KINETICS OF LEACHING AND BINDING OF FUMONISIN B_1 IN SOIL MICROCOSMS

by

LONNIE DWAYNE WILLIAMS

(Under the direction of Dr. Ronald T. Riley)

ABSTRACT

Fumonisin B_1 (FB_1) is a water-soluble mycotoxin produced by *Fusarium verticillioides*. Research objectives were to determine the leaching of FB_1 through soil and FB_1 binding in soil. Leachate and microcosm columns were used to determine the movement of FB_1 through soil. FB_1-contaminated corn screenings or water extracts containing FB_1 were placed on the surface of soil columns. In 100% sand columns FB_1 leaching was only slightly retarded, whereas, at 50%, 75% and 100% Cecil sandy loam, approximately 60%, 50% and 20% of the FB_1 was recovered in the column leachate, respectively. The FB_1 retained on the 100% Cecil sandy loam column was tightly bound. However, approximately 75% of the bound FB_1 was released with 5% formic acid and 5% formic acid:acetonitrile (1:1) suggesting that binding was ionic. The results suggest that FB_1 is quite stable in soils and, while tightly bound, under certain environmental conditions could be released.

INDEX WORDS: Fumonisin B_1, Cecil sandy loam, *Fusarium verticilliodes*, corn

KINETICS OF LEACHING AND BINDING OF FUMONISIN B_1 IN SOIL MICROCOSMS

by

LONNIE DWAYNE WILLIAMS

B.S., Albany State University, 1999

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2002

KINETICS OF LEACHING AND BINDING OF FUMONISIN B_1 IN SOIL MICROCOSMS

by

LONNIE DWAYNE WILLIAMS

Approved:

Major Professor: Dr. Mary A. Smith

Committee: Dr. Ronald T. Riley
Dr. Charles W Bacon
Dr. Roger D Wyatt

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
May 2002

DEDICATION

Dedicated to my family and friends, thank you all.

ACKNOWLEDGEMENTS

I would like to thank God for giving me the opportunity to reach my goals and for giving me strength. I would like to thank my research advisor, Dr Ronald Riley, for giving me guidance and support, and for teaching about life. I would also like to thank Jency Showker and the staff of TMRU for their technical assistance. I would like to thank Dr. Charles Bacon, Dr. Mary A. Smith, and Dr. Roger Wyatt for being on my committee and being very supportive. Last but not least, I would like to thank my family and friends for always believing in me and pushing me to be the best that I can be.

TABLE OF CONTENTS

Page

INTRODUCTION AND LITERATURE REVIEW

I. The Plant-Fungus Association

The fungus *Fusarium verticilliodes* Sheldon (syn. = *F. moniliforme*) produces a number of toxic chemicals that include the fumonisins (Bacon and Nelson, 1994). *F. verticilliodes* Sheldon is a nonobligate plant pathogen that is commonly associated with corn (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. verticilliodes* does not follow this pattern because a large percentage of the isolates get their nutrition from living cells, they are therefore biotrophic (Bacon and Hinton, 1996). The development of disease symptoms and death of infected corn 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. verticilliodes belongs to the section *Liseola* and can be separated into two mating populations known as populations A and F of *Gibberella fujukuroi* (Klittich and Leslie, 1992). Fungi of mating population A are primarily associated with corn, while fungi of mating population F are usually associated with sorghum. *F. proliferatum* (Matsushima) Nirenberg in mating population D, is also commonly found on corn and can produce large amounts of the fumonisins (Munkvold and Desjardin, 1997).

F. verticilliodes 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 corn because this fungus may produce a wide variety of chemically different mycotoxins on corn (Bacon and Hinton, 1996). Because of its non-obligatory nature, this parasite is able to attack many different plants and plant parts, and produce and secrete a large number of nonspecific phytotoxins or extracellular enzymes that can affect metabolic pathways or cellular functions common to many plants (Bacon and Hinton, 1996). The nature of the interaction between isolates of *F. verticilliodes* and corn varieties is diverse (Munkvold and Desjardins, 1997). Some isolates are virulent, while others are non-virulent, but highly infective and associated with corn without producing any sign of disease to the plant (Thomas and Buddenhajen, 1980 and Foley, 1962). This aspect of the fungal-plant interaction is referred to as symptomless association (Bacon and Hinton, 1996).

Diseases of corn associated with *F. verticilliodes* 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. verticilliodes*, 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). The mesocotyl near the ground level with other tissues in the leaves appear water-soaked. Root rot may be initiated as a part of the seedling blight disease, but persists into the matured corn 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. verticilliodes* 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. verticilliodes* 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 15 different fumonisins have been reported and other minor metabolites have been identified, although some of them do not occur naturally (Abbas & Shier, 1997; Musser & Plattner, 1997; Plattner, 1995). Fumonisin B_1 (FB_1) is the most abundant of the fumonisins, which occur naturally (Abbas & Shier, 1997; Musser & Plattner, 1997; Plattner, 1995). 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., 1993a; 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 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 C18 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 corn cultures of *F. verticilliodes* in southern Africa (Bezuidenhout et al., 1988). Since this discovery, the fumonisins have been isolated from corn and corn-based food and feedstuffs naturally contaminated with this fungus in many countries including the United States, Canada, China, Egypt, Argentina, Nepal, Peru and Brazil (see review of Bacon and Nelson, 1994). These locations follow the geographic distribution of both the fungus and corn production, suggesting that fumonisins will be found wherever corn is grown (Bacon and Nelson, 1994).

The precise timing of fumonisin formation and accumulation patterns in the vegetative parts of corn seedlings is known (Bacon et al., 2001). Recently fumonisin has

been shown to appear soon after infection of field corn (1 week after anthesis) and peaked within 2 weeks after it first appeared (Payne et al., 2001). A second peak in fumonisin production occurred late in the season and was not associated with an increase in kernel infection (Payne et al., 2001). Fumonisins have been detected in corncobs; glumes of old corn florets (bee wings), kernels and corn screenings (Bacon and Nelson, 1994). The highest concentrations have been detected in bee wings (reviewed in Riley et al., 1993). However corn screenings have higher concentrations than whole corn and have been associated with farm animal diseases in swine and horses (Riley et al., 1993). Fumonisins also are found in corn and corn-based products for human consumption (Chu et al., 1995; Sydenham et al., 1991; Thiel et al., 1991; Richard et al., 1993). Sound corn or "symptom-less" kernels can contain fumonisins, however, visually damaged kernels often contain much higher levels of fumonisin (Desjardin and Plattner, 1998).

Fusarium verticilliodes is also saprophytic (Bacon and Nelson, 1994). *F. verticilliodes* is considered a field fungus because it normally produces its toxin under field conditions (Bacon and Nelson, 1994). The high levels of fumonisin production under culture conditions suggest that corn 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 corn storage in the United States has not been demonstrated. Laboratory studies have established that the optimum temperature range for growth of isolates of *F. verticilliodes* 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). Corn 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.

III. Mycotoxin-Animal Association

There are several diseases associated with *F. verticilliodes* and fumonisins. These include equine leucoencephalomalacia (ELEM), porcine pulmonary edema syndrome (PPE), poultry toxicity, as well as a suggested link between fumonisins and human esophageal cancer (reviewed in Marasas et al., 2000). 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 (reviewed in Riley et al., 1993). In 1902, ELEM was experimentally produced by feeding horses moldy corn obtained from a field in Kansas (Butler, 1902). The disease was known as “moldy corn poisoning,” but early

attempts to identify the responsible fungus failed. In 1971 Wilson and Maronpot isolated *F. verticilliodes* as the predominant contaminant of moldy corn that had caused many cases of ELEM and they reproduced ELEM by feeding horses *F. verticilliodes* corn 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. verticilliodes* 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. verticilliodes* was found to be the predominant contaminant of the corn (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). Reports have been published suggesting that *F. verticilliodes* contamination of feed is related to disease of poultry (Bryden et al., 1987). Studies have also confirmed that *F. verticilliodes*; *F. proliferatum,* fumonisin B_1 and moniliformin are toxic to broiler chicks (Brown et al., 1992; Dombrink-Kurtzman et al., 1993, Henry et al., 2000) and chicken embryos (Bacon et al., 1995, Henry et al., 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 corn as a staple as well as consuming a beer brewed with corn and a non-alcoholic fermented drink made with corn (Rose, 1982; Segal, 1988). The corn was locally grown, stored in open cribs and was often visually moldy, with the moldiest ears hand selected for use in beer brewing (Marasas, 1979). *F. verticilliodes* 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 (reviewed in Marasas et al., 2000).

F. verticilliodes corn-culture material, corn naturally contaminated with *F. verticilliodes*, and pure FB_1 are hepato-carcinogenic in rats (reviewed in Marasas et al., 2000). Recently completed long-term feeding studies have shown that fumonisin B_1 is both a liver and kidney carcinogen in rodents (National Toxicology Program, 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 cause morphological changes and lead to decreased cell growth and increased cell death, in both animals and plants including corn (Riley et al., 1996). There is considerable supporting evidence that fumonisin-induced disruption of sphingolipid metabolism is an important event in the cascade of events leading to altered cell growth, differentiation and cell injury observed both *in vitro* and *in vivo* (Riley et al., 1998).

V. Rationale for Research

F. verticilliodes and the fumonisins have become areas of great concern for corn producers, processors, and consumers. Contamination of corn with fumonisins is worldwide and can have varied effects on animals. Fumonisins pose a threat for humans in developing countries because low quality corn is often consumed in large amounts when food is scarce. Safe levels for human exposure have not been set because of limited data, however risk assessments by regulatory agencies are presently being conducted to address this issue.

F. verticilliodes and the fumonisins have also become areas of great concern for regulators. The worldwide occurrence and contamination of corn can also possibly have adverse health effects on humans (IARC, in press). Recently, FB_1 was evaluated by the

International Agency for Research on Cancer and was found to be carcinogenic in rodents and possibly carcinogenic to humans (IARC, in press). In addition, nothing is known about the environmental fate of fumonisins and therefore other routes of human exposure are possible (Marasas et al., 2000). 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/of body weight/day (Bolger et al., 2001). The U.S. Food and Drug Administration final guidance for Industry was issued in 2001. These recommendations are intended to minimize exposure and prevent adverse health effects from consumption of FB_1 contaminated corn-based foods and feeds. However, nothing 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). The symptomless association between the fungus and the corn plant is important because the absence of overt signs of infection increases the possibility that fumonisin-contaminated plant residues could enter, undetected, into the soil environment. Unfortunately information regarding the level of FB_1 contamination in plant debris in the field is limited. The World Health Organization, International Programme on Chemical Safety has recommended that research needs to be conducted on the environmental fate of fumonisin (Marasas et al., 2000). Diseased corn parts often containing high levels of mycotoxins are usually not harvested and contribute to the surface-soil plant litter. Therefore it is possible that fumonisin levels in soils and water could be quite high given that 1) fumonisins are heat and light stable (Dupuy et al., 1993a; Howard et al., 1998; IARC, 1993), 2) fumonisins can accumulate to high levels in moldy corn parts and plant debris,

and 3) the majority of fumonisins consumed in contaminated feeds are rapidly excreted by farm animals.

While the amount of fumonisin that enters the environment from plant debris is unknown, it is possible to estimate the amount of FB_1 that enters the environment from farm animal waste. Corn represents a large portion of the feed ration for many commercially produced livestock commodities. Livestock production plays an important role in the economy of the U.S., so a tremendous amount of corn and other grains are produced annually to support these enterprises. The total corn crop for 1998 was 9.76 billion bushels, which translates to 250,000,000 metric tons of corn (Corn Refiners Association, 1999). Assuming that 1-2 g of fumonisin is produced per metric ton of corn (1-2 ppm), the annual total of fumonisin contained in the corn crop in 1998 is estimated to be 250 to 500 metric tons. Of the 250,000,000 metric tons of corn produced, 60% (150,000,000 metric tons) was used for animal feed, 20% was exported, and the remaining 20% was used to make cereal and other products (Corn Refiners Association, 1999). The 60% used for animal feed could have possibly contained 150-300 tons of fumonisin contamination. Potentially 90% of the fumonisin consumed by livestock ends up in litter, sewage, or on the ground each year (130-270 metric tons), since fumonisin B_1 is 1) a water-soluble mycotoxin, 2) poorly absorbed, rapidly eliminated from circulation and 3) excreted in feces. There is limited data suggesting that fumonisins can be metabolized by soil microorganisms, however, little is known about the environmental fate of fumonisins after they are excreted. Therefore, this research is necessary to determine if the potentially large amount of fumonisins entering the soil annually pose a significant threat to the ecosystem. In addition a large amount of corn plant debris is left

in the field or is plowed into the soil. In south Georgia, contamination of corn with fumonisin is a persistent problem with levels commonly exceeding 5 ppm (Mubatanhema, in press). During drought periods, as has been the case in recent years, much of the corn is not harvested and remains in the field. These drought stressed plants could have very high levels of fumonisin contamination.

Hypotheses and Objectives

The hypotheses of this research project are: 1) fumonisin is bound by constituents in soil matrices; 2) some fumonisin will pass readily through soils and be recovered in water leachate. The overall objective of this research was to develop a better understanding of the fate of fumonisin B_1 in soil ecosystems. The specific objectives of this research are to determine: 1) if FB_1 is bound in the soil, and 2) the leaching of FB_1 through two soil types.

VI. Approach

Use soil columns (leachate columns) and varying soil types to develop a model system.

The soil matrix contains many biological and physical constituents that have the potential to interact with chemicals. The biological constituents include bacteria, fungi, algae, microscopic animals, and many other creatures (Moor, 1998). Millions of bacteria live in the soil, many play important roles in the soil ecosystem. Some bacteria can take animal excrement and purify it, while others can take nitrogen from the air and in the soil and convert it into nitrates that higher plants use for growth (Moor, 1998). Soil microorganisms also can breakdown a wide variety of complex chemical compounds, for example naphthalene (Moor, 1998). Fungi are important in topsoil because they help

break down woody organic matter. Some can be parasitic or saprophytic, and others are mutualistic with plant roots, which allow both the plant and fungi to thrive (Moor, 1998). Algae are important because they take up carbon dioxide from the surface air and convert it to oxygen, which is used by humans and animals (Moor, 1998). Microscopic animals such as amoebae, protozoa and nematodes also are inhabitants of the soil (Moor, 1998). Another important soil inhabitant is the earthworm. Earthworms are important in mixing organic matter from the surface into lower depths of the soil, and they also help to aerate the soil by the burrows they produce (Moor, 1998).

In addition to the biological component, soils are both physically and chemically complex. Differences in physical and chemical properties of soils can have a profound influence on the fate of chemicals in the soil ecosystem. Soil type is distinguished by the size of its non-living or mineral content (Michaels, 2000). There are three main types of soil: clay, silt, and sandy (Michaels, 2000). Clay is the densest because of its small particle size (Michaels, 2000). Silt has medium density, and sandy soil is the least dense and most porous of the soil types (Michaels, 2000). However, mixing humus (partially decomposed, chemically stabilized organic material) into the soil creates loam, which is the best growth medium for plants (Michaels, 2000). Other factors such as pH and acidity are important soil characteristics. These factors combined determine how and at what rate the soil will react with chemicals and materials entering the matrix. Because of the amphipathic-zwitterionic chemical nature of fumonisins of the "B" series it is possible that FB_1 could react chemically with soil constituents. In particular, the reactivity of the tricarboxylic acids and the primary amino group could form ionic interactions with charged soil constituents and this would be influenced by soil pH and

the ionic composition of the soil. Two extremely different soils, and various mixtures of the two soils will be used in this study. Washed fine sand will be used to model the most simple soil system because it contains very little organic material or mineral nutrients. The second soil will be a local (Clarke County, Georgia, USA) soil type known as Cecil sandy loam. Samples of Cecil sandy loam and washed sand will be analyzed by The University of Georgia soil testing and plant analysis laboratory (Athens, GA). They will be tested for pH, organic matter (%), potassium, calcium, magnesium, zinc, manganese, and phosphorous. The Cecil sandy loam will be used in the leachate columns either as cored field samples or it will be ground in a mortar so as to produce a more homogenous mixture. The washed fine sand will be used by pouring it directly into the column and never obtained as a core.

a) *Fumonisin release from corn debris*

In order to assess the interaction of fumonisin in soils, it is important to determine the total amount of fumonisin extractable by rainfall. To conduct this experiment, corn screening contaminated with fumonisins (approx. 173 ppm total) have been obtained from P. Frank Ross (APHIS, NVSL, Ames, IA). A time course study will be conducted to compare the extraction of FB_1 using H_2O to FB_1 extraction using acetonitrile:H_2O (1:1) to determine if FB_1 can be completely extracted using H_2O alone.

Corn screenings naturally contaminated with FB_1, or water extracts of this material, will be used in studies to simulate leaching of FB_1 from corn debris through the soil, and subsequently into the ecosystem. The total amount of water-extractable FB_1 present in the contaminated corn will be determined by placing 1 g samples of FB_1 contaminated corn screenings in 125 ml Erlenmeyer flasks and adding 25 ml of H_2O.

These solutions will be capped and placed on a rotary shaker (Eberbach Corp.) for 6-12 hrs. After shaking, the extracts will be filtered through Whatman #1 filter paper, and analyzed by high performance liquid chromatography (HPLC).

b) *Fumonisin interaction with various soil models*

To better understand the dynamics of fumonisin in soils, miniature leachate columns and soil microcosms will be used. The miniature columns will be constructed of 60 ml disposable syringes attached to a BIO-RAD model 2110 fraction collector. Each miniature column will be filled with 30g of each soil type and a thin layer of glass wool will be placed on the surface of the soil to minimize surface disturbances. Extracts of fumonisin-contaminated corn screening (30 ml per column) will used to saturate the mini-columns and 1 ml fractions will be collected and analyzed by HPLC for fumonisin. Soil microcosms will be constructed of 10 x 20 cm PVC columns to be filled with test-substrates or cored soil samples. The test-substrates/soil types used to conduct this experiment will be pure sand and Cecil sandy loam. Approximately 50g of the fumonisin contaminated corn screening will then be placed on the surface of the soil in each core, each with a known level of fumonisin contamination. The rainfall simulator will then be used to apply 190 ml of water to each soil column for 5 days. The effluent from each soil column will then be collected and analyzed by HPLC for detectable levels of fumonisin.

REFERENCES

Abbas, H. K. and Shier W. T. (1997) Phytotoxicity of australifungin and fumonisins to weeds. In: Proceedings of the 1997 Brighton Crop Protection Conference-Weeds. Croydon, United Kingdom, British Crop Protection Council, pp. 795-800.

Bacon, C. W. and Hinton, D. M. (1996) "Fusaric acid and pathogen interactions of corn and non-corn isolates of *Fusarium moniliforme,* a nonobligate pathogen of corn". *Adv Exp Med Biol.* **392**:175-191.

Bacon, C. W., and Nelson, P.E. (1994) "Fumonisin production in corn by toxigenic strains of *Fusarium moniliforme* and *Fusarium proliferatum*". *J Food Pro*. **57**:514-521

Bacon, C.W., Porter, J.K., and Norred, W.P. (1995) "Toxic interaction of fumonisin B_1 and fusaric acid measured by injection into fertile chicken eggs". *Mycopathologia*. **129**:29-35.

Bacon, C.W., Yates, I.E., Hinton, D.M., and Meredith, F.I. (2001) "Biological control of *Fusarium moniliforme* in maize". *Environ Health Perspect*. **109**:325-332.

Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C.P., Horak, R.M., Marasas, W.F.O., Spiteller, G., and Vleggaar, R. (1988) "Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*". *J Chem Soc Chem Commun*. **1988**:743-745.

Blackwell, B. A., Gilliam, J. T., Savard, M. E., Miller, J. D. and Duvick, J. P. (1999) "Oxidative deamination of hydrolyzed fumonisin B_1 (AP(1)) by cultures of *Exophiala spinifera*". *Nat Toxins*. **7**:31-38.

Brown, T. P., Rottinghaus, G. E., Williams, M. E. (1992) "Fumonisin mycotoxicosis in broilers: performance and pathology". *Avian Dis*. **36**:450-54.

Bryden, W. L., Love, R. J. Burgess, L. W. (1987) "Feeding grain contaminated with *Fusarium graminearum* and *Fusarium moniliforme* to pigs and chickens". *Aust Vet J*. **64**:225-226.

Bolger, M., Coker, R.D., Dinovi, M., Gaylor D., Gelderblom, W.C.A., Paster, N., Riley, R.T., Shephard, G. and Speijers, J.A. (2001) Fumonisins. In: *Safety evaluation of certain mycotoxins in food*. Food and Agriculture Organization of the United Nations, paper **74**. World Health Organization food additives, series **47** pp. 103-279.

Butler, T. (1902) "Notes on a feeding experiment to produce leucoencephalitis in a horse, with positive results". *Am Vet Rev.* **26**:748-51.

Chu, F. S., Huang X., Maragos, C. M. (1995) "Production and characterization of anti-idiotype and anti-anti-idiotype antibodies against fumonisin B_1". *J Agric Food Chem*. **43**, 261-267.

Cook, R. J. Water relations in the biology of *Fusarium*. In *Fusarium: Diseases, Biology, and Taxonomy*; Nelson, P.E., Toussoun, T. A., Cook, R. J., Eds., The Pennsylvania State University Press: University Park, 1981, pp 236-244.

Corn Refiners Association. (1999) "Frequently Asked Questions." <http://www.corn.org /web/faq.htm>.

Desjardins, A. E., Plattner, R. D. (1998) "Distribution of fumonisins in maize ears infected with strains of *Fusarium moniliforme* that differ in fumonisin production". *Plant Dis*. **82**:953-958.

Dombrink-Kurtzman, M.A., Javid, T., Bennett, G.A., Richard, J.L., Cote, L.M., and Buck, W.B. (1993) "Lymphocyte cytotoxicity and erythrocytic abnormalities induced in broiler chicks by fumonisins B_1 and B_2 and moniliformin from *Fusarium proliferatum*". *Mycopathologia.* **124**: 47-54.

Dupuy J, Le bars P, Boudra H, and Le Bars J. (1993a) "Thermostability of fumonisin B_1, a mycotoxin from *Fusarium moniliforme*, in corn". *Appli Environ Microbiol*. **59**: 2864-2867.

Duvick, J. (2001) "Prospects for reducing fumonisin contamination of maize through genetic modification". *Environ Health Perspect*. **109**:337-342.

Foley, D. C. (1962) "Systemic infections of corn by *Fusarium moniliforme*". *Phytopathology*. **52**:870-872.

Harrison, L. R., Colvin, B. M., Greene, J. T., Newman, L. E., and Cole, R. J. (1990) "Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*". *J Vet Diagn Invest*. **2**:217-221.

Henry, M.H., and Wyatt, R.D. (2000) "The toxicity of purified fumonisin B_1 in broiler chicks". *Poult Sci*. **79**:1378-1384.

Henry, M.H., and Wyatt, R.D. (2001) "The toxicity of fumonisin B_1, B_2, and B_3, individually and in combination, in chicken embryos". *Poult Sci*. **80**:401-407.

Howard, P.C., Churchhill, M.I., Couch, L.H., Marques, M.M., and Doerge, D.R. (1998) "Formation of *N*-(carboxymethyl) fumonisin B_1, following the reaction of fumonisin B_1 with reducing sugars". *J Agri Fd Chem*. **46**:3546-3557.

IARC, International Agency for Research on Cancer (1993) "IARC Monographs on the Evalution of Carcinogenic Risk to Humans". IARC, Lyon, France. **56**: 445-466.

IARC (in press) "IARC Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Humans, Vol. 82, Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene", in press.

Javed, T., Bennett, G. A., Richard, J. L., Dombrink-Kurtzman, M. A., Cote L.M., and Buck W.B. (1993a) "Mortality in broiler chicks on feed amended with *Fusarium proliferatum* culture material or purified fumonisin B_1 and moniliformin". *Mycopathologia*. **123**:171-184

Javed, T., Richard, J. L., Bennett, G. A., Dombrink-Kurtzman, M. A., Bunte, R. M. (1993b) "Embryopathic and embryocidal effects of purified fumonisin B_1 or *Fusarium proliferatum* culture material extract on chicken embryos". *Mycopathologia*. **123**:185-193

Joffe, A. Z., Palti, J., and Arbel-Sherman, R. (1973) "*Fusarium moniliforme* Sheld. In Israel (*Gibberella fujikuroi* [Saw.] Wollenw.)". *Mycopath Mycol Appl*. **50**:85-107.

Koehler, B. (1938) "Fungus growth in shelled corn as affected by moisture". *J Agri Res*. **56**:291-307.

Kriek, N. P. J., Kellerman, T. S., Marasas, W. F. O. (1981) "A comparative study of the toxicity of *Fusarium verticilliodies* (=*F*. *moniliforme*) to horses, primates, pigs, sheep and rats". *Onderstepoort J Vet*. **48**:129-131.

Klittich, C. J., Leslie, J. F. (1992) "Identification of a second mating population within the *Fusarium moniliforme* anamorph of *Gibberella fujikuroi*". *Mycologia.* **84**: 541-547.

Kommedahl, T., Windels, C. E. Root-,stalk-, and ear-infecting *Fusarium* species on corn in the USA. In *Fusarium*: Diseases, Biology and Taxonomy; Nelson, P. E.; Toussoun, T. A., Cook, R. J., Eds., The Pennsylvania State University Press: University Park, 1981; pp 94-103.

Marasas, W. F. O., Kellerman, T. S., Gelderblom, W. C. A., Coetzer, J. A. W., Thiel, P. G. (1988) "Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme*" *Onderstepoort J Vet Res.* **55**:197-203.

Marasas, W. F. O., van Rensberg, S. J., Mirocha, C. J. (1979) "Incidence of *Fusarium* species and the mycotoxins, deoxynivalenol and zearalenone, in corn produced in esophageal cancer areas in Transkei". *J Agric Food Chem*. **27**:1108-1112.

Marasas, W.F.O., Miller, J.D., Riley, R.T., and Visconti, A. (2000) *Environmental Health Criteria 219: fumonisin B_1*, International Programme on Chemical Safety, United Nations Environmental Programme, the International Labour Organization and the World Health Organization. Geneva, 150 pp.

Michaels, Patricia. (2000) "Soil Types." <http://environment.about.co…y/weekly/blsoil2,htm?iam=mt&terms=%2Bsoil.htm>.

Miller, M. A., Honstead, J. P., and Lovell, R. A. (1996) "Regulatory aspects of fumonisins with respect to animal feed". *Adv Exp Med Biol*. **392**:363-368.

Moor, Fred. (2000)"Soil Inhabitants." <http://homepages.which.net/~fred.moor/soil/ formed/f0105.htm>.

Mubatanhema, W., Jurjevic, Z., Wilson, D.M., Widstrom, N.W., Meredith, F., and Evans, B. (in press) "Fumonisins and aflatoxins in preharvest corn in south Georgia: A five year survey". *Mycopathologia*.

Munkvold, G. P.,Desjardins, A.E. (1997) "Fumonisins in maize: Can we reduce their occurrence?" *Plant Dis*. **81**:556-565.

Musser S.M., and Plattner, R.D. (1997) "Fumonisin composition in cultures *of Fusarium moniliforme, Fusarium proliferatum, and Fusarium nygami*". *J Agri Fd Chem*, **45**:1169-1173.

NTP Technical Report. (1999) "Toxicology and carcinogenesis studies of fumonisin B_1 on f344/N rats and B6C3F1 mice." NTP TR 496 NIH Publication No. 01-3955.

Osweiller, G.D., Ross, P.F., Wilson, T.M., Nelson, P.E., Witte, S.T. (1992) "Characterization of an epizootic of pulmonary edema in swine associated with fumonisin in corn screenings". *J Vet Diag Invest*. **4**:53-59.

Plattner, R.D. (1995) "Detection of fumonisins produced in *Fusarium moniliforme* cultures by HPLC with electrospray MS and evaporative light scattering detectors". *Nat Toxins*. **3**:294-298.

Rheeder, J.P., Marasas, W.F.O, Thiel, P.G., Sydenham, E.W., Shephard, G.S., and van Schalkwyk, D.J. (1992) "*Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei". *Phytopathology*. **82**:353-357.

Richard, J.L., Bennett, G.A., Ross, P.F., Nelson, P.E. (1993) "Analysis of naturally occuring mycotoxins in feedstuffs and food". *J Anim Sci*. **71**, 2563-2574.

Riley, R.T., Norred, W.P., and Bacon, C. W. (1993) "Fungal toxins in foods: recent concerns". *Annu Rev Nutr*. **13**:167-189.

Riley, R.T., and Norred, W.P. (1996) "Mechanism of mycotoxicity". *The Mycota VI*. **10**:194-211.

Riley, R.T., Voss, K.A., Norred, W.P., Wang, E., and Merrill, A.H. Jr. (1998) "Fumonisin: mechanism of mycotoxicity". *Rev Med Vet*, **149**:617-626.

Rose, E. F. Esophageal cancer in Transkei—The pattern and associated risk factors. In *Cancer of the Esophagus*, pp. 19-29. Boca Raton, Fla: CRC Press, 1982.

Segal, I., Reinach, S. G., de Beer, M. (1988) "Factors associated with oesophageal cancer in Soweto, South Africa". *Br J Cancer* **56**:681-86.

Shephard, G.S., and Snijam, P.W. (1999) "Elimination and excretion of a single dose of the mycotoxin fumonisin B_2 in non-human primate". *Food Chem Toxicol*. **37**:111-116

Shurtleff, M. C. *Compendium of Corn Diseases*, 2nd ed., The American Phytopathological Society: St. Paul, MN, 1980.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Marasas, W.F.O. Stockenstrom, S. (1991) "Fumonisin contamination of commercial corn-based human foodstuffs". *J Agric Food Chem.* **39**:2014-2018.

Thiel, P.G., Shephard, G.S., Sydenham, E.W., Marasas, W.F.O., Nelson, P.E. and Wilson, T.M. (1991) "Levels of fumonisin B_1 and B_2 in feeds associated with confirmed cases of equine leukoencephalomalacia". *J Agric Food Chem*. **39**:109-111.

Thomas, M. D., Buddenhagen, I. W. (1980) “Incidence and persistence of *Fusarium moniliforme* in symptomless maize kernels and seedlings in Nigeria”. *Mycologia*. **72**:882-887.

Tuite, J.F., Haugh, C.G., Issacs, G.W., and Huxsoll, C.C. (1967) “Growth and effect of molds in stored high-moisture corn”. *Trans Am Soc Agric Eng*. **10**:730-737.

Van Rensberg, S. J. (1985) “Recent studies on the etiology of oesophageal cancer”. *S Afr Cancer Bull*. **29**:22-31.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., and Merrill, A.H. Jr. (1991) “Inhibition of sphingolipid biosynthesis by fumonisins: implications for diseases associated with *Fusarium moniliforme*”. *J Biol Chem*. **266**:14486-14490.

Wilson, B.J., Maronpot, R.R. (1971) “Causative fungal agent of leucoencephalomalacia in equine animals”. *Vet Rec*. **88**:484-486.

KINETICS OF LEACHING AND BINDING OF FUMONISIN B_1 IN SOIL MICROCOSMS[1]

[1]Lonnie D. Williams, Charles W. Bacon, Filmore I. Meredith, Alan J. Franzluebbers, Mary A. Smith, Roger D. Wyatt and Ronald T. Riley. To be submitted to the *Journal of Agriculture and Food Chemistry*.

ABSTRACT

Fumonisin B_1 (FB_1) is a water-soluble mycotoxin produced by *Fusarium verticillioides*. Research objectives were to determine the leaching of FB_1 through soils and FB_1 binding in soil. Leachate columns were used to determine the movement of FB_1 through soil. FB_1-contaminated corn screenings or water extracts containing FB_1 were placed on the surface of soil columns. In 100% sand columns FB_1 leaching was only slightly retarded, whereas, at 50%, 75% and 100% Cecil sandy loam, approximately 60%, 50% and 20% of the FB_1 was recovered in the column leachate, respectively. The FB_1 retained on the 100% Cecil sandy loam column was tightly bound. However, approximately 75% of the bound FB_1 was released with 5% formic acid and 5% formic acid:acetonitrile (1:1) indicating the nature of the interaction was probably ionic. The results suggest that FB_1 is quite stable in soils and, while tightly bound, under certain environmental conditions could be released.

INDEX WORDS: Fumonisin B_1, Cecil sandy loam, *Fusarium verticilliodes*, corn

INTRODUCTION

Fumonisins are mycotoxins produced by the fungus *Fusarium verticilliodes* (syn = *F. moniliforme*). *F. verticilliodes* 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, sugar cane, several forages and many other commercially important crops and plants (1). Extensive research has been done on the parasitism of corn because this fungus may produce a wide variety of chemically different mycotoxins on corn (2). Diseases of corn associated with *F. verticilliodes* contamination include: seed rot, seedling blight, root rot, stalk rot, and kernel or ear rot (3-5). At present, at least 15 different fumonisins have been reported and other minor metabolites have been identified, although some of them do not occur naturally (6-8). Fumonisin B_1 (FB_1) (Fig. 1) is the most abundant of the fumonisins, which occur naturally (6-8). The pure substance is an amphipathic zwitterion and is water soluble, and heat and light stable (9-11).

Animal diseases associated with FB_1 include equine leukoencephalomalacia, porcine pulmonary edema, and liver and kidney carcinogenesis in rodents. There have also been studies, which suggest that FB_1 plays a role in increased incidence of esophageal cancer in humans in southern Africa (11).

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 (11). Thus, most of the fumonisin consumed by farm animals is rapidly returned to the ground intact, or as their partially hydrolyzed metabolites.

F. verticilliodes and the fumonisins have become areas of concern for corn producers, processors, consumers, and regulators. The occurrence and contamination of corn is worldwide and can have health effects on animals and possibly humans (12). Recently, FB_1 was evaluated by the International Agency for Research on Cancer and the evaluation was that there is sufficient evidence in experimental animals for the carcinogenicity of FB_1. The overall evaluation was that FB_1 is possibly carcinogenic to humans (Group 2B) (12). In addition, nothing is known about the environmental fate of fumonisins and therefore other routes of human exposure are possible (11). 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 (13). The U.S. Food and Drug Administration final guidance for Industry was issued in 2001. These recommendations are intended to minimize exposure and prevent adverse health effects from consumption of FB_1 contaminated corn-based foods and feeds. However, since nothing is known about the environmental fate of fumonisins, other routes and sources of exposure could contribute to the total fumonisin intake by humans and animals and the human exposure could be underestimated. For this reason, the World Health Organization, International Programme on Chemical Safety has recommended that research needs to be conducted on the environmental fate of fumonisin (11).

Diseased corn parts infected with *F. verticilliodes* that are not harvested could contribute to the surface-soil plant litter. It is possible that fumonisin levels in soils and water could be quite high given that 1) fumonisins are highly water soluble, heat and light stable (9-11), 2) fumonisins can accumulate to high levels in moldy corn parts and plant

debris (11), and 3) the majority of fumonisins are rapidly excreted by farm animals (11). Thus, FB_1 contamination of ground water and airborne dust could contribute to human and animal exposure to fumonisin.

While the amount of fumonisin that enters the environment from plant debris is unknown, it is possible to estimate the amount of FB_1 that enters the environment from farm animal waste. Corn represents a large portion of the feed ration for many commercially produced livestock commodities. Livestock production plays an important role in the economy of the USA, so tremendous amounts of corn and other grains are produced annually to support these enterprises. The total corn crop for 1998 was 9.76 billion bushels, which translates to 250,000,000 metric tons of corn (14). Assuming that 1 to 2 g of fumonisin is produced per metric ton of corn (1-2 ppm), the annual total of fumonisin estimated in the 1998 corn crop could be 250 to 500 metric tons. Of the 250,000,000 metric tons of corn produced, 60% (150,000,000 metric tons) was used for animal feed, 20% was exported, and the remaining 20% was used to make cereal and other products (14). The 60% used for animal feed could have contained 150 to 300 tons of fumonisin contamination. Since FB_1 is 1) a water-soluble mycotoxin, 2) poorly absorbed, rapidly eliminated from circulation, and 3) excreted in feces. Potentially 90% of the fumonisin consumed by livestock ends up in litter, sewage, or on the ground each year (130-270 metric tons).

There is limited data suggesting that fumonisins can be metabolized by soil microorganisms, however, little is known about the environmental fate of fumonisins after they are excreted. In addition a large amount of corn plant debris is left in the field or is plowed into the soil. During drought periods, much of the corn grown is not

harvested and remains in the field. These drought stressed plants could have very high levels of fumonisin contamination. The purpose of this study was to determine if fumonisins entering the soil could also enter the ground water and contribute to human, animal, and plant exposure. Two model systems were used. The first was miniature columns packed with soils and the second was intact soil cores. The specific objectives were to determine 1) the leaching of FB_1 through soils, and 2) if FB_1 is bound in the soil.

MATERIALS AND METHODS

Fumonisin(Test Material). Corn screenings naturally contaminated with FB_1, or water extracts of this material, were used in studies to simulate leaching of FB_1 from corn debris through the soil, and subsequently into the ecosystem. The total amount of water-extractable FB_1 present in the contaminated corn was determined by placing 1 g samples of FB_1 contaminated corn screenings in 125 ml Erlenmeyer flasks and adding 25 ml of H_2O. These solutions were capped and placed on a rotary shaker (Eberbach Corp.) for 6 h. After shaking, the extracts were filtered through Whatman #1 filter paper, and analyzed by (to be described later) high performance liquid chromatography (HPLC). Water was used as the extraction solution and this was compared to the FB_1 extraction efficiency of acetonitrile:H_2O (1:1) in a time course study (15 min to 96 h).

When miniature columns were used, the test material was water extracts of the corn screenings containing FB_1 and when intact soil cores were used (to be described later) FB_1 contaminated corn screenings were placed directly on the surface of the soil and water was allowed to percolate through the screenings extracting the FB_1 and allowing it to enter the soil.

HPLC Method. Samples of the extracts (100 µl) were combined with 500 µl *ortho*-phthalaldehyde (OPA, Sigma Chemical Company) derivatizing reagent and 500 µl of acetonitrile:water (1:1). Samples and reagents were maintained at 4^{o}C, and mixing of reagents and derivatization was accomplished at 4^{o}C using a Shimadzu model SIL-9A programmable auto-injector. The derivatized samples (50 µl) were injected 3 min after mixing with the OPA reagent, and separation was accomplished using a Microsorb^{tm} C18 column (3 µm particle size, 4.6 mm ID x 5 cm L Rainin Instrument Company, Woburn, MA), maintained at 27^{o}C with a mobile phase of methanol:1% phosphoric acid in water (66:34) and a flow rate of 0.8 ml/ min. OPA-positive substances were detected using a Shimadzu RF-551 spectrofluorometric detector at 335 nm excitation and emission cutoff filter at 440 nm. FB_1 standard (>96%) (15) was injected after every three unknown samples and FB_1 concentration in unknown samples was quantified based on the areas under the peaks.

Test Soils used in Leachate Columns. Two extremely different soils, and various mixtures of the two soils were chosen for study. Washed fine sand was chosen to model the most simple soil system and contained very little organic material or mineral nutrients (Table 1). The second soil was a local (Clarke County, Georgia) soil type known as Cecil sandy loam. Samples of Cecil sandy loam and washed sand were analyzed by The University of Georgia soil testing and plant analysis laboratory (Athens, GA). Samples were tested for pH, organic matter (%), potassium, calcium, magnesium, zinc, manganese, and phosphorous. Cecil sandy loam contains a mixture of silt, clay and sand and a much higher content of organic material and mineral nutrients than the washed fine sand (Table 1). The Cecil sandy loam was used in the leachate columns either as

cored field samples (see Fig. 2A) or it was air dried, pebbles and rocks removed, and the remaining material was gently ground in a mortar so as to produce a more homogenous mixture. The washed fine sand soil was always used by pouring directly into the column and never obtained as a core.

Miniature Leachate Columns, FB_1 Binding to Soil. To determine if FB_1 interacts with soil constituents, miniature leachate columns (60 cc syringes) (Fig. 2B) were used with either washed sand alone, or a homogenous mixture of washed sand and Cecil sandy loam. Mixtures of 50, 75, and 100% Cecil sandy loam soil and washed sand were used. Water extracts of the FB_1 contaminated corn test material (previously described) were percolated through the miniature columns (n=3) and fractions were collected (1 ml each) over a 24-h period and analyzed to determine if FB_1 interacted with the soil constituents so as to retard or otherwise affect the retention or recovery of FB_1 in the eluate from the leachate columns.

The FB_1 recovery based on HPLC analysis was compared to the movement of bromophenol blue (Bb), a dye that moved freely through sand columns and the sand and soil mixtures. A stock solution of Bb dye was prepared by mixing 0.5 g Bb dye with 200 ml H_2O; the absorbance at 590 nm was approximately one. Aliquots (20 to 30 ml) of the dye solution were allowed to percolate through the small columns containing the previously described sand and sand/soil mixtures and 1 ml fractions were collected over a 24 h-period. The absorbance of these fractions was measured using a Beckman DU-65 spectrophotometer, and the values were compared to the absorbance of the stock dye solution. The absorbance values for the 1 ml fractions were greater than 80% of the maximum absorbance, which suggested that the dye had a relatively low affinity for the

sand or sand/soil mixtures used in the miniature columns. Void volume was estimated by weighing 30 g samples of 100% sand, 50% sand/50 % Cecil sandy loam and 100% Cecil sandy loam and mixing the soil with 30 ml of water and then centrifuging at 240 relative centrifugal force (rcf) for 60 min and measuring the volume of the supernatant. The void volume was calculated based on the water retained in the soil. The estimated void volumes in 30 g of 100% sand, 50% sand/50 % Cecil sandy loam and 100% Cecil sandy loam were 7.5±0.5, 10.5±0.5, and 14.5±0.5 ml (n=6), respectively.

Intact Soil Core Columns. To determine the interaction of FB_1 with soils as they occur in the field, a model soil microcosm was constructed, which consisted of cores (Fig. 2A) of Cecil sandy loam soil. PVC columns filled with washed sand were also prepared and used in a similar manner. The cores were obtained from a local USDA field station in Watkinsville, Georgia. The soil core columns consisted of three 10 x 20 cm PVC columns of cored soils with fine nylon screens at the bottom. A funnel was attached to the bottom of the columns, which allowed the eluate to be collected into 500 ml Erlenmeyer flasks. Rainfall (2.4 $cm^3/cm^2/day$) was simulated using a 10 x 5 cm PVC column with a PVC base perforated with 1/16" holes in a 1 cm^2 grid pattern. Finely ground FB_1 contaminated corn screenings were placed on the surfaces of the cores (50 g per core). The rainfall simulator was used to apply 190 ml H_2O to the surface of each core, which allowed the H_2O and any FB_1 it eluted to percolate through the soil. The H_2O was applied at hourly intervals for 10 h to simulate 10 consecutive days of rainfall (Fig. 5). The effluent from each application was collected and analyzed by HPLC for the presence of FB_1. Leaching of FB_1 through soil cores was compared to leaching of FB_1 through PVC columns filled with washed sand.

To determine if FB_1 is bound to soil constituents, an acid displacement procedure was developed using mixtures of sand and soil. The procedure consisted of mixtures (30 g) containing 100% sand, 50% sand/ 50% Cecil sandy loam soil, or 100% Cecil sandy loam, which were mixed in 50 ml culture tubes with FB_1 aqueous solutions containing either 188 μg FB_1/ml or 33 μg FB_1/ml. The tubes were shaken for 12 hr and centrifuged at 240 rcf, supernatants removed and aliquots analyzed by HPLC for FB_1. The soil samples were then consecutively extracted with acetonitrile:H_2O (1:1), 5% formic acid, and acetonitrile:5% formic to determine if the bound FB_1 could be extracted using more non-polar or more polar solvents. Formic acid was used because it was hypothesized that there might be a strong ionic interaction between FB_1 and the soil. Acetonitrile 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, centrifuged and FB_1 in the supernatant determined by HPLC. Each sample was similarly extracted with 5% formic acid and then acetonitrile:5% formic. HPLC analysis of Cecil sandy loam extracted with acetonitrile:water (1:1) or 5% formic acid before adding FB_1 revealed no OPA-positive substances other than the peak for OPA itself which elutes within a minute of injection (data not shown).

Statistical Analysis. Statistical analysis was done using Sigma Stat software (Jandel Scientific, San Rafael, CA). One way analysis of variance (ANOVA) was used followed by tests for post hoc multiple comparisons where appropriate. All data were expressed as mean $\pm$ SD, and differences among means were considered significant if the probability was <0.05. For the time course study the data were analyzed by non-linear regression analysis and ANOVA.

RESULTS AND DISCUSSION

Fumonisin B_1 is rapidly and completely extracted from corn screenings using either pure water or acetonitrile:water (1:1) (Fig. 3). After 24 h extraction with water there was evidence of microbial growth as determined by microscopic examination and culture on potato dextrose agar (PDA) plates. This increased microbial growth was associated with a significant time-dependent decrease in the average concentration of FB_1 extractable by water based on linear regression analysis, although there were no significant differences among groups based one-way analysis of variance (Fig 3A). These results alleviated the need for extensive extraction periods because it showed that maximum extraction of FB_1 was achieved within 4 h by either method.

Fumonisin B_1 is able to move rapidly through both simple and more complex soils intact. Elution of FB_1 through the miniature columns, applied to the surface of washed sand as water extracts of the corn screenings (123.15 ± 3.59 μg FB_1/30 ml), was slightly retarded relative to the elution of Bb dye (Fig. 4A). The calculated void volume of the miniature columns filled with sand was approximately 7.5 ml. Thus, both the Bb dye and the FB_1 first appeared in the void volume but did not attain equilibrium until after the void volume had passed through the column. After 20 ml of the FB_1 solution had passed through the column, the concentration of FB_1 in the leachate was approaching the concentration that was in the original water extracts. The average cumulative total of FB_1 recovered in the leachate was 98 μg FB_1, which was 80% of the total FB_1 calculated in 20 ml of water extract. Both Bb dye and FB_1 leaching exhibited a short initial lag

phase, rapid elution phase, and a slowly eluted terminal phase, kinetics consistent with a three-compartment model.

Elution of FB_1 through miniature columns containing 50, 75, and 100% Cecil sandy loam showed a decrease in FB_1 elution as soil concentration increased (Fig 4B, C and D). At 50, 75, and 100% Cecil sandy loam, the concentration of FB_1 in the leachate was approximately 60, 50, and 20% of the originally applied FB_1 concentration, respectively, after 25 to 30 ml of the solution had passed through the columns (Fig. 4B, C, and D). Leaching of Bb dye through the Cecil sandy loam (Fig, 4B, C, and D) columns followed a pattern similar to that seen with pure sand indicating that Bb dye did not interact with the soil in the same manner as FB_1. Nonetheless, both Bb dye and FB_1 leaching included a rapid elution phase followed by a slowly eluted terminal phase, kinetics consistent with a two-compartment model. The elution profiles of FB_1, applied to the surface of leachate columns containing 50, 75, and 100% Cecil sandy loam (Figs. 4B, C and D), as water extracts of the corn screening, indicated that FB_1 was either chemically altered or was more tightly retained by the Cecil sandy loam relative to either washed sand alone (Fig. 4A) or Bb dye (Fig. 4A, B, C and D).

In order to determine if the behavior of FB_1 in a field soil sample would mimic that seen in the miniature columns, FB_1 leaching through PVC columns filled with washed sand was compared to FB_1 leaching through intact field cores (n=3) of Cecil sandy loam soil (Fig. 5). Approximately 40% of the applied FB_1 was eluted from the soil cores in the first 10 applications, and after the fifth application, the elution profile appeared to be reaching a steady state. However, FB_1 passed rapidly through the

columns filled with sand, and 100% of the FB_1 applied to the columns was recovered in the leachate after 10 water applications (Fig. 5).

In order to determine the nature of the interaction between Cecil sandy loam and FB_1, soil samples were mixed with aqueous solutions of FB_1 and then extracted with various solutions as described in the methods. FB_1 did not bind appreciably to sand (Fig. 6A) but was tightly bound by the Cecil sandy loam soil (Fig. 6B and C) as evidenced by the fact that very little free FB_1 was detected in the water or acetonitrile:water supernatants (Fig. 6B and C). The fact that FB_1 was not extractable from Cecil sandy loam with acetonitrile:H_2O suggests that the interaction between FB_1 and soil is not hydrophobic. However, extracts using either 5% formic acid or acetonitrile:5% formic acid (Fig. 6B and C), released approximately 75% of the FB_1; suggesting that the interaction between the Cecil sandy loam and FB_1 was ionic.

The result of this study indicates that FB_1 is probably quite stable in the soil environment and suggests that while FB_1 is tightly bound, it could be released and become biologically available under certain environmental conditions. If FB_1 is mobilized by rainwater then it can enter and move through both simple and more complex soils and possibly be recovered chemically intact in the leachate. Therefore, it is possible that FB_1 could alter the biological activity in the soil flora (6, 16) and fauna. It is also possible that FB_1 from corn debris in field situations could enter the groundwater. In addition, the more complex the soil, the more likely that FB_1 will be retained in the soil matrix, however, this does not preclude that it is not biologically available. Also, acid conditions could facilitate the mobilization of FB_1 bound presumably via ionic interactions with soil constituents.

SAFETY

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

ABBREVIATION

FB_1, fumonisin B_1; Bb, Bromophenol blue

ACKNOWLEDGEMENTS

I would like to thank P.F. Ross for supplying the corn screenings used as the test material. and J.L. Showker for technical assistance.

LITERATURE CITED

1. Bacon, C. W. and Nelson, P. E. Fumonisin production in corn by toxigenic strains of *Fusarium moniliforme* and *Fusarium proliferatum*. *J Food Protect*. **1994,** 57:514-521.

2. Bacon, C. W. and Hinton, D. M. Fusaric acid and pathogen interactions of corn and non-corn isolates of *Fusarium moniliforme,* a nonobligate pathogen of corn. *Adv Exp Med Biol.* **1996**, 392:175-191.

3. Cook, R. J. Water relations in the biology of *Fusarium*. In *Fusarium: Diseases, Biology, and Taxonomy*; Nelson, P.E., Toussoun, T. A., Cook, R. J., Eds., The Pennsylvania State University Press: University Park, PA, **1981**, pp 236-244.

4. Shurtleff, M. C. *Compendium of Corn Diseases,* 2nd ed., The American Phytopathological Society: St. Paul, MN, **1980**.

5. Kommedahl, T.; Windels, C. E. Root-,stalk-, and ear-infecting *Fusarium* species on corn in the USA. In *Fusarium*: diseases, biology and taxonomy; Nelson, P. E.; Toussoun, T. A.; Cook, R. J., Eds., The Pennsylvania State University Press: University Park, **1981**; pp 94-103.

6. Abbas, H. K. and Shier W. T. Phytotoxicity of australifungin and fumonisins to weeds. In: Proceedings of the 1997 Brighton Crop Protection Conference-Weeds. Crydon, United Kingdom, British Crop Protection Council, **1997**, pp. 795-800.

7. Musser, S.M. and Plattner, R.D. Fumonisin composition in cultures *of Fusarium moniliforme, Fusarium proliferatum, and Fusarium nygami*. *J Agric Food Chem*. **1997,** 45:1169-1173.

8. Plattner, R.D. Detection of fumonisins produced in *Fusarium moniliforme* cultures by HPLC with electrospray MS and evaporative light scattering detectors. *Nat Toxins*. **1995**, 3:294-298.

9. Dupuy, J.; Le bars, P.; Boudra, H. and Le Bars, J. Thermostability of fumonisin B_1, a mycotoxin from *Fusarium moniliforme*, in corn. *Appli Environ Microbiol*. **1993a,** 59:2864-2867.

10. Howard, P.C.; Churchhill, M.I.; Couch, L.H.; Marques, M.M. and Doerge, D.R. Formation of *N*-(carboxymethyl) fumonisin B_1, following the reaction of fumonisin B_1 with reducing sugars. *J Agric Food Chem*. **1998**, 46:3546-3557.

11. Marasas, W.F.O.; Miller, J.D.; Riley, R.T. and Visconti, A. *Environmental Health Criteria 219: fumonisin B_1,* International Programme on Chemical Safety, United Nations Environmental Programme, the International Labour Organization and the World Health Organization. Geneva, **2000**, 150 pp.

12. IARC (in press) IARC Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Humans, Vol. 82, Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene, in press

13. Bolger, M.; Coker, R.D.; Dinovi, M.; Gaylor D.; Gelderblom, W.C.A.; Paster, N.; Riley, R.T.; Shephard, G. and Speijers, J.A. Fumonisins. *In Safety evaluation of certain mycotoxins in food*. Food and Agriculture Organization of the United Nations, paper 74. World Health Organization food additives, **2001** ; series 47., pp. 103-279.

14. Corn Refiners Association. "Frequently Asked Questions." <http://www.corn.org /web/faq.htm>. **1999**.

15. Meredith, F.I.; Bacon, C.W.; Plattner, R.D. and Norred, W.P. Preparative LC isolation and purification of fumonisin B_1 from rice culture. *J Agric Food Chem*. **1996**, 195-198.

16. Abbas H.K.; Tanaka, T.; Duke, S.O.; Porter, J.K.; Wray, E.M.; Sessions, A.E.; Wang, E.; Merrill, A.H. Jr.; and Riley, R.T. Fumonisin-and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. *Plant Physiol.* **1994**, 106:1085-1093.

FIGURES LEGEND

Figure 1. Chemical structure of fumonisin B_1.

Figure 2. Example of a leachate column for obtaining soil cores from the field (A), and a leachate column for testing homogenous mixtures of soils (B). Briefly, column "A" is fabricated from a 10 x 20 cm piece of thick walled PVC pipe with a bevel cut at the bottom to facilitate obtaining field soil cores. Field cores were obtained by forcing the columns down into the soil using approximately 2 tons of pressure. The columns were carefully removed by excavating around the base of the columns. The soil cores obtained in this manner were tightly adherent to the inside surface of the PVC columns. To simulate rainfall, water was added to the rain simulator (shown to the left) and allowed to drain into the PVC leachate column. The rain simulator is a flat piece of perforated PVC glued to a piece of PVC pipe. The column shown in "B" (60 cc syringe) was used in experiments where FB_1 was in water solution and was allowed to pass through homogenous sand or sand/soil mixtures. Syringes containing glass micro-fiber filters, were filled with either washed sand or the homogenous mixture of sand and soil (30 g). The filled columns were placed in an elevated clamp attached to a ring stand and tubing connected the columns to a BIO-RAD model 2110 fraction collector.

Figure 3. Comparison of the extraction of FB_1 from 1 g of finely ground (1 mm screen in a Thomas Wiley mill) naturally contaminated corn screening by distilled H_2O and 1:1 acetonitrile:water. The pH of the acetonitrile extracts were adjusted to 4.5 with 6N HCl. The values are expressed as the means±SD (n=3 to 5). One way analysis of variance

indicated that there were no significant differences among groups in either the water or acetonitrile:water extractions groups. However, linear regression analysis of the means with time indicated that there was a significant decrease of FB_1 content with time in the water extracted sample ($F = 7.9$, $p=0.009$, $df = 26$).

Figure 4. Comparison of the FB_1 concentration (open diamonds) and the bromophenol blue (Bb) absorbance at 590 nm (solid circles) in column leachate expressed as a percent of the FB_1 concentration (123.15 ± 3.59 μg FB_1/30 ml) or the Bb absorbance at 590 nm, respectively, of the solutions originally placed on the soil surface of the columns. Columns were loaded with washed sand (A) or homogenized mixtures of 50% (B), 75% (C), or 100% (D) Cecil sandy loam. FB_1 was water extracted from 1 g of corn screenings and Bb was dissolved directly into water and added on top of the sand or soil mixture. A total of 20 to 30 one-milliliter fractions of the eluate were collected and analyzed for FB_1 or in the case of Bb, absorbance at 590 nm, and these values were compared to the total FB_1 placed on the columns and the maximum absorbance of the stock dye solution. The results are expressed as the means±SD (n=3).

Figure 5. Elution of FB_1 from Cecil sandy loam field cores (open diamonds) was compared to FB_1 elution through columns filled with washed sand (solid circles). Intact field cores of Cecil sandy loam soil were obtained as described in Figure 2, and 50 g samples of FB_1 contaminated corn screenings were placed on the surface of both Cecil sandy loam and washed sand columns. Rainfall (10 days) was simulated by hourly application of 190 ml H_2O for 10 hrs. After which, the collected eluates from the soil

cores and sand columns were analyzed by HPLC for FB_1. The values were expressed as a percentage of the total FB_1 applied to the columns.

Figure 6. The ability of soils to bind FB_1. Briefly, conical tubes (50 ml) were filled with 100 % sand (A), 50% sand/50% Cecil sandy loam (B), and 100% Cecil sandy loam (C), and used to test the ability of soils to bind FB_1 and test the ability of the various extraction solutions to extract any FB_1 bound to the soil. The extraction solutions used were water, acetonitrile:water (ACN:H_2O), 5% formic (F) and ACN:5% F. Aliquots (30 ml) of an FB_1 water-extracted solution were added to each tube, the tubes were then shaken for 12 hrs and centrifuged at 240 rcf and the supernatant removed. The retention of FB_1 in the soils was determined by comparing the FB_1 samples taken from the supernatant with the FB_1 content of the original solutions. The soil samples were then consecutively extracted with acetonitrile:H_2O (1:1), 5% formic acid, and acetonitrile:5% formic to determine if the bound FB_1 could be extracted using more non-polar or more polar solvents. Acetonitrile 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, centrifuged and FB_1 in the supernatant determined by HPLC. Each sample was similarly extracted with 5% formic acid and then acetonitrile:5% formic. Values are means±SD (n=3) and ND indicates no FB_1 was detected. One-way analysis of variance indicated that within each group (low and high) that there were significant ($p<0.05$) differences among extraction solutions for each soil type. Statistical differences among groups were not determined.

Table 1. Comparison of the chemical analysis of washed sand and Cecil sandy loam.[a]

Sample	Sand	Cecil sandy loam
pH	6.0	5.8
Organic Matter (%)	0.2	2.6
Ca (lbs/acre)	13	700
K (lbs/acre)	8	86
Mg (lbs/acre)	1.6	108
Mn (lbs/acre)	0.3	49
P (lbs/acre)	1.8	35
Zn (lbs/acre)	0.7	1.4

[a]Samples of Cecil sandy loam soil and washed sand were analyzed by the University of Georgia soil testing and plant analysis laboratory (Athens,

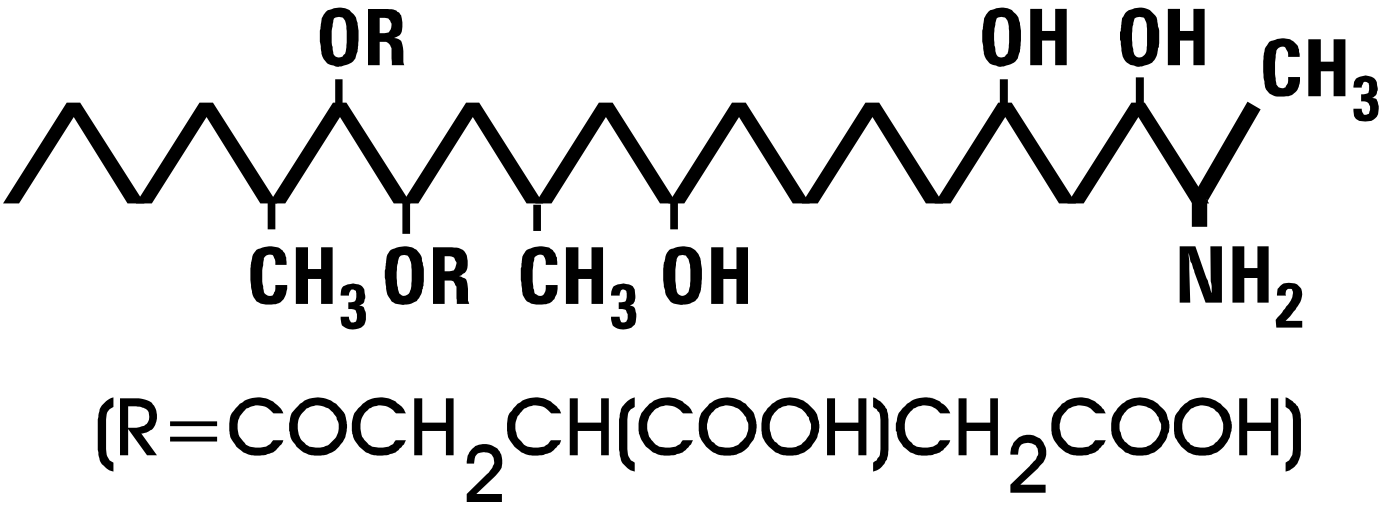
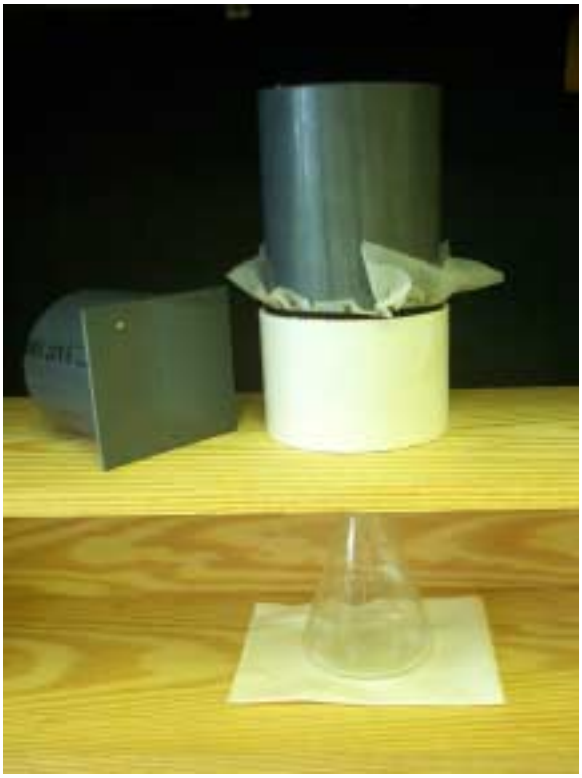


Figure 1

A



B

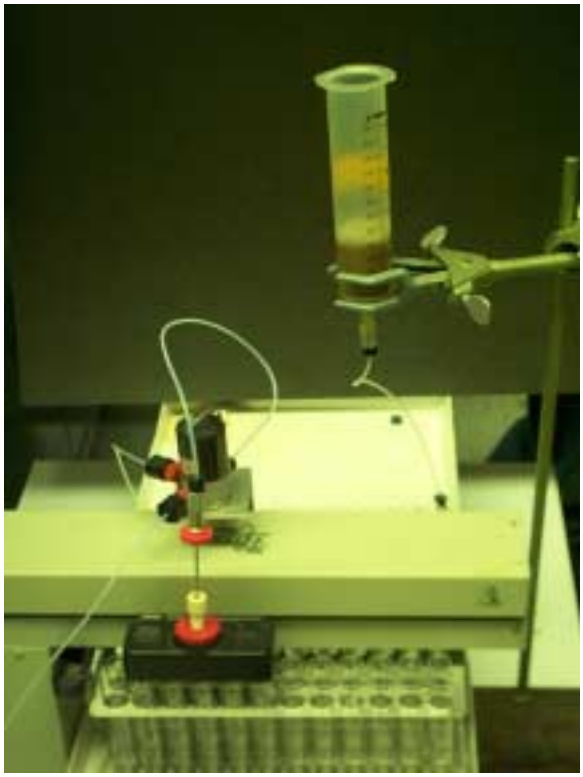


Figure 2

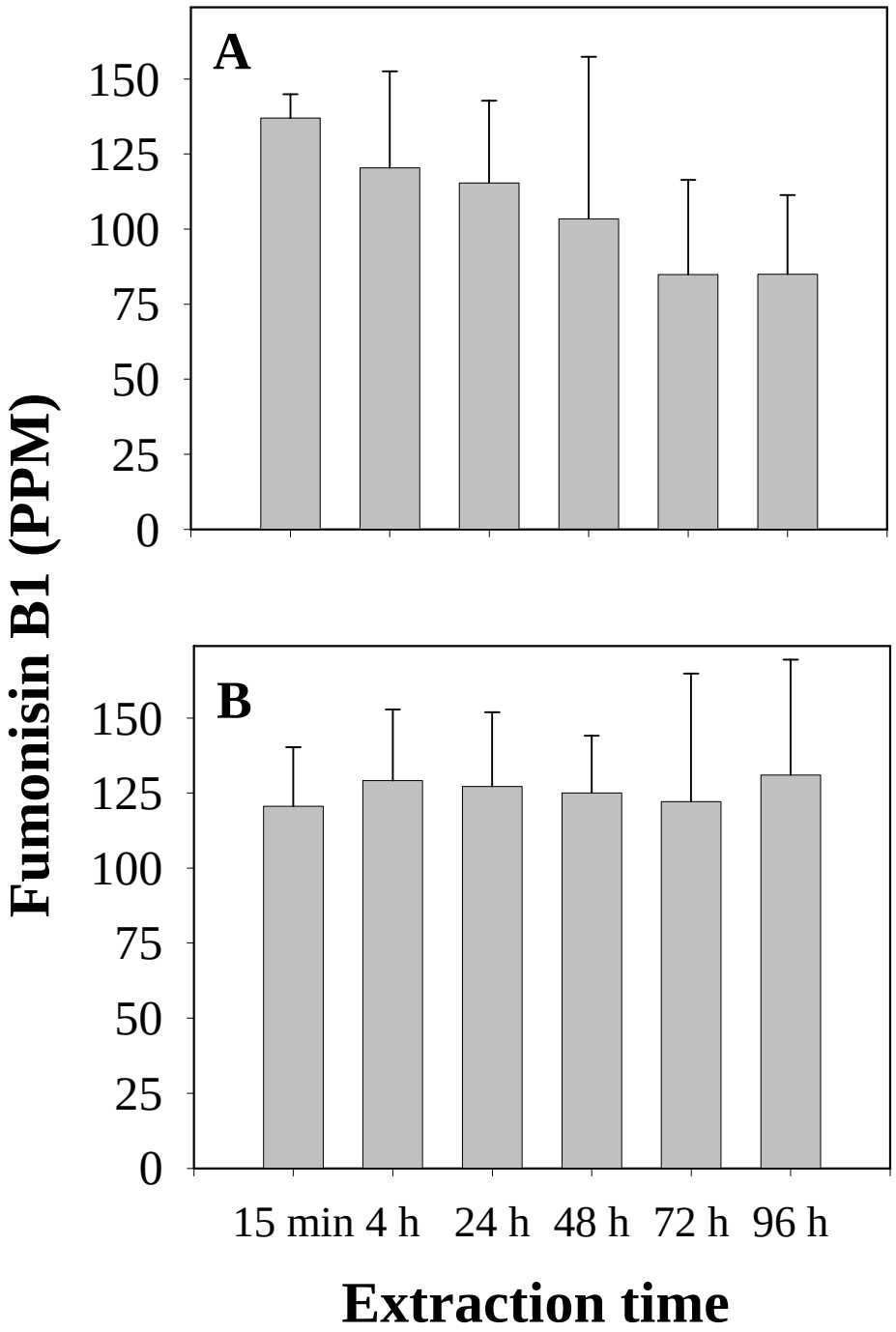

A
B
150
125
100
75
50
25
0
Fumonisin B1 (PPM)
15 min 4 h 24 h 48 h 72 h 96 h
Extraction time

Figure 3

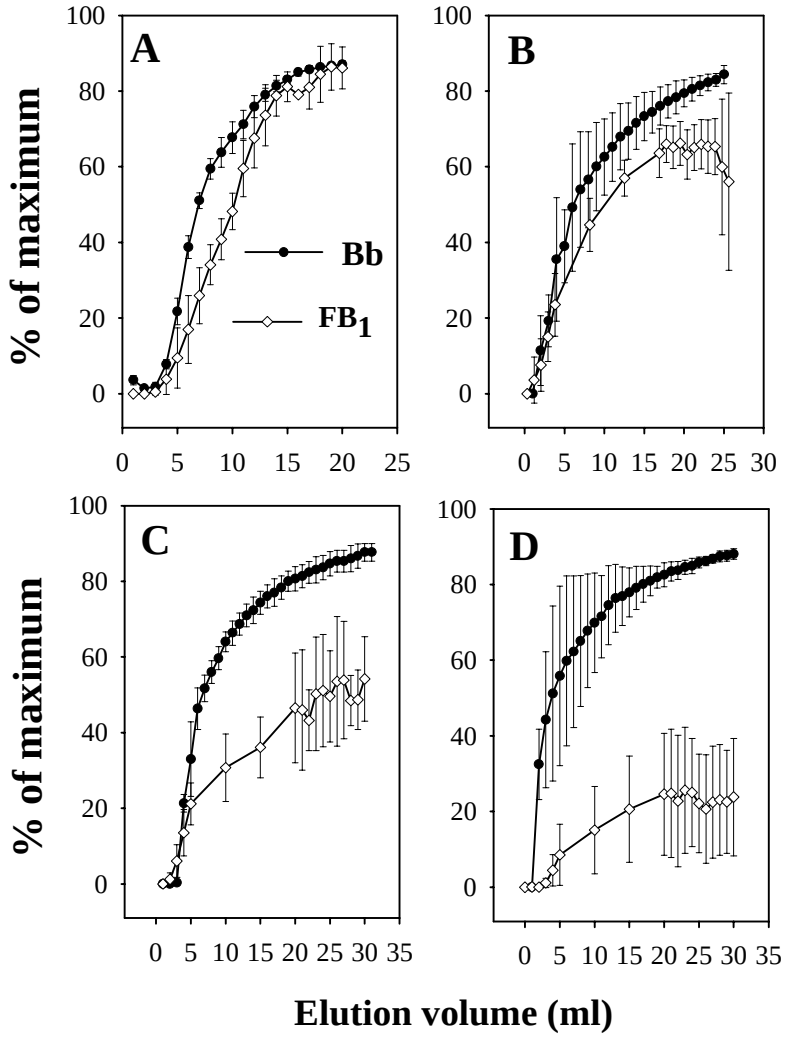


Figure 4

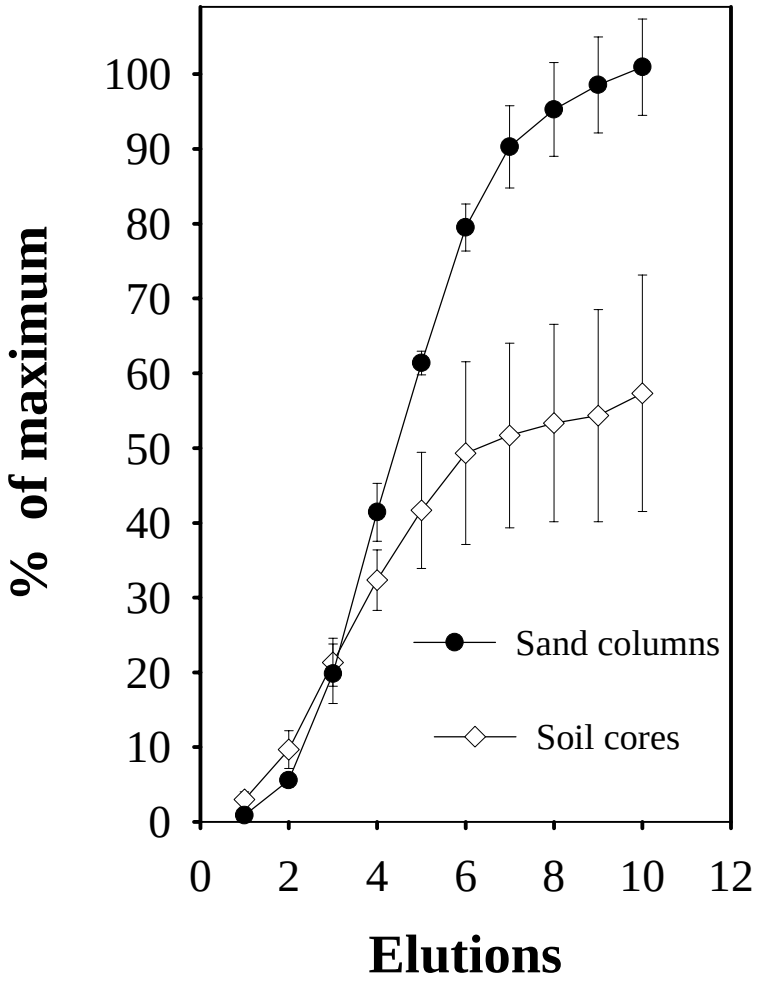

% of maximum
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Elutions
Sand columns
Soil cores

Figure 5

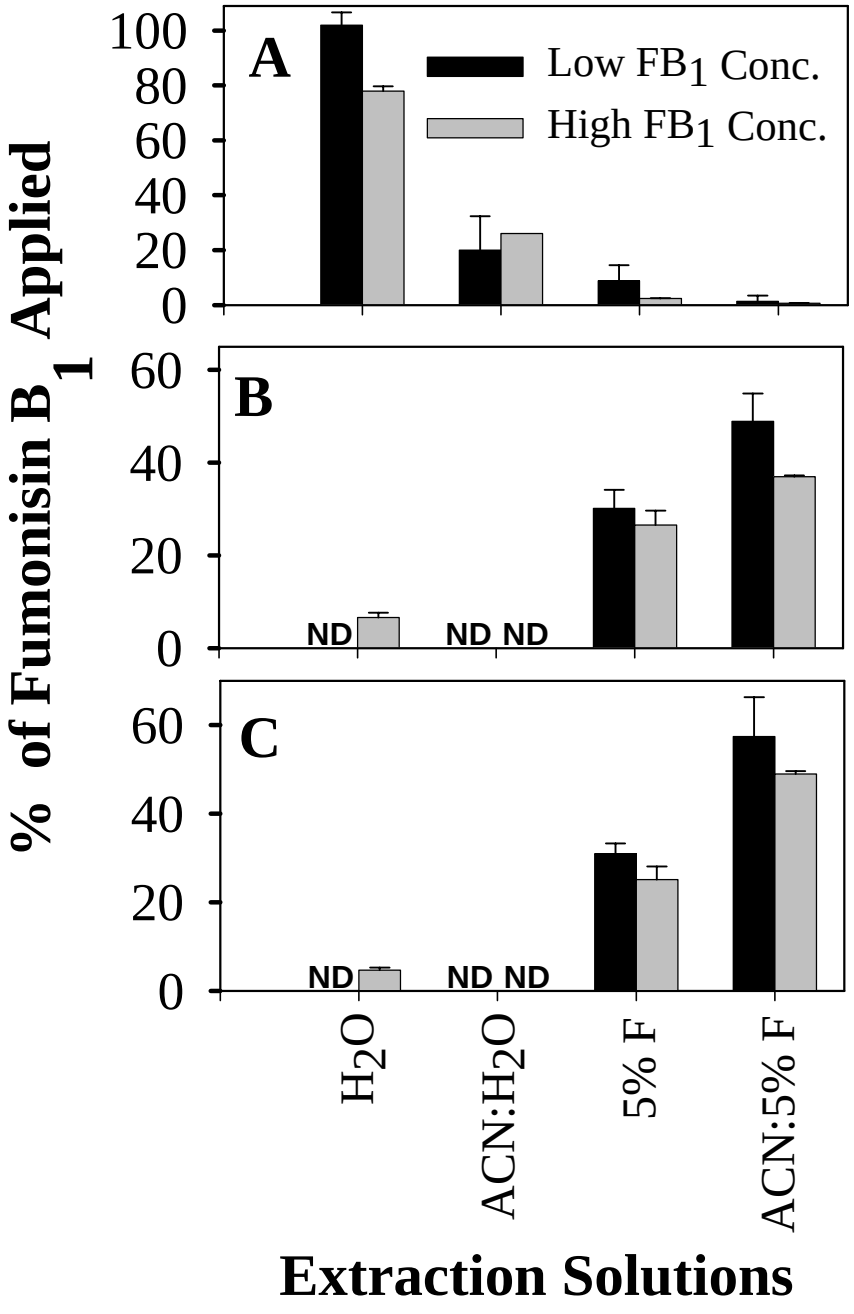

A
B
C
Low FB1 Conc.
High FB1 Conc.
100
80
60
40
20
0
ND
ND ND
% of Fumonisin B1 Applied
H2O
ACN:H2O
5% F
ACN:5% F
Extraction Solutions

Figure 6

SUMMARY AND CONCLUSIONS

Fumonisin B_1 (FB_1) is a water soluble, carcinogenic mycotoxin produced by the fungus *Fusarium verticilliodes*, which is parasitic to corn plants. The fungus can be found in the ear, roots, and stalks of corn plants. It is estimated that 90% of the FB_1 consumed by livestock is excreted in feces. The objectives of this research were to determine 1) the kinetics of FB_1 leaching in soil and 2) if FB_1 is bound to soil. Leachates from mini-columns were used to determine the movement of FB_1 through soil matrices comprised of washed sand and 0%, 50%, 75%, or 100% Cecil sandy loam soil. The movement of FB_1 was compared to that of bromophenol blue (Bb), a dye that moved freely through the soil columns. FB_1-contaminated corn screenings or extracts containing FB_1 were placed on the surface of the soil columns. The 100% sand columns slightly retarded the efflux of FB_1 relative to Bb but did not appear to chemically alter the FB_1. The recovery of FB_1 decreased with increasing concentrations of Cecil sandy loam soil. At 0%, 50%, 75% and 100% Cecil sandy loam, approximately 80%, 60%, 50% and 20% of the FB_1 was recovered in the column leachate, respectively. The FB_1 retained on the 100% Cecil sandy loam column was tightly bound as evidenced by the fact it could not be extracted using acetonitrile:water (1:1). However, approximately 75% of the bound FB_1 was released with 5% formic acid and 5% formic acid:acetonitrile (1:1) indicating that the nature of the interaction was probably ionic. When corn screenings were used as the source of FB_1, a rain simulator was attached to the top of PVC columns filled with sand and intact soil core columns of Cecil sandy loam soil. Water was allowed to

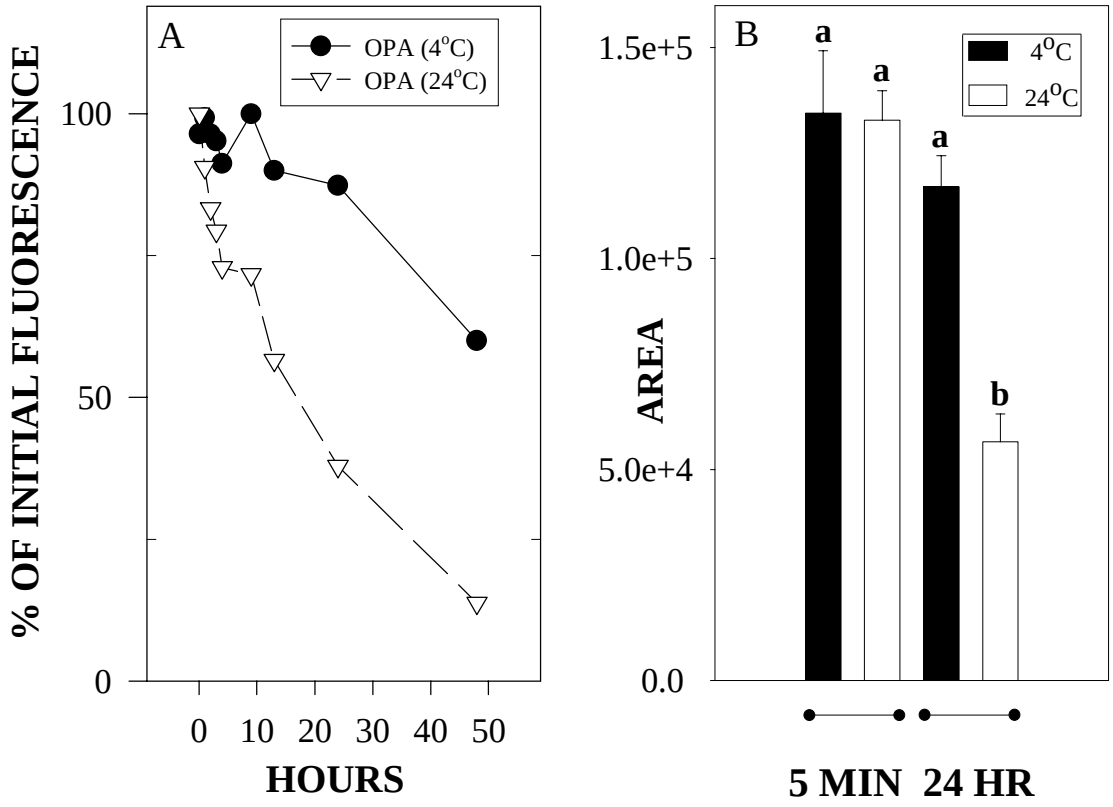
saturate the corn screenings (extracting the FB_1) and percolate through the columns eluting the FB_1. The sand columns did not appreciably bind the FB_1, evidenced by the fact that 100% of the applied FB_1 was recovered in the leachate. However, in the intact soil core columns, approximately 50% of the applied FB_1 was recovered in the leachate.

The result of this study indicates that FB_1 is probably stable in the soil environment. It is also possible that FB_1 from corn debris in field situations could contaminate groundwater. If FB_1 is mobilized by acid rainwater then it can enter and move through both simple and more complex soils and can be recovered chemically intact in the leachate. Therefore, it is possible that FB_1 could alter the biological activity of soil flora and fauna. The more complex the soil, the more likely that FB_1 will be retained in the soil matrix, however, this does not preclude that it is not biologically available. Finally, acid conditions could facilitate the mobilization of FB_1 bound presumably via ionic replacement of soil chemical constituents.

APPENDICES

Figure 1. To determine the stability of the OPA-FB_1 deriviative formed when FB_1 is combined with OPA, samples consisting of 500 µl refrigerated OPA derivatizing reagent, 500 µl 1:1 acetonitrile:water solution and 100 µl FB_1 standard (0.00178 µg/ul water), were placed into a Shimadzu SIL-9A Auto Injector, under refrigerated conditions (4^oC). The samples were analyzed via HPLC at hourly intervals for 48 hr, to determine the change in emitted fluorescence with time. This experiment was repeated at 24^oC, and the percentages were recorded and compared to determine the effect of temperature on the stability of the OPA-FB_1 derivative. To verify these findings, this experiment was repeated with triplicate samples analyzed at 5 min and 24 hr after mixing the OPA, FB_1 standard, and 1:1 acetonitrile:water solution.

After 48 hr at 4^oC and 24^oC, the emitted fluorescence of the OPA-FB_1 derivative had decreased by approximately 40% and 90%, respectively . However, after 24 hr at 4^oC and 24^oC the decrease was 10% and 65%, respectively. At 24 hr the decrease in emitted fluorescence of the OPA-FB_1 derivative at 4^oC was not statistically significant ($p>0.05$). These results show that if the derivative is refrigerated at 4^oC, it is much more stable, which allows for a more consistent, reproducible experimental method



Appendices, Figure 1