) and placed into scintillation vials for 14 days. Strain JFLA04516, which has a mutation in a key gene in the fumonisin gene cluster, and strains NRRL25059 and AEG1-1-57, which lack the fumonisin gene cluster, did not produce fumonisins when grown on maize culture (Figure 6.5). Analysis of maize kernels from cultures inoculated with strains AEG3-A3-6 and MRC826, which both possess the fumonisin gene cluster, revealed that both produced fumonisins. MRC826 produced considerably more fumonisins than AEG3-A3-6 (Figure 6.5). In a virulence study, maize seeds were inoculated with the two fumonisin producing strains or the three non-producing strains of *F. verticillioides* and grown in sterile potting soil for 21 days (10 seeds/pot). Only the seedlings grown from seeds inoculated with strains AEG3-A3-6 and MRC826 exhibited all the symptoms of *F. verticillioides* maize seedling disease and fumonisins were only detected in the soils used to grow seeds inoculated with AEG3-A3-6 and MRC826 (Table 6.1). MRC826 produced considerably more fumonisins that AEG3-A3-6 in the soil, which was correlated with the degree and severity of disease symptoms (leaf lesions, stunted aerial growth, abnormal leaf development and reduced root development) seen in maize seedlings grown from seeds inoculated with AEG3-A3-6 and MRC826 (Table 6.1). While strain NRRL25059 had no significant effects on root or stalk growth, strains JFLA04516 and AEG1-1-57 were able to cause significant reductions in root and stalk growth when compared to un-inoculated control seedlings (Table 6.1). However, the effect on root and stalk growth were significantly less than in the

producing strains and like the un-inoculated control seedlings, none of the seedlings grown from seeds inoculated with the non-producing strains developed leaf lesions or abnormal leaf development. These findings provide support for the hypothesis that the ability of *F. verticillioides* to produce fumonisin is essential in the development of the full spectrum of *F. verticillioides* maize seedling disease symptoms.

Table 6.1. Summary of phytotoxic effects and FB_1 in roots and soil from virulence assay with fumonisin producing (MRC826 and AEG3-A3-6) and non-producing (NRRL 25059, AEG1-1-57, and A04516) strains of *F. verticillioides* at 21 days.

Treatment	Survival incidence[a]	Leaf lesion[b]	Mean height (cm)[c]	Root weight (mg)	FB_1 in soil ($\mu g\ g^{-1}$)
Control	50/50	0/5 (0/50)	54.6^{d}	736^{d}	ND
NRRL 25059	49/50	0/5 (0/50)	$54.0^{d,e}$	$678^{d,e,f}$	ND
A04516	50/50	0/5 (0/50)	$49.2^{e,f}$	$540^{e,f,g}$	ND
AEG1-1-57	50/50	0/5 (0/50)	$53.0^{d,e}$	$516^{f,g}$	ND
AEG3-A3-6	50/50	5/5 (23/50)	47.2^{f}	442^{g}	11.5^{d}
MRC 826	48/50	5/5 (48/50)	37.6^{g}	264^{h}	35.3^{e}

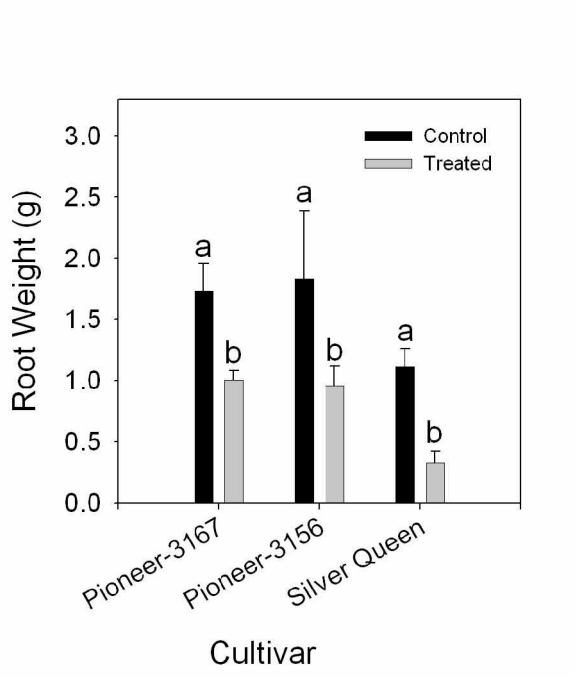
[a] Indicates the total number of seedlings harvested on day 21 over the number of seeds planted (10/pot).

[b] Indicates the number of pots that had at least 1 seedling exhibiting leaf lesions over the total number of pots per treatment (n=5). In parentheses is the number of plants exhibiting leaf lesions/total plants. Based on Chi-Square analysis, there was a statistically significant difference ($p<0.001$) in the incidence of leaf lesions between the fumonisin producing strains (AEG3-A3-6

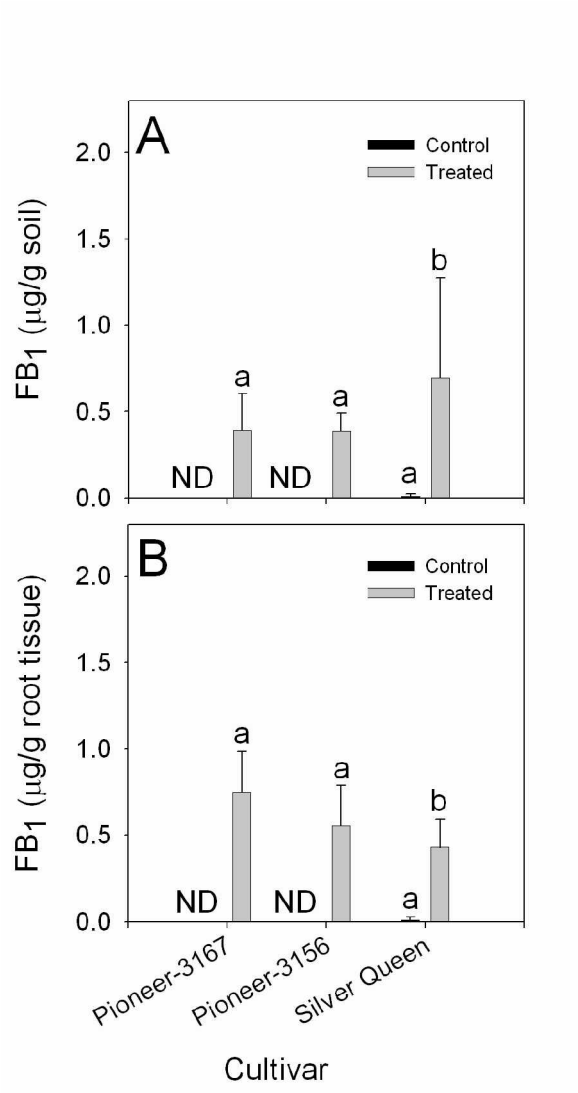
and MRC 826) and the non- producing strains (NRRL 25059, A04516 and AEG1-1-57) and there was also a statistically significant difference ($p<0.001$) in the incidence of leaf lesions between the two fumonisin producing strains ($p<0.001$).

[c] Value is the mean ± standard deviation (n=5) of the average height of all plants harvested on day 21 from each treatment group.

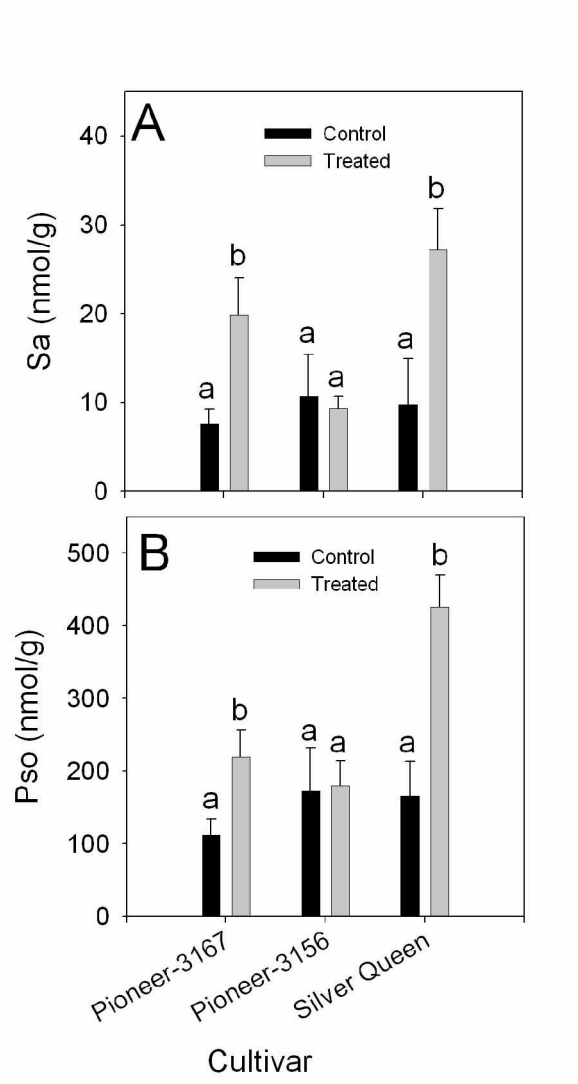
[d-h] Means within columns with differing superscripts are significantly ($p \leq 0.05$) different



Supplemental data, Figure 6.1



Supplemental data, Figure 6.2



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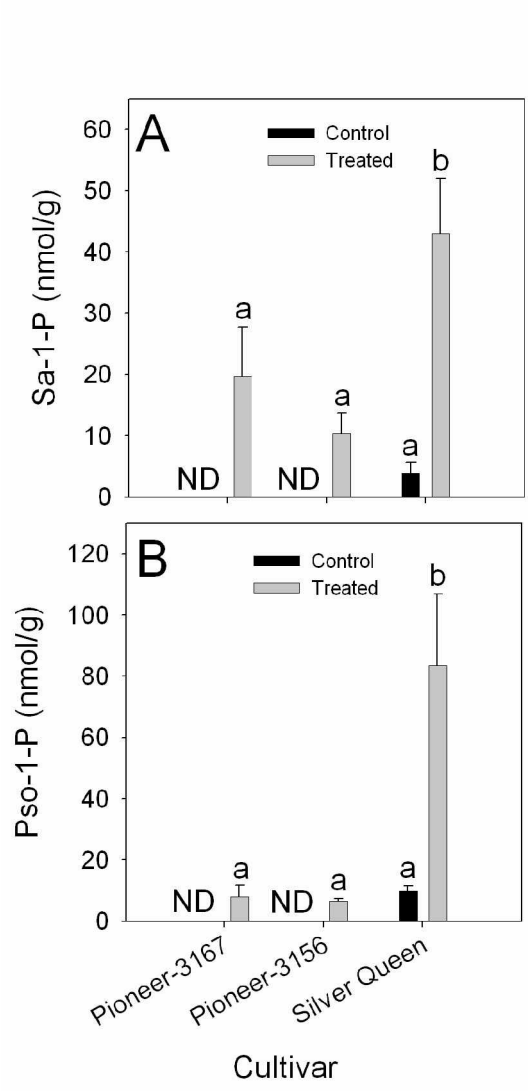
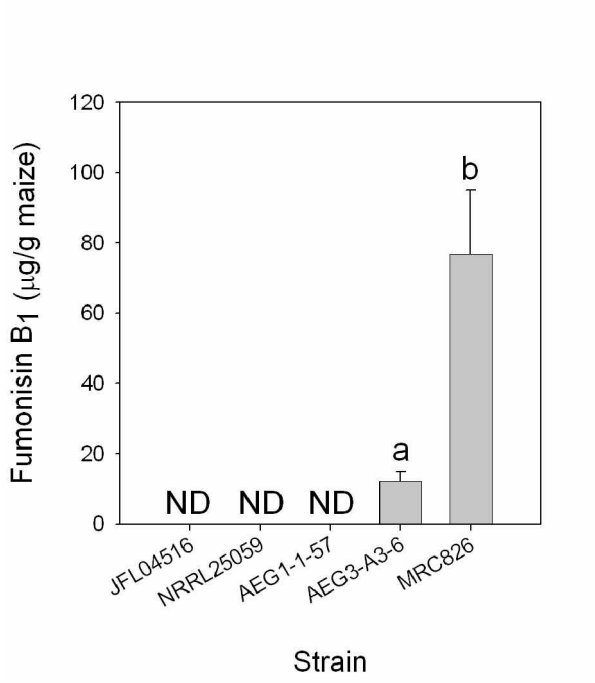


Supplemental data, Figure 6.4



Supplemental data, Figure 6.5