ABSTRACT

Title of Dissertation / Thesis: Development of Detection and Reduction Methods for Mycotoxins in Corn and Herbal Plant Material

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This is a study of methods to reduce, eliminate or prevent fumonisins and aflatoxins in corn and to survey botanical supplements for aflatoxins. Gamma (Cobalt^{60}) and electron beam irradiation were used to eradicate fumonisin B_1. Fumonisin B_1 in water was irradiated with 0.5 to 30.0 kGrays. The minimum dosage (0.5 kGray) reduced fumonisin 99%. When naturally contaminated whole and ground corn was exposed to irradiation (10.0-100.0 kGray), *Aspergillus* and *Fusarium* fungi were killed; whereas, the level of fumonisins was not changed.

Transgenic Bt and non-Bt (NBt) 2000 and 2001 corn were inoculated with *Fusarium verticillioides* and *Aspergillus flavus* at silking. At harvest, corn was rated for insect damage, mold populations and levels of fumonisins and aflatoxins. There were little aflatoxins in 2000 corn and none in 2001 corn. Levels of fumonisins in 2000 Bt corn were not significantly lower than the level found in Bt corn; however, 2001 Bt corn showed significantly higher levels of fumonisins than those found in NBt corn.

Whereas irradiated herbal material contained no microorganisms, non-irradiated material was contaminated with *Aspergillus*, *Penicillium*, and *Rhizopus*. All *A. flavus* isolates from herbal material produced aflatoxin B_1 on rice cultures. Twenty-four of 25 irradiated and non-irradiated herbals contained AFB_1, ranging from trace levels to 450 ng/g.

An analytical method to detect aflatoxins in herbal products was developed for ginseng root, *Panax quinquefolius*. Aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2 were added to toxin free ginseng at 2 ng/g, 4 ng/g, 8 ng/g and 16 ng/g. Based on HPLC analysis, mean recoveries ranged from 77% to 92 %.

This method was used to survey wild simulated and cultivated ginseng roots harvested after a dry growing season (2002) and a wet growing season (2003). Root samples were from UMD field plots, a commercial source, and from Appalachian growers. Levels of 5.5-32.0 ng/g AB_1 were found in 4 samples from a wild simulated grower. One of nine cultivated samples contained trace amounts (0.10 ng/g) AB_1 from the 2002 season, and no aflatoxins were found in 2003 samples. In the commercial fresh root sample 16 ng/g of AB_1 was found.

THE DEVELOPMENT OF DETECTION AND REDUCTION METHODS FOR MYCOTOXINS IN CORN AND HERBAL PLANT MATERIAL

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2005

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DEDICATION

To Jaime J. Vázquez Saldaña, my heart, my life, my deep gratitude and my love always.

ACKNOWLEDGEMENTS

My most profound gratitude goes to my husband, Jaime, who has given me unconditional, unquestioned support over these many years even when things became very difficult and stressful.

I would like to thank the many undergraduate student research assistants whose hard work and dedication contributed greatly to the success of this research effort: Stephen Johnson, Elizabeth Burguieres, Jeremy Tilstra, Erin Horn, Meredith Bilek, Angie Bryl, John Salogiannis, Ameet Joshi, Nancy Nguyen, Sali Mahmoud, Felicia Conrad, Eva Lee, Angela Bien, Sang Jo Kim, PJ Ghorbani, James Heeres and Ning Lu.

I would also like to extend my immense appreciation for the advice and assistance afforded me from a number of graduate students in the Department of Nutrition and Food Science: Lynda Brown, Cindy Cole, Juanfra DeVillena, Lorraine Niba, Cecilia Fernandez and David Ingram.

I would like to express my gratitude to the CBMG Department for supporting me for the last 4 years as a Graduate Teaching Assistant and to the many graduate students with whom I have taught who have touched my life in a positive way. My thanks to Tammatha O'Brien, Kerry Fitzpatrick, Linda Pope and D.B. Poli for all of their assistance.

My most sincere thanks to Rosana Peña Freyre for her faith and trust in me while working on a novel research study together while becoming a great friend. I will always remember our first face-to-face meeting in Lima, Peru and

knowing that we would always be connected somehow, someway through that collaborative effort.

There are many professors that have assisted me along this journey that I wish to recognize. Dr. Angela Caines, I appreciate the words of wisdom that you imparted. As well, I would like to send my thanks to Dr. Donald Schlimme for his advice and assistance when I was lost. I appreciate all of the time and resources spent by Dr. Galen Divley on my behalf during my two-year Bt corn field plot study. In addition, I would like to extend my appreciation to my dissertation committee, specifically my advisor Dr. George Bean, for pushing me to go beyond my self-imposed limitations and Dr. Marla McIntosh for her many contributions to my research development and for being a significant mentor and role model. I am very grateful for having as my special committee member Dr. Mary Trucksess and want to thank for her constant support and encouragement without which I would not have been able to get through this process. I wish to praise my committee as a whole for their contributions throughout this research and give my thanks to Dr. Jianghong Meng and Dr. Bruce Jarvis for the many times they were available to me.

There are many individuals at the Food and Drug administration who gave me their assistance over the years who I would like to praise: Michael Stack, Robert Eppley, Valorie Tornes, Carol Weaver, and Mamen Diaz Westpaul.

I am very grateful to my brothers and sister, Mike, Steve and Theresa, and my parents, Lou and Mary, for their support during this long journey.

As well, I want to thank my nieces and nephew, Gabrielle, Alexandra, Olivia, Nicholle, Samantha, Madison, Hope, Amy and Brian for their constantly shining light my way when things were difficult. They are my angels.

Gratefulness goes out to my dear friend Yusuan C. Smithson, for being such good listener and helping me break out of graduate student hibernation now and then and to my angel, Sonia E. Ruiz, for sending positive energy my way.

To my best friend, Carey A. Grow, thanks for always being there when I needed a boost and continuously reminding me that this was my destiny.

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Chapter 1: Methods of Elimination and/or Prevention of Mycotoxins in Foods, Feeds and Botanical Supplements

1.1 General Background

Mycotoxins are secondary metabolites produced by filamentous microfungi that are important entities in diseases of animals and humans (CAST, 2003). They are a relatively large, diverse group of naturally occurring compounds, and many have been implicated as chemical agents of toxic disease syndromes including liver tumors and kidney tumors in humans. Knowledge of the occurrence and distribution of mycotoxins in foods is important for determining the level of human exposure to bioactive agents and may help to explain the etiology of some chronic diseases. The usual route of human exposure to mycotoxins is ingestion as food contaminants. Mycotoxins, ingested in high amounts, can cause severe health problems that can ultimately lead to death. Whereas prolonged exposure to small quantities of mycotoxins can cause chronic disease symptoms. Low-level exposure is particularly problematic for foods and supplements considered high quality and, therefore, presumed to contain a lesser quantity of mycotoxins.

The common fungal species present in grains are *Fusarium verticillioides* and *F. proliferatum*, *Aspergillus flavus*, *A. ochraceus* and *A. parasiticus*, and *Penicillium verrucosum* and *P. commune* (CAST, 2003). The major mycotoxins produced by these fungal species that pose the most serious toxicological risk to both animals and humans are the aflatoxins, ochratoxins, patulin, trichothecenes and fumonisins. Fumonisin $B_{1,}$ B_2 and B_3 are produced by *F. verticillioides* and *F. proliferatum*. Animal diseases associated with consumption of grains containing the fumonisins are

equine leukoencephalomalacia (ELEM), porcine pulmonary edema (PPE) and to cause liver cancer in rats (Thiel, et al., 1991). Human disease associated with consumption of corn-based foods containing fumonisins are esophageal cancer found in Northern of China, Northeastern Italy, and in areas of South Africa (Marasas et al., 1988).

The major mycotoxin produced by *A. flavus* and *A. parasiticus* in grains are the aflatoxins (B1, B2, G1, G2). Animal mycotoxicoses caused by aflatoxins include liver diseases, immune system deficiencies, decreased breeding efficiency, and hypoprotenemia. Human diseases caused by aflatoxins include hepatocellular cancer (HCC) (CAST, 2003).

The widespread prevalence of aflatoxins and fumonisins in grains combined with the range of toxic effects that they produce have created a need to investigate these mycotoxins in foods, feeds and more recently botanical supplements. Determining the approximate levels and identities of these mycotoxins in the commodities investigated requires precise and reliable analytical equipment and appropriate solvent extraction. The current methods utilized for determination of aflatoxins and fumonisins in foods and feeds are thin layer chromatography (TLC), immunoaffinity column (IAC), enzyme-linked immunosorbent assay (ELISA), and reverse phase high performance liquid chromatography (RFLC), which provide collectively tools for accurate detection and quantification of these mycotoxins (Trucksess, 2000b).

Studies have been done on the processing effects as well as the stability of fumonisins in corn and corn-based products, however none have been done on the

irradiation effects on the levels of fumonisins in whole and ground field corn. Thermal processing effects on fumonisins have found it to be heat stable to 190ºC. When using higher temperatures (above 190ºC) during extrusion processing with reducing sugars, fumonisin levels are reduced in processed corn products (Bullerman, et al., 2002).

Aflatoxin stability has also been researched in corn and corn-based products, and it has been found that conventional processing such as cooking, roasting, frying, and baking are not able to completely eliminate aflatoxins since they are relatively heat stable (Christensen, et al., 1977). Aflatoxins were not significantly degraded in spiked corn after a gamma irradiation dose of 20 kGray (Hooshmand & Klopfenstein, 1995).

It is vital to prevent, reduce or eliminate the levels of fumonisins and aflatoxins in commodities due to their hazards to humans and animals and as well the economic impact. It is estimated that the amount of corn-based product consumed today in the U.S. is 36 lb/year (NCGA, 2004a).

1.2 Irradiation Study to Eliminate Toxigenic Fungi/Mycotoxins

There are no known studies to determine the effects of gamma irradiation and electron beam irradiation of fumonisin levels in whole and ground corn. Effects of irradiation on aflatoxin B_1 have been studied and the results indicate that although production by *Aspergillus flavus* of aflatoxin B_1 is reduced as irradiation dosage increases the molecule itself is difficult to destroy (Frank, et al., 1971). It was found that doses as high as 182 kGray were unable to destroy more than 10% of the aflatoxin molecule in dry material (Aibara & Miyaki, 1970). The dosage required to

totally eliminate aflatoxin B_1 from foods and feeds would be so high that it would cause a significant deterioration of quality thus changing the organoleptic properties of the irradiated product. In a later study, with grains such as rice, high doses of irradiation at 30 kGray had no effect on the compound (Frank, et al., 1970). In a related study, corn, wheat and soybean were irradiated at doses from 5 to 20 kGray and the results demonstrated that the highest dose had no affect on aflatoxin B_1 however, the trichothecene toxins such as T-2, zearalenone and deoxynivalenol, produced by *Fusarium* species of molds, were significantly reduced at 7.5 kGray (Hooshmand & Klopfenstein, 1995). Given this result, it may be feasible to irradiate foods and feeds containing trichothecenes or other *Fusarium* produced mycotoxins including fumonisins.

Fumonisins are omnipresent in field corn. Higher levels are usually found during seasons when the weather is very dry during grain fill and very wet in the latter part of the growing season (Cotton & Munkvold, 1998). Fumonisin B_1 is a water-soluble mycotoxin. Given that the effects of irradiation are to create free radicals from the water present in the material undergoing irradiation, fumonisins in corn could be removed by irradiation of the product. With ground and whole corn, the 12% moisture level present may be adequate to allow for the water present to react under irradiation conditions and form the free radicals and effectively react with the fumonisin molecule, causing the molecule to be broken down.

Among the products designated for human consumption, fumonisins are found in corn-based cereals, corn flakes, tortillas, tortilla chips sweet corn and popcorn (Stack, 1997: Shepard, et al., 1996). Since field corn is typically contaminated with

fumonisins, it is important to determine a method to decontaminate the corn before it is processed. It is known that FB_1 is omnipresent in corn therefore finding a method to effectively reduce or eliminate this mycotoxin in corn and corn-based products is of paramount importance given the risks associated with the consumption of corn products naturally contaminated with FB_1.

1.3 Objectives of the Irradiation Study

The objectives of the irradiation study were to determine if gamma irradiation and electron beam irradiation-processing methods were effective in reducing or eliminating fumonisins in whole and ground corn. In addition, it was also investigated whether irradiation could eliminate the fungus, *F. verticillioides* from whole corn and determine the optimum dose (kGray), to treat the corn. Hypothesis of this study is that fumonisin B_1 would be effectively eliminated from ground and whole kernel corn when subjected to gamma irradiation or electron beam irradiation. To test this hypothesis, this study examined 1) whether FB_1 could be eliminated in an aqueous solution; 2) whether FB_1 could be eliminated in ground corn; 3) whether FB_1 could be eliminated in whole kernel corn; and 4) the dose at which the fungus, *F. verticillioides* could be eliminated in both ground and whole kernel corn.

1.4 Bt Corn Field Plot Study

This was a classic study to improve food safety of corn by studying the interaction of microbial pathogens in Bt corn and how this affects the levels of toxins they produce. It involved the study of two toxigenic fungi, *A. flavus* and *F. verticillioides*. *Aspergillus flavus* is a producer of the compound, aflatoxin B_1 which is classified as a

human carcinogen according to IARC (International Agency for Research on Cancer, 1993a). The second fungus, *Fusarium verticillioides,* is wide spread throughout the world, occurring in numerous corn food products. *F. verticillioides* is a producer of numerous toxins, the one of most concern are the fumonisins. Fumonisins have been frequently found in the U.S., in corn and corn products including corn tortillas and corn flour (Stack, 1997). Fumonisins are a food safety concern because they have been associated with gastric cancers in humans, leukoencephalomalacia in horses, and pulmonary edema in swine (IARC, 1993b; Thiel, et al., 1991). This study will contribute significantly to our food safety because Bt corn inhibits insect damage afforded by expression of Bt protein in the silks and kernel tissue which should result in a reduction of growth of *F. verticillioides* and *A. flavus* and reduce mycotoxin production. The effect of the Bt protein is indirect by limiting insect feeding damage which reduces fungal infection compared to conventional (non-Bt expressing) corn (Munkvold, 1999).

There have been few studies looking at the interaction of toxigenic fungi in Bt corn under field conditions. In his field plot study of 1999, Dively (UMCP), observed that under optimal conditions for corn ear rot, there was a high incidence of *Fusarium* infection and a lower incidence for infection *by Aspergillus* in Bt 11 corn, which he had not expected. These findings indicated a competitive inhibition by *Fusarium* against *Aspergillus.* The drought conditions of 1999 in addition to high insect infection levels should have created an environment favoring both high *Fusarium* and *Aspergillus* infection in the ears. It can be concluded from these studies that additional investigations looking at the interactions *of Fusarium* and *Aspergillus* in Bt corn are justified, especially since the author did not

determine the interaction of both fungi and their effects on the levels of aflatoxins and fumonisins in Bt corn.

1.5 Objectives of Bt Corn Field Plot Study

Fumonisins have been found in a number of commodities destined for human consumption (Munkvold & Desjardins, 1997, Desjardins et al, 1998). It is also known that the fungus *Fusarium* is omnipresent in corn within the kernels (Trofa & Bean, 1985). In contrast, *Aspergillus* is present on the exterior of the seed and toxic metabolites are produced when the corn is growing under stress conditions such as unfavorable weather conditions and heavy insect infestation. Thus if we can prevent the incidence of insect damage to corn by the use of transgenic corn, we should affect the levels not only of aflatoxins but other toxic metabolites produced by *Fusarium* since both toxigenic fungi can occur simultaneously in corn.

The purpose of this experiment was to study the incidence of *F. verticillioides* and *A. flavus* and their toxic metabolites, fumonisins and aflatoxins, in Bt corn. This was accomplished as a field investigation in which the infection of the two fungi were manipulated and their toxin production monitored in near iso-genic corn hybrids with and without Cryl Ab genes by determining if the production of their toxic metabolites or growth of the two microbes were affected by this relationship. The objectives were to specifically determine if the crystalline protein in Bt corn reduced insect damage to the extent that there was an indirect affect on mold counts and mycotoxin levels. It was hypothesized that the reduction in damage by insects to the Bt corn would result in lower aflatoxins and reduced fumonisin levels when compared to non-Bt corn. In addition, it was also hypothesized that

the interactions of the two fungi would also affect the mycotoxin levels depending on the treatment, for example, once the fungus is established on the corn it may out-compete the other for growth substrate and resulting toxin production.

1.6 Toxigenic Fungi/Mycotoxins in Peruvian Herbals

Recently, interest has been focusing on assessing the safety and efficacy of botanical supplement plant materials which included studies by investigating the occurrence of toxigenic fungi and/or their mycotoxins in these products especially when grown and stored under less than optimal conditions. Molds found in herbal drug plants include *A. flavus, A. ochraceus, Penicillium commune, Penicillium spp., Fusarium oxysporum, Fusarium* spp., and *Alternaria* (Efuntoye, 1999; Halt, 1998). Mycotoxins found to be present in the medicinal plant material were aflatoxin B_1, ochratoxin A, citrinin, and zearalenone (MacDonald and Castle, 1996, Patel, et. al., 1996, Thirumala-Devi, et. al., 2001). Currently, there are no federal restrictions or regulations that govern importation of herbals which frequently originate from third world countries where quality control is often lacking. Thus, mycotoxins could pose a potential risk to those consuming these products which frequently occurs in the U.S.

As stated previously, irradiation is an effective means to eliminate fungi from various commodities such as whole kernel corn, ground corn, soybeans, wheat and other plant materials. However, in our study of whole and ground corn, although the material becomes sterile after irradiation, the mycotoxin present is unaffected. The same situation may occur in mycotoxin contaminated herbal plant material.

1.7 Objectives of Peruvian Herbal Study

This study was an investigation of 29 herbal samples originating from a Peruvian firm which specializes in the sales of “100% Natural”, irradiated herbal products. Since it is known that herbs and spices in the United States are either irradiation processed or high steam heat processed to sterilize them, it would be important to see the effects of the irradiation processing technique on the quality of plant products treated outside the U.S.

Irradiated herbals would be compared to non-irradiated herbals relative to the microflora found, aflatoxigenic strains of *A. flavus* present, and level of aflatoxin contamination. It is hypothesized that if toxigenic strains of *A. flavus* were present prior to irradiation, then aflatoxins would be found, in similar amounts in both the irradiated and non-irradiated samples.

1.8 Determination of Aflatoxins in Ginseng

The focus of this project will be on the development of analytical methods for the determination of aflatoxins in ginseng. The goal will be to optimize conditions of extraction, isolation, purification, derivatization and determination of the toxin in the herbal ginseng. Aflatoxins will be isolated and identities of the toxin(s) confirmed by chemically derivatized or mass spectrometric analysis. The method parameters such as limit of detection, limit of quantitation, precision and accuracy should be similar to approved AOAC official methods for aflatoxins in grains. The method, once developed, will be subjected to peer evaluation and validation. The method will be used in a survey of aflatoxins in ginseng grown under widely variable production as well as differing post harvest practices and processes. The data from the survey

should be an indicator of whether aflatoxins in ginseng are a health concern. Results will suggest whether HACCP needs to be implemented in the processing and marketing of ginseng and other dietary supplements. By estimating the exposure levels to the toxins traceable to consumption of these botanicals, the FDA can also ensure that various botanical supplements are within the aflatoxin safety limits within science-based risk assessments.

1.9 Objectives of Aflatoxins in Ginseng Study

In the previous study on Peruvian herbals, it was determined that aflatoxins were produced by *A. flavus* isolated from the plant material and that aflatoxins were presumably present as well. However, due to high levels of interfering pigmentation in the herbals it was difficult to qualify and quantify aflatoxins when spiked into the plant material. In addition, when methodologies for isolating aflatoxins from other plant materials, were applied in the herbal analysis the results were inconsistent. Thus, a methodology had to be developed in order to investigate the occurrence of aflatoxins in botanical supplements. Ginseng was chosen as the model plant material to use in this study because of its popularity, its lack of pigmentation, and the ease in obtaining samples. It was hypothesized that the ginseng would have little pigmentation interference and being a root crop it most likely would be contaminated with aflatoxins if improperly harvested, dried, processed and stored.

The goal of this study was to develop an analytical method for aflatoxins and to investigate the occurrence of aflatoxins in ginseng. The objectives were to: 1) develop analytical methods for aflatoxins in ginseng. 2) conduct a survey of

aflatoxins in fresh and processed ginseng using the developed method. 3) and suggest a HACCP for avoiding mycotoxins in botanical supplements.

Chapter 2: Literature Review

2.1 History of Toxigenic Filamentous Fungi

Filamentous fungi such as *Aspergillus spp*. and *Fusarium spp*. are widespread and found in a number of foods and feeds (Bullerman, 1986). All fungi produce secondary metabolites, but some produce secondary metabolites such as the mycotoxins that are toxic to humans and animals (Turner, 1978; Turner & Alderidge, 1983). The most notorious of these toxigenic fungi include *Aspergillus*, *Fusarium*, *Stachybotrys*, *Penicillium* and *Alternaria*. Well over 100 years ago, there was emerging evidence that fungi growing on feeds produced toxic compounds. The yellow rice outbreak in Japan first revealed that ethanol extracts from *Penicillium* molded grain were toxic to animals (Miyake et al., 1940). This was followed by studies whereby extracts of the mold itself were shown to be toxic to animals (Alsberg & Black, 1913). Mycotoxins became a recognized major problem after the death of thousands of poults (turkey chicks) and other birds in England in 1960. Massive hepatic necrosis, parenchymal cell degeneration, and bile duct proliferation characterized the disease that affected poults (Blount, 1961). The cause of these deaths was eventually traced to the ground peanut meal in the feed that contained the toxin-producing fungus, *Aspergillus flavus*. The mycotoxins that caused the disease was subsequently identified and named aflatoxins (Allcroft & Carnaghan, 1963; Austwick & Ayerst, 1963; Sargent, et al., 1963).

2.2 Toxigenic Aspergillus spp.

Almost 50 species of *Aspergillus* have been identified capable of producing toxic secondary metabolites (Cole and Cox, 1981). The mycotoxins produced by various species of *Aspergillus* are diverse in number as well as in their range of toxicities. The most toxic of the *Aspergillus* mycotoxins are aflatoxins produced by *A. flavus, A. paraciticus* and *A. nomius*; ochratoxin A produced by *A. ochraceus, A. carbonarius*, and occasionally *A. niger*; steringmatocystin, produced primarily by *A. versicolor* and *Emericella* species; and cyclopiazonic acid produced by *A. flavus* and *A. tamarii*. In addition, citrinin, patulin, penicillic acid and the tremorgenic toxins are also produced by *Aspergillus* species and cause a wide range of effects (Flannigan & Pearce. 1994; Kozakiewicz, 1994; Scott, 1994).

2.3 Aflatoxins

The most widespread and notorious of the Aspergillus mycotoxins are aflatoxins. At least 4 forms of naturally occurring aflatoxins have been identified: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Asao, T., et al, 1963; Mirocha & Christensen, 1982).

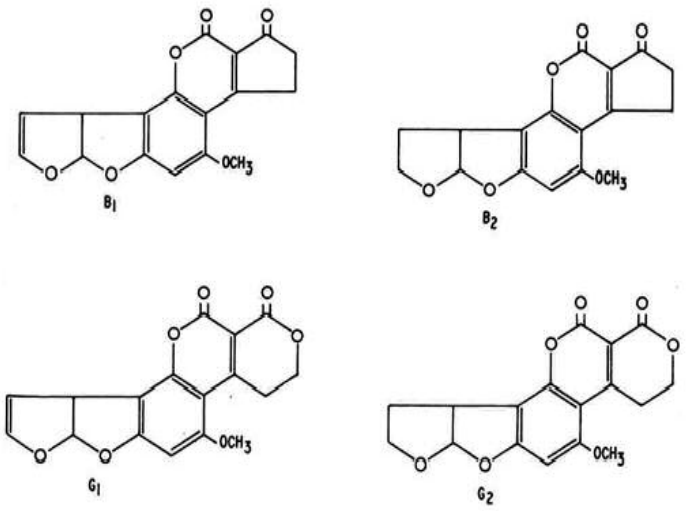


Figure 1: Aflatoxin B_1, B_2, G_1, and G_2 chemical structures.

The "G" aflatoxins are identified due to their green fluorescent color under ultraviolet light on silica gel TLC plates and the "B" aflatoxins for their blue fluorescence. Aflatoxins primarily target the liver in acute toxicity tests. Whereas aflatoxin B_1 is also a powerful tetratogenic, mutagenic, and hepatogenic-causing compound (Castegnaro & Wild, 1995). Because of this, the Agency for Research on Cancer (IARC) has classified the aflatoxins as group 1 human carcinogens (Castegnaro & Wild, 1995).

2.3.1 Aflatoxin producing strains of *Aspergillus* spp.

The ubiquity of the aflatoxigenic molds, *A. flavus* and *A. paraciticus*, is demonstrated by their presence on nuts, spices, corn, rice, and other grains pre-harvest, during and post-harvest (Gams, et al, 1985). Cereals and spices are common substrates for *A. flavus*, and subsequent aflatoxin production in these foodstuffs is almost always due to poor drying, handling, or storage (Arim, 1995; Dhavan). Thus,

aflatoxin levels are normally higher in tropical countries where crops, such as corn, ground nuts, and other nuts and oilseeds are often grown under marginal conditions where drying and storage facilities are limited (Choudary, 1995; Lubulwa & Davis, 1994).

2.4 Aspergillus flavus

Infection of corn by *A. flavus* is the best understood of all of the possible susceptible hosts the fungus can infect. *Aspergillus* spp. can colonize all stages of corn growth including infection of the developing embryo by the fungus by growing down the silk tissue directly into the ear. Infection can also occur in kernels before harvest as well as in corn after harvest (Marsh & Payne, 1984). Although kernel surface contamination by *A. flavus* can be extensive, internal infection will normally not occur unless the seed's morphological barriers are compromised. Factors that can result in internal colonization by the fungus are insect injury, drought stress, and high temperatures. Insects can cause infection of corn kernels by transporting the inoculum into the ears, moving inoculum from the silks into the ears, disseminating inoculum throughout the ear, or else by facilitating colonization and infection of the kernels by causing injury to the kernels themselves facilitating entry of the fungus into the kernels (Payne, 1992; Smart, 1990). Other factors such as high temperature and low moisture can result in cracks in the seed and subsequent invasion by the fungus. Temperature and moisture are the dominant factors that affect aflatoxin contamination of corn. Environmental conditions most favorable for maximum growth and aflatoxin production by *A. flavus* are temperatures greater than 30ºC, maximum relative humidity of greater than 85%, and water activity of 0.98 to 0.99

(Payne, et al, 1988). Thus, *A flavus* can infect with proper moisture/temperature conditions during storage almost any stored product (Payne, 1992).

2.4.1 Pathogenicity and Occurrence of *A. flavus*

Aspergillus flavus is considered an opportunistic pathogen of plants. In the field, high *A. flavus* populations occur continuously but fungal populations increase during dry and hot weather conditions (McMillian, 1983). Infection propagules may be conidia, sclerotia or mycelia. Since *A. flavus* is also a saprophyte, it can grow well on crop debris, dormant tissues, and/or damaged or weakened crops (Ashworth et al., 1969; Stephensen and Russel, 1974).

2.4.2 Prevention of A. flavus

Pre-harvest strategies that are currently applied to prevent aflatoxigenic strains of fungi from infecting plants in the field, include its biological control utilizing non-toxigenic strains of *A. flavus* to prevent the growth of toxigenic strains (Cotty, 1994; Cotty et al., 1994; Daigle, & Cotty. 1995; Dollear, et al., 1999). Other techniques under investigation are the development of anti-fungal compounds that can block the molecular interaction of the fungus with the host plant, genetic engineering to increase antifungal compounds in host plants, and the enhancement of current plant fungal resistance mechanisms (Smith, 1997).

Post-harvest control of *Aspergillus flavus* and aflatoxin formation emphasizes improvement of storage conditions which include better drying practices, maintaining low moisture levels and temperatures and maintaining good aeration of the stored commodity. When proper storage facilities are not available, the use of chemical preservatives has been proposed. For example, propionic acid and sorbic acid are

reported excellent preserving agents on agricultural commodities to prevent aflatoxin contamination (Al-Hilli & Smith, 1992).

2.5 Detection of aflatoxins

Thousands of methods have been published for aflatoxin analysis. They can be classified as chemical and immunochemical methods. The Food and Drug Administration as well as other government agencies utilize AOAC approved procedures in order to detect aflatoxins in various commodities. Two frequent chromatography methods employed to detect aflatoxins involve chemical analysis of commodities are HPLC and TLC.

2.5.1 Chromatography Methods

Traditional chromatographic techniques such as thin layer chromatography (TLC), and liquid chromatography (LC) and gas chromatography (GC) are widely employed for separation and quantitation of the mycotoxins. The enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column (IAC) assays are most commonly used. AFL has a strong UV absorbance with a maximum range from 350-365 nm for AFL when measured in methanol, acetonitrile, toluene-acetonitrile, or benzene-acetonitrile. AFB_1 and AFB_2 exhibit a blue fluorescence whereas the AFG_1 and AFG_2 emit a blue-green fluorescence when exposed to long-wave UV light. In all TLC methods and most of the LC methods used for AFL separation and quantitation, the toxin concentration is based on the intensity of the fluorescence observed. ELISA and IAC assays are in general not as sensitive and precise as TLC and LC methods (Trucksess, 2003a).

2.5.2 Thin Layer Chromatography

TLC, also known as flat bed chromatography or planar chromatography, is one of the most widely used separation techniques. TLC separation includes sample application, plate development, visual observation and quantitation (Trucksess, 2000b). Since TLC is simple and could be economical it is the most useful technique for mycotoxin analysis in the developing countries. For aflatoxins in corn, the method employed is AOAC Official Method 993.17; Aflatoxins in Corn and Peanuts (Trucksess, 2003a)

2.5.3 Reverse Phase Liquid Chromatography

Reversed phase HPLC is the most widely used procedure for separation and determination of AFL. When HPLC methods are used the fluorescence of AFB_1 and AFG_1 is quenched by the mobile phase used for reversed-phase LC, therefore it is necessary to prepare derivatives of the two toxins to over come this quenching. This is done by pre-column derivatization with TFA/acetic acid/water, or post-column derivatization with iodine or bromine, or cyclodextrins can be used to eliminate quenching problems. A programmable HPLC fluorescence detector is used for the detection of the AFL. The identity of the AFL could be confirmed by chemical derivatization. AFB_1 and AFG_1 are converted to a water adducts which changes their chromatographic properties on TLC and HPLC. Other approaches such as thermospray MS, LC/MS, and MS/MS are also used for confirmation of identity of the AFL.

2.6 Prevention and/or Elimination of aflatoxins

A number of studies on the prevention of aflatoxin contamination in foods and feeds have been done which include using physical, chemical and biological methods. A first choice in the reduction and/or prevention of aflatoxins in feeds is to physically sort the kernel/seed/nut that appears damaged or contaminated form the non-damaged appearing product. This can involve cleaning, sorting and hand picking (Dickens and Whitaker, 1975. Visual inspection of the commodity for the presence of *Aspergillus flavus* is also done however the presence of *A. flavus* does not always correspond to the presence of aflatoxins (Hocking, 2001). As a result of the wet milling processing of corn for various product preparations, aflatoxin B_1 is mainly found in the steep water (39-42%) and the fiber fractions, (30-38%), with lesser amounts found in the gluten (13-17%), germ (6-10%), and starch (1%) fractions however little of the aflatoxin was destroyed under this processing method (Wood, et al., 1982; Bennett and Anderson, 1978). Similarly, in dry milling processing of corn aflatoxins will be found in varying proportions of the corn fractions, with the greater amounts found in the germ and hull, with only 6-10% of the aflatoxins found in the grits, low-fat meal and low-fat flour. Detoxification of aflatoxins was reported in milk using Phyllosilicate Clay for 12 to 14 days (Phillips et al., 1995). Another study reported total destruction of aflatoxins in peanut oil of at least 160ppb in glass or plastic jars using local clay and exposure to sunlight for 18 to 24 hours in Senegal (Kane et al., 1996). Under high heat conditions, aflatoxins are very stable and cannot be completely eliminated when boiled or autoclaved. Even under various typical thermal food processing conditions, aflatoxins remain relatively unchanged

(Christensen, et al., 1977). Roasting is one thermal condition that shows a reduction in aflatoxins in peanuts including oil and dry roasted as well as microwave roasted peanuts (Peers, 1975; Luter, et al., 1982).

2.6.1 Irradiation to Eliminate Aflatoxins

Another method which has been investigated to inactivate aflatoxins is the use of gamma irradiation, which is considered a cold temperature process. For example, sterilization of commodities which contain *Aspergillus flavus* has been accomplished through irradiation. In a Peruvian study involving the irradiation of botanical supplements, a dose of 8 kGray was sufficient to eliminate all microorganisms, including *Aspergillus,* from the plant material (Castro Gamero, 1995). In maize kernels, doses ranging from 1 to 2 kGray were required to eliminate *Aspergillus* spp. (Gero, et al., 1986). However, in an additional irradiation study, there was no effect on aflatoxins present in maize, wheat and soybean exposed to 20 kGray (Hooshand, 1995). However, exposure of a toxigenic strain of *Aspergillus flavus* on inoculated yellow corn to microwaves showed that the rate of aflatoxin destruction increased as the exposure time to microwaves increased (Farag, 1996). The moisture content of the yellow corn that showed a reduction of aflatoxins was at least 17%; whereas, the aflatoxin content was not affected in the dry model system of pure aflatoxins coated on a silica gel. In addition to physical methods, biological methods have been attempted. Biological methods that have been examined in order to decontaminate grains include the use of microorganisms under various conditions. During the fermentation process of beer brewing, aflatoxin B_1 can be reduced by as much as 70-80% in the starting materials and is not found in the alcohol portion of the product

(Chu, et al., 1975). Destruction and inactivation of aflatoxins can be accomplished by a number of chemical treatments. The most effective and practical method is the use of anhydrous ammonium gas which when applied at elevated temperatures (80 - 120˚C) and pressure (30-50 psi), resulting in a 95 to 98% reduction in total aflatoxins in peanut meal (Van Egmond, 1991).

2.7 Regulations

In the U.S., the Food and Drug Administration regulates the allowable levels of aflatoxin in various commodities. The "action level" (Table 1) is 20μg of total aflatoxins (B_1, B_2, G_1, & G_2) per kilogram in food for humans including corn, peanuts and peanut products with varying allowable levels for animal feeds up to 300μg/kg for corn and peanut products intended for finishing (i.e., feedlot) beef cattle (Lubulwa & Davis, 1994).

Table 1: "Action levels" for aflatoxins in foods and feeds (FDA, 2000).

Commodity	Action Level
Animal Feeds	
Corn and peanut products intended for finishing (i.e., feedlot) beef cattle	300*
Cottonseed meal intended for beef, cattle, swine, or poultry (regardless of age or breeding status)	300
Corn and peanut products intended for finishing swine of 100 pounds or greater	200
Corn and peanut products intended for breeding beef cattle, breeding swine, or mature poultry	100
Corn, peanut products, and other animal feeds and feed ingredients but excluding cottonseed meal, intended for immature animals	20
Corn, peanut products, cottonseed meal, and other animal feed ingredients intended for dairy animals, for animal species or uses not specified above, or	20
Brazil nuts	20
Foods	20
Milk	0.5 (aflatoxin M1)
Peanuts and Peanut products	20
Pistachio nuts	20

*ppb (ng/g) total aflatoxins

2.8 Toxigenic *Fusarium* spp.

The second most commonly found toxigenic fungi in corn are species of *Fusarium*. The first reported case of moldy-corn poisoning of horses linked to *Fusarium* fungi was reported in the U.S. in the early part of the 1900's and the disorder identified as equine leukoencephalomalacia (ELEM), a disease of the central nervous system that affects horses, mules, and donkeys (Butler, 1902; Schwarte et al., 1937). Since 1928, there have been many reports of field outbreaks of mycotoxicoses caused by *Fusarium* damaged corn being fed to animals (Tuite et al., 1974). Outbreaks of the *Fusarium* related disease ELEM have also occurred in South Africa (1970) (Marasas, et al., 1984). Also, in the fall of 1989 and winter of 1990 horses exhibiting ELEM symptoms and pigs diagnosed with porcine pulmonary oedema (PEM), characterized by "changes in oxygen consumption caused by hypoxic vasoconstriction which reduces the mechanical efficiency of the left ventricle, thus, pulmonary oedema in pigs is due primarily to acute left-sided heart failure" (Constable et al., 2000; Smith et al., 1999; Smith et al., 2000). In both instances the animals were fed *Fusarium* contaminated corn (Marasas, et al., 1984). The fungus isolated, *Fusarium verticillioides* (*F. moniliforme*), was shown to be carcinogenic and hepatocarcinogenic in rats in 1984 (Wilson, et al., 1985). In 1988, the chemical structure of the compound which causes ELEM and PEM was identified and named fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) (Bezuidenhout, et al., 1988; Gelderblom, et al., 1988).

2.8.1 Taxonomy of *Fusarium* spp.

Over the last 25 years there has been intensive research and studies on the taxonomy and toxigenic potential of *Fusarium* species. Correlating species of *Fusarium* with toxin production is somewhat unclear due to the misidentifications of fungal species and toxin characterization (Burgess, et al., 1981; Marasas et al., 1984). However much of the confusion has been eliminated following the introduction of a *Fusarium* taxonomic system developed by Nelson in 1983 (Burgess, et al., 1981; Marasas et al., 1984). It is now estimated that approximately 20 species of*Fusarium* can occur in maize and other grains, with a few being of great importance due to their high pathogenecity, frequency and toxicigenic capabilities. These include *F. culmorum*, *F. graminearum*, *F. subglutinans*, and *F. verticillioides* (Burgess, et al., 1981; Marasas et al., 1984).

2.8.2 Pathogenicity and Occurrence of *Fusarium* spp.

Fusarium species are the most encountered toxigenic contaminant in human foods including cereal grains, beans and oil seeds (Chelkowski, 1989a). In corn, *Fusarium* species are both plant pathogens as well as soil saprophytes (Chelkowski, 1989b). There is increasing concern about fumonisins in corn because they are omnipresent in all corn products, including corn-based breakfast cereals, cornmeal, corn flakes, tortillas, tortilla chips, popcorn and sweet corn (Stack, 1997; Shephard, *et al*., 1996). They have also been found in a survey of tortilla chips and sweet corn from retail outlets in Italy (Doko & Visconte, 1994). Although there are more *Fusarium* mycotoxins found in corn than in any other crop due to the numerous species of *Fusarium* that can attack the plant, *F. verticillioides* is the most common

soil-borne pathogen found in corn in all regions of the world. Frequently, *Fusarium* can cause both symptom less infection of corn plants as well as grain infection (Marasas et al., 1984b). It is not unusual to find 100 percent internal kernel infection by *F. verticillioides* (Marasas et al., 1984b). The fungus is transmitted "vertically and horizontally" to the next generation of plants via clonal infection of seeds and plant debris (Foley, 1962). The means that the fungus infects the host plant "horizontally" is from outside inoculum sources such as conidia or mycelia present in plant debris. In the endophytic phase, the fungus infects the plant vertically, starting from the fungus in the inter-cellular tissues of the seeds spreading throughout the plant during the growing season without showing signs of infection. Thus *F. verticillioides* is a seed transmitted pathogen that produces no symptoms of infection (Kendera, et al., 1994; Bacon & Hintin, 1996; Bacon et al., 2001; Desjardins, 1998). Environmental factors that favor kernel infection by *Fusarium* are warm temperatures (20-30˚C) and rainfall shortly before silks emerge (Lacey, 1989). Plants remain susceptible for 7 to 10 days after silking occurs. Other environmental factors which increase the likelihood of infection are physical damage to the maturing ear such as from birds or insects, drought stress, warm, dry climates, and the presence of other fungal diseases (Miller, 2001).

2.9 Production of Fumonisins by *Fusarium* spp.

Fusarium verticillioides, currently the most prominent of the *Fusarium* species, produces a number of mycotoxins, the most significant being the fumonisins. There are more than 10 fumonisins, but only three, FB_1, FB_2 and FB_3, occur naturally (Richard, et al, 1993).

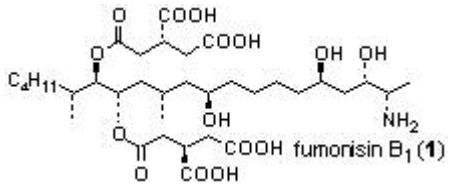


Figure 2. Fumonisin B_1 chemical structure.

The Agency for Research on Cancer (IARC) evaluated the fusarium toxins fumonisins as Group 2B carcinogens i.e. possibly carcinogenic to humans (IARC 1993b). Fumonisin B_1 is considered a serious threat to human and animal health and is responsible for a number of diseases including ELEM and PEM in test animals and more recently hepatic carcinogenicity in rats (Dawlatana, *et al*, 1995). Studies on the toxicological nature of fumonisins indicate that FB_1 and FB_2 may possibly be carcinogenic to humans. For example, fumonisins are believed associated with oesophageal cancer in two regions in the world; the Transkei region in South Africa (Gelderblom *et al*., 1988, 1992, Theil *et al*., 1992, Marasas, 1995) and in Linxian county, China (Chu & Li, 1994, Groves,et al., 1999). In a study done by the FDA, fumonisin B_1 added to the diets of male rats caused renal tubule tumors and in female rats induced hepatic tumors (Howard, et al., 2001).

2.10 *Fusarium* spp. Control

Elimination of the toxigenic fumonisins in foods and feeds is of paramount importance because of widespread consumption of corn and the frequent occurrence of fumonisins in corn. There are a number of studies which have looked at various control and prevention strategies for preventing growth and toxin production of *Fusarium* on crops still in the field (Miller, 2001). Other studies are focusing on

elimination of fungal growth during storage while a number of researchers are examining post-harvest removal of the fumonisins from the commodity itself (Bacon, et al., 2001; Bullerman, et al., 2002). The primary strategy for pre-harvest control of *F. verticillioides* is to prevent the fungus from ever infecting the plant. However, this is difficult as *Fusarium* species are capable of invading the endosperm of the corn kernels by perforating the seed coat after enzymatic changes in the outer areas of the seed coat (Chelkowski, 1989). In addition, *Fusarium* can colonize the silk to reach the developing kernel. Since *F. verticillioides* is ubiquitous in corn it is important to improve resistance of corn to *Fusarium spp*. by developing resistant varieties. One of the most important environmental causative factors leading to *F. verticillioides* infection in corn are crops grown outside their area of adaptation which are at greater risk for fumonisin accumulation due to the increased stress placed on the plant (Shelby, et al., 1994; Visconti, 1996).

2.10.1 Pre-harvest prevention of *Fusarium verticillioides*

An important signal-controlling factor for cereal diseases caused by *Fusarium* species is temperature. *Fusarium verticillioides* growth and subsequent fumonisin production of in corn occurs optimally in an environment with temperatures well above 26˚C, a water activity above 0.87. Infection of corn by other *Fusarium* fungi such as *F. graminearum* also predisposes the corn to *F. verticillioides* and fumonisin accumulation (Miller, 2001). Insect damage also increases infection by *F. verticillioides*; as the number of insect herbivory on corn increases, *Fusarium* disease increases leading to greater amounts of fumonisins (Lew, et al., 1991). A strategy currently being utilized to reduce the incidence of insect damage is through the use of

transgenic crops developed which contain insect resistance proteins. Biological control of *Fusarium verticillioides* in corn has also been attempted using the bacterium *Bacillus subtillis* as an inhibitor of the fungal growth during its endophytic growth phase (Bacon, et al., 2001). Other methods of control include burning crop debris prior to planting to destroy the fungus (Trenholm, et al., 1989). Crop rotation which has been shown to reduce insect infestation of wheat and corn, using urea fertilizer instead of ammonium nitrate fertilizer for nitrogen sources will reduce *Fusarium* infection. Application of fungicides during saphrophytic phase on crop debris, planting early and effective weed control should aid in the reduction of *Fusarium* infection of crops.

2.10.2 Post-harvest control of *F. verticillioides*

Using *Trichoderma* fungal species for post-harvest control of the saphrophytic phase of growth and toxin accumulation of *F. verticillioides* on corn in storage has been proposed (Bacon, et al., 2001). Proper storage conditions should also reduce growth and fumonisin production of *F. verticillioides*. Riley reported that maintaining silage and kernel moisture content at less than 22% reduced or prevented fumonisin production during storage (Riley, et al., 1993). It has been reported that FB_1 production rate in storage was maximum at 20˚C and then sharply decreased at temperatures: 10, 15, 25, and 30˚C (Le Bars, et al., 1994). Fumonisin production is minimal when there is a low oxygen tension and the nitrogen and/or carbon dioxide levels in the atmosphere are modified. Irradiation prior to storage may preserve foods in storage by eliminating the spoilage fungi (Cuero, et al., 1986).

Microwaving has been used to sterilize field corn naturally contaminated with *Fusarium*, *Aspergillus*, and other naturally occurring field fungi. At 2600 watts for 16 seconds, 100 grams of corn placed in a 600 mL beaker was rendered completely sterile (Wetter, 1996).

2.11 Occurrence of fumonisins

Fumonisins appear to be wide spread in U.S. corn (Bullerman, 2001). Surveys of 1,300 maize samples collected in the central United States from 1988 through 1995 indicated low levels of fumonisin B_1 in the majority of samples, with levels ranging from 5 to 38 μg/g (Munkvold & Desjardins, 1997). Significant fumonisin accumulation in maize occurs when weather conditions favor *Fusarium* kernel rot (*F. graminearum*), and the severity of ear infection is a good indicator of fumonisin levels (Pascale, et al., 1997). Warm, dry years result in greater concentrations of fumonisins than cooler years (Murphy, et al., 1993).

2.12 Food processing and its affect on fumonisins

The destruction of fumonisins does not occur during standard food processing methods. Although it had been shown that after wet milling, starch prepared from fumonisin contaminated corn was free of the toxin, the gluten and fiber fractions were still highly contaminated with FB_1 (Bennett and Richard, 1996). Ammoniation, although effective for removal of other mycotoxins, is not an effective method for the removal of fumonisins from corn (Norred, et al., 1991). However, more severe methods have been shown to reduce fumonisins. For example, the treatment of corn with lime and water in corn products (tortillas) can reduce levels of fumonisins

(Sydenham, et al., 1991). Corn contaminated with fumonisins and treated with a simulated nixtamalization ($Ca(OH)_2$) alone or in addition with $NaHCO_3 + H_2O_2$ produced 100% reduction of FB_1 (Park, et al., 1996).

2.13 Using irradiation to eliminate fumonisin

Currently, there appear to be no studies using irradiation on grains, including corn, to eliminate fumonisins. Food irradiation is the controlled application of energy in the form of ionizing radiations such as gamma rays, X-rays or accelerated electrons to food products (Sharma and Nair, 1994; Urbain, 1986). Upon irradiation of foods, the primary reaction is the ionization of water molecules. Upon exposure to ionizing radiation, the water molecule is split into a positively charged water radical and a negative free electron. The water radical then decomposes into a hydroxyl radical and a hydrogen ion. The reaction progresses until the end products of hydrated electrons, hydroxyl radicals, hydrogen ion and hydrogen atoms are formed (Murano, 1995). A study conducted by Cuero, et al., (1986) looked at the effects of gamma irradiation on maize seed microflora before and after treatment. They found that at 120 Krad, *Aspergillus* and *Penicillium* were eliminated from the corn, whereas *Fusarium* spp. eradication was not obtained until a level of 1200 Krad was reached. Sensitivity of microbes to irradiation was also influenced by water content of the maize seeds. Microorganisms were more susceptible to destruction at moisture levels of 22% than at 15% (Cuero, et al., 1986).

2.14 Sampling Protocols for Fumonisin in Grain

Fumonisin levels are detectable in cereal grains and processed grain-based foods (Bullerman, 2001), using a protocol that includes size reduction and mixing, subsampling, extraction, filtration, cleanup, concentration, separation of components, detection, quantification and confirmation (Bullerman, 1987; Steyn, et al., 1991).

2.15 Methods for detection of fumonisins

A number of methods have been published for fumonisin analysis. They can be classified as chemical and immunochemical methods (Musser, et al., 2002). The Food and Drug Administration as well as other government agencies utilize AOAC approved procedures in order to detect fumonisins in various commodities. Two frequent chromatography methods employed to detect fumonisins involve chemical analysis of commodities using enzyme-linked immunosorbent assay (ELISA) and HPLC (LC).

2.15.1 Chromatography Methods to Detect Fumonisins

Traditional chromatographic techniques such as thin layer chromatography (TLC), and liquid chromatography (LC) and gas chromatography (GC) are widely employed for separation and quantitation of the mycotoxins (CAST, 2003). HPLC is the most commonly used (Trucksess, 1998). For fumonisin, the most common extraction methods are ELISA or LC with fluorescent detection separation and quantitation; the toxin concentration is based on the intensity of the fluorescence observed (Trucksess, 2003b). Fumonisin after derivatization with OPA reagent has a UV absorbance with a maximum range from an excitation of 335 nm, to an emission

of 440 nm for fumonisin B_1 when measured in acetonitrile-water-acetic acid (5:5:1). ELISA and IAC assays are in general not as sensitive and precise as TLC and LC methods.

2.15.2 Thin Layer Chromatography

TLC separation technique for fumonisins in corn also uses derivatization of the free amine group usually with fluorescamine spray F1-y[c] (Trucksess, 2000b). The extraction solvent used is ethanol + water+ acetic acid (65:35:1). The sample cleanup employs strong anion exchange and solid phase extraction (SAX, SPE). The sample is dissolved in 100 μL of acetonitrile/water (l:l), spotted on a C18 TLC plate along with fumonisin B1 standards (5,10, and 100 ppm) dissolved in acetonitrile/water (1:l). The TLC plate is developed in a mobile phase of methanol + 4% aqueous KC1 (3:2), air-dried, and sprayed with sodium borate buffer followed by fluorescamine in acetonitrile. The plate is sprayed with 0.01 M boric acid + acetonitrile (40:60). The TLC plate is typically air-dried at room temperature and examined under long wave ultraviolet light. Fumonisin levels are usually estimated by visual comparison with standards.

2.15.3 Reverse Phase Liquid Chromatography

Reversed phase LC is the most widely used procedure for separation and determination of fumonisins. When LC methods are used since the fumonisins do not naturally fluoresce under UV detection, the amine group must be derivatized with the reagent *O*-phthaldiadehyde. This is done by pre-column derivatization. The autoinjector is programmed to mix 50.0μl standard or sample solution with 200.0μl OPA reagent. A programmable LC fluorescence detector is used for the detection of

fumonisins. The most common method employed for detection and quantification of fumonisins is high performance liquid chromatography (Richard, et al., 1993).

2.16 Fumonisin Regulations

In the U.S., the FDA has established that fumonisins cannot be completely eliminated from the food supply. The goal of the FDA therefore is to establish a safe Acceptable Daily Intake level for fumonisins in humans and animals "that would result in negligible risk or a reasonable certainty of no harm" (Troxell, 1996). The FDA has established the following guidance levels for fumonisins (FB1+FB2+FB3) in foods (Table 2).

Table 2: "Guidance levels" for fumonisins in foods (Henry K. & Lovell, R. 2001).

Guidance Levels for Fumonisins in Foods	Total Fumonisins
Degermed dry milled corn product	2 ppm*
Whole/partly degermed dry milled corn product	4 ppm
Dry milled corn bran	4 ppm
Cleaned corn intended for popcorn	3 ppm
Cleaned corn for masa production	4 ppm

*ppm (µg/g) total fumonisins

2.17 Transgenic Corn

The development of an effective method to prevent toxigenic fungi from infecting corn is very desirable but to date, very difficult to achieve. A number of studies on the secondary effects of reduced insect damage in transgenic corn showed reduced levels of fumonisins produced by *F. verticillioides* (Munkvold, et al., 1999). This discovery was vital as it conforms to the widespread belief by mycotoxicologists that prevention of fungal infection and the production of mycotoxins in grains is the fundamental goal of mycotoxin management (Trenholm, et al., 1989). Transgenic plants are those plants whose cells have successfully incorporated the foreign DNA

into their genome (Sloderbeck, 1995). This transformation is mediated by a soil bacterium, *Agrobacterium tumefaciens* which is pathogenic to plants, causing crown gall tumors at wound sites. This transformation is accomplished by transferring DNA via a tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is integrated into the plant cell's genome, and subsequently transcribed. The bacterial plasmid that naturally infects plants is the vector used in the transfer foreign DNA into a plant's genome (de la Riva, 1998). Bt transgenic corn contains a gene that encodes an insecticidal protein from the soil bacterium *Bacillus thuringiensis* (Bt). The gene codes for δ-endotoxins, which protect the plant from *Lepidopteran* insects. The insect eats the crystalline protein, protoxin, that is modified in the insect's midgut. The converted endotoxin then binds to a specific epithelial receptor, which causes the cells to rupture. Paralysis of the gut occurs next, leading ultimately to the insect's death. The corn *Lepidopteran* insects controlled by the Bt insecticidal gene are the European corn borer, Southwestern corn borer, and other corn stalk borers.

Safety of the Bt transgenic corn was thoroughly tested in the field, greenhouse and laboratory as to their environmental, ecological and health safety (Witkowski, 1997). Three U.S. government agencies were involved in the safety testing. The USDA/APHIS performed an environmental assessment and found no significant impact, concluding that it was safe to grow. The FDA conducted a study and found no harmful affect to animal or human health, so it was safe to eat. The EPA judged the environmental safety of the transgenic plants and the protein active ingredient under the category of a "new use of pesticides" and found that it was safe for the environment. Hence, Bt transgenic corn was approved for use as a field crop by

regulators in 1995. Bt transgenic corn does, however, require a permit from the EPA to grow it, as it is considered a plant pesticide, or a plant incorporated protectant (PIP).

2.17.1 Use of Bt corn to control Insects

The major targeted insect in corn is the European corn borer, which feeds mainly on the pollen in leaf axils and tunnels through the stalk. Control attempts and damage from the European corn borer is estimated to cost farmers one billion dollars annually, or a loss of over 40 million tons of corn annually (Powell, 1999). The European corn borer has two generations in U.S. corn (Rice, 1998). The first generation tunnels through the corn stalk, while the second generation occurs during the blister or dough-stage corn. The development of Bt transgenic corn should help solve the problem of European corn borer damage to the plant and the ears, while reducing use of insecticides (Russell, 1992). Ongoing studies have been done to assess the efficacy of the Bt cry1Ab protein in Bt corn in controlling and/or reducing the European corn borer and corn earworm. In 1997, studies were done in Nebraska on Bt 11 and non-Bt corn and inoculated with European corn borer. The results showed significantly higher yields of corn with the Bt 11 corn compared to the non-Bt corn (Clark, et al, 1999). Additional studies yielded similar results demonstrating that Bt corn showed significantly lower insect damage compared to non-Bt corn (Pilcher, et al., 1997; Rice, et al., 1997, & Rice, et al., 1998). *F. verticillioides*, *F. proliferatum* and *F. subglutinans* stalk infections were evaluated in field experiments done in 1998, 1999 and 2000 to determine the effect of Bt corn on the level of European corn borer damage and mold infection (Gatch and Munkvold, 2002). There

were significant differences in the mean percentage of stalk infection between Bt and non-Bt corn in 1998 and 1999, but not in 2000.

2.17.2 Reduced Mycotoxin levels in Transgenic Field Corn

High populations of *Lepidoptera* insects in corn can cause an increase in the levels of toxigenic fungi, leading to corn ear rots. Therefore, an ongoing investigation has been in progress analyzing the influence of Bt corn on the development of two, commonly occurring, toxigenic fungi, *A. flavus* and *F. verticillioides*. The purpose of these experiments was to study the incidence of *F. verticillioides* and *A. flavus*and their toxic metabolites, fumonisins and aflatoxins, in Bt corn. It has been investigated whether or not transgenic corn can provide an effective means of eliminating toxigenic fungi from corn, thus subsequently eliminating their toxic metabolites. Reduced kernel injury and larvae movement in kernels of Bt corn ears has been shown to lower the risk of mycotoxin contamination (Munkvold, 1999). In a similar field plot studied done in 1997 and 1998 using Bt 11 corn infected with European corn borer (*Ostrinia nubilalis*) and inoculated with *A. flavus* and another fumonisin producer, *F. proliferatum*, the Bt corn showed a 30 to 40 fold lower fumonisin level compared to non-Bt (Dowd, 2000). Furthermore, fewer significant differences in fumonisin levels were found between Bt and non-Bt when the corn was also infestation with corn earworm (*Helicoverpa zea*). In addition, the *F. proliferatum* levels were not significantly different between Bt and non-Bt corn. Studies done in 1998 and 1999 on the association of insect damage with mycotoxin levels in corn and ear molds showed that there were some significant differences between Bt and non-Bt corn concerning fumonisin levels and significant

correlations between insect damage and fumonisin levels suggesting that reduced insect damage was causing lower fumonisin levels (Dowd, 2001). However, Bt corn in different sites, years and different hybrids showed variability between the correlations. In a 2000-2002 study of Bt hybrids in 107 locations in the U.S. including Nebraska, Iowa, Illinois and Kansas, there were lower fumonisin levels in the Bt hybrids compared to the non-Bt hybrids (Hammond, et al., 2004). It was found in a 2000 and 2001 Illinois study that Bt corn with European corn borer damage showed reduced fumonisin levels compared to non-Bt corn but not when damaged by corn earworm (Clements, et al., 2003). Bt corn did not show as dramatic a reduction in aflatoxin levels compared to non-Bt corn as wasseen in the levels of fumonisins (Munkvold & Hellmich, 2000). *A. flavus* infection and aflatoxin concentrations have been reduced such that there were no significant differences among hybrids in studies done in Iowa and Illinois. In contrast, aflatoxin concentrations in the Mississippi and Texas studies were very high. In studies in the southern US in Mississippi and Texas, southwestern corn borer insect damage actually increased aflatoxin amounts well above the FDA action level of 20 ppb due to infection by *A. flavus* in Bt corn, however there were significantly lower levels in the Bt corn compared to the non-Bt corn (Windham, et al., 1999).

2.17.3 Co-inoculation Studies using Transgenic Field Corn

In any crop of field corn, *Fusarium* is the most dominant species present, followed by *Aspergillus* species. Since these fungi can grow together on the same ear of corn, it is important to study their relationship with one another as it affects fungal growth and toxin production. A study performed in Mississippi in 1989, reported that

Aspergillus and *Fusarium* fungi growing together on hybrid corn resulted in a synergistic relationship; whereas *Fusarium* affects *Aspergillus* growth and aflatoxin production, *Aspergillus* did not affect growth and toxin production by *Fusarium* (Zummo and Scott, 1992). This study used a co-inoculation procedure where the fungal spores are manually applied to the emerging silks on the ears. These authors, (Zummo and Scott) reported that ears inoculated with both *A. flavus* and *F. verticillioides* have significantly less aflatoxin than ears inoculated with *A. flavus* alone. In a following study, Marín, et al. 1998, it was found that *Fusarium* species inhibited the growth of *Aspergillus* and the production of aflatoxins.

It has been demonstrated in a number of field studies that Bt corn under certain conditions may reduce the intensity of *Fusarium* infection and fumonisin concentrations. In other studies, *A. flavus* infection and aflatoxin concentrations were too low to distinguish a significant difference between Bt and non-Bt corn in Iowa and Illinois. Still other studies showed that there were extremely high aflatoxin concentrations in Mississippi and Texas (Munkvold & Hellmich, 2000). Studies looking at the competitive colonization and direct interaction between *A. flavus* and *F. verticillioides* and mycotoxin production of the two fungi in Bt corn have been limited. It is important to determine if one fungus is able to invade and establish a competitive advantage over the other more efficiently given varying inoculation patterns, given that both fungi are naturally present.

2.18 Resistant non-transgenic Corn lines

Commercial corn hybrids have been evaluated for inherent insect resistance since the early 1970's as well as for reduced aflatoxin levels from *Aspergillus flavus*

natural field infection and researchers have found significant differences in aflatoxin levels among hybrids samples from non-infested plots (Widstrom, et. al, 1978). Studies were continued by a number of research groups on inbred corn lines which demonstrated resistance to aflatoxin production and *Aspergillus flavus* infection as well as insect resistance throughout the late 1980's and 1990's. Cultivars were identified in a southeastern U.S. study that were resistant to corn ear infesting insects compared to control corn hybrids (Barry, et. al., 1991). In a later study inbreds were highly and consistently resistant to *Aspergillus* ear rot, kernel infection and production of aflatoxins (Campbell, 1995a). It was determined that the resistance seen in the inbreds in addition to other resistant inbreds, was likely inherited and therefore part of the plant genome. The latest work known on *Aspergillus* ear rot and aflatoxin accumulation resistance was published in 2002 examining 6 resistant inbreds and found that 2 had the lowest levels of aflatoxins and were most resistant to ear rot (Naidoo, et. al., 2002). These inbreds were selected for molecular mapping to determine their genetic composition.

In the early 1990's, work on a sweet corn line resistant to *F. verticillioides* kernel infection, was being conducted to determine genetic factors as the potential mechanism of resistance specifically examining maternal tissues such as the silk, pericarp and closing layer (Headrick, 1991). The field inoculation study determined that silk actively growing after pollination inhibited *F. verticillioides* from infecting the kernel and indicated the potential use of this inbred as the possible parent for use in seed production. In 2001, corn genotypes known for *A. flavus* infection and aflatoxin contamination were examined for resistance to *F. verticillioides* growth

(Brown, et. al., 2001). Both wounded and non-wounded kernels were inoculated with *F. verticillioides* transformed with a reporter gene to easily quantify fungal growth. It was determined that non-wounded kernels of the resistant hybrid were less susceptible to *F. verticillioides* infection.

Studies were also conducted on corn lines to examine resistance to *A. flavus* and aflatoxin contamination. They focused on specific genotypes that were determined to have ears which contained kernels that were fungus-free under evaluation (Widstrom, et. al., 1987). The kernels on the ear were morphologically characterized based on color and observable *A. flavus* sporulation; kernels without *A. flavus* infection were utilized for the field plot inoculation study. The results showed that the kernels showing no infection had significantly less aflatoxin accumulation. In another experiment, aflatoxin resistant maize genotypes were compared to drought tolerant genotypes as to cuticle and wax layers in the kernel (Tubajika, 2001). In this experiment, SDS-PAGE was employed to determine the presence of proteins in the kernel as possible sources of resistance (Tubajika, 2001). The results suggested that the pericarp wax layer in the kernels imparted resistance to *A. flavus* infection as this genotype had very high levels according to microscopic observations. Results also showed a 14 kDa protein in both drought tolerant and control genotypes, however amounts were greater in the drought tolerant maize. This protein was characterized as a trypsin inhibitor, believed to inhibit fungal α-amylase thus affecting growth. The fact that drought tolerant genotypes were more resistant may indicate an association between the two.

The specific kernel resistance mechanisms were examined in kernels resistant to post-harvest aflatoxin contamination by *A. flavus* as well as fungal growth (Brown, et. al., 1993). Only autoclaving, crushing or wounding of the embryo caused a loss of aflatoxin accumulation resistance. The study authors concluded that the metabolic activities of the living embryo conveyed the resistance to aflatoxin contamination in the kernel. Plant antifungal proteins were identified in the seeds of corn and other grains (Vigers, et, al., 1991). Antimicrobial proteins known to be present in seeds are chitinases, β-1, 3-glucanases, proteinase inhibitors, and ribosome-inactivating proteins. Seeds were found to contain an antifungal zeamatin like 22kDa protein, which caused the cell membrane to become permeable, demonstrating like activity to zeamatin.

In a multi-lab study, highly resistant maize hybrid lines were screened for proteins conveying antifungal activity against *A. flavus* and *F. verticillioides* during germination of corn kernels (Guo, et. al., 1997). Using SDS-PAGE gel electrophoresis, antifungal proteins were examined in both germinated and non-germinated kernels. Higher concentrations were found in the germinated kernels compared to controls, and when Western Blot analysis was conducted, the 22kDa zeamatin-like protein was present as well as two ribosome inactivating proteins (RIP), of 11-kDa and 9-kDa in size. In the non-germinated kernels, a 32-kDa proRIP-like form and an 18kDa peptide were found. When purified zeamatin and RIP were tested on *A. flavus*, growth of the fungus was inhibited, and plant extracts from germinated kernels inhibited both *A. flavus* and *F. verticillioides*. In another similar study, a >100 kDa protein was found to inhibit aflatoxin biosynthesis, and a 28-kDa protein

similar to pathogenesis-related proteins-5 (PRs), which is very similar to thaumatin, was determined to inhibit growth (Huang, et. al., 1997). Dry kernel extracts were tested for antifungal activity from a resistant population and were found to contain a 14-kDa protein which was determined to both inhibit *A. flavus* growth and produce trypsin activity causing the spore to rupture and hyphae to develop abnormally. (Chen, et, al., 1998). This same corn trypsin inhibitor was tested for inhibition against both *A. flavus* and *F. verticillioides* and was found to be an effective inhibitor of both fungi simultaneously (Chen, 1999). Five additional proteins were determined to be associated with resistance to aflatoxin production in resistant corn populations and are also stress-related proteins (Chen, et. al., 2001; Chen, et, al., 2002). Globulin-1 and globulin-2 embryo storage proteins are high in glycine and are associated with kernel resistance. Late embryogenesis abundant proteins (LEA) related to drought, water or osmo-stress related proteins (WSI18 and aldose reductase), and heat stress related proteins (HSP16.9). The determination of the dominant alleles in *A. flavus* and aflatoxin resistant inbreds to be transferred to commercial inbreds has also been investigated (Maupin, et. al., 2003). Potential resistance to fumonisin accumulation and *Fusarium* Ear rot in corn in currently under study, with a number of dominant genes to be determined conveying resistance as well as the potential to transfer these alleles to commercial hybrids (Clements, et. al., 2004).

2.18.1 In-Vitro Non-transgenic corn co-inoculation studies

In a plant growth room controlled study, aflatoxin production was lower in maize ears infected with *F. moniliforme* and *A. flavus* (Wicklow, et al., 1988). In a

similar study the same results occurred when *A. flavus* was in direct competition with *F. moniliforme* and *Penicillium oxalicum* (Lillehoj, et al., 1982). Studies comparing growth proficiency and mycotoxin formation *A. flavus* and *F. verticillioides* have been conducted on maize *in vitro*. It was observed that under ideal conditions of temperature and moisture, *F. verticillioides* was the dominant species, out competing *A. flavus* and *Penicillium* species (Marin, et al., 1998). In mixed cultures of *A. flavus* and *F. proliferatum* on irradiated maize seed under optimal growth conditions in vitro, the interaction produced an inhibition of aflatoxin B_1 and a stimulation of fumonisin B_1 (Picco, et al., 1999). Growth of *A. flavus* is suppressed by interactions with *Rhizopus stolonifer*. Aflatoxin production is decreased by *A. niger, Penicillium viridicatum* and *Fusarium graminearum* (Lacey, 1989).

2.19 Botanical Supplements

In the US, herbals are becoming increasingly popular in the U.S and are readily available in health stores, pharmacies, and grocery stores. In a 2001 survey commissioned by the Dietary Supplement Education Alliance, 23% of the 1027 Americans surveyed regularly use herbs and specialty supplements (Harris Poll, 2001). In 1994, herbals were legally defined as dietary supplements by the Dietary Supplement Health and Education Act (DSHEA). The DSHEA exempted manufacturers of dietary supplements from having to prove safety or efficacy before marketing and placed the burden of proof on the FDA to show that a supplement is unsafe. On March 10, 2003, the FDA proposed labeling standards for dietary supplements. This ruling would establish federal standards to ensure that dietary

supplements are not adulterated with contaminants or contain other harmful substances and are labeled to accurately reflect the ingredients found in the products.

The FDA has relied on “an inefficient system of voluntary reporting of adverse events” (Lashof, et. al., 2002). A recent body of research on many herbals regarding the safety of their active components and the presence of toxic adulterants from plant sources added to the herbal product as fillers has assisted the FDA with ensuring the safety of dietary supplements. However, little information exists on mycotoxins and their potential risks in herbals. Herbal delivery systems are quite variable and include capsules, tablets, teas, tonics, and food ingredients. Also, herbals may be consumed fresh, dried, or processed.

Many herbs are imported from countries where quality control may be deficient. The materials may be moldy due to exposure to adverse weather conditions, insect damage, improper harvesting procedures, transportation, inadequate drying and poor storage facilities. The growing mold may produce toxic secondary metabolites such as mycotoxins.

Contamination of herbal plant material may occur during one or a number of phases of the production process, and the mold and/or mycotoxin will be present regardless of the finished product appearance. Mycotoxin contamination is often an additive process, beginning in the field and escalating during the harvest, drying and storage processes. Fungi can either colonize in the field, (field fungi) or can contaminate stored plant material under the right environmental conditions (storage fungi) (Christensen, 1974). Environmental conditions, particularly temperature and moisture, are major determinants of fungal colonization of field crops. Mycotoxin

contamination, and especially aflatoxin contamination is favored by drought conditions coupled with above average temperature. Insect and animal damage can also contribute to mold and mycotoxin damage in the field.

Factors affecting the probability of stored plant material being contaminated by mycotoxins include water activity, substrate aeration, and temperature, inoculum concentrations, microbial interactions, mechanical damage and insect infestation (Ominiski, et. al., 1994). As in the field, temperature and water availability and distribution are the key factors determining fungal contamination and mycotoxin production of stored plant material (Wilson & Abramson, 1992). This is an important consideration for herbals because many are imported from countries where quality control is deficient. The plant materials may be moldy due to exposure to adverse weather conditions, insect damage, improper harvesting procedures, transportation, inadequate drying and poor storage facilities. Much of the herbal plant material is imported from countries with tropical or semi-tropical climates, which adds to the potential for mold colonization and growth, and mycotoxin contamination, especially during the post-harvest phase of production. This can be a major problem if inadequate drying has occurred, or if temperature, humidity and moisture are too high during storage or transport of the plant material, leading to continued fungal growth and mycotoxin formation after the plant material processing and finishing has been completed and quality assured.

2.20 Occurrence of Toxigenic Fungi in Botanical Plant Materials

There are over 100,00 species of fungi, although most are not toxin producers. The most common of the known toxigenic species fall into three recognized genera:

Aspergillus, *Penicillium*, and *Fusarium* (CAST, 2003). These genera produce a majority of the agriculturally important mycotoxins. The fungi of the genus *Aspergillus*, are distributed world wide, but are predominantly found in subtropical and warm temperate climates. They are generally considered saprophytes, and due to their growth at high temperatures and low water activity they are able to colonize a variety of crops and crop debris under favorable conditions. These conditions include above average temperature and below average rainfall as well as insect damage. The fungi are able to grow and produce toxins on most any stored substrate. *Aspergillus* species produce a number of mycotoxins including aflatoxins, ochratoxins, versicolorins, steringmatocystin, gliotoxin, citrinin, CPA, patulin, citreoviridin, and tremorgenic. Aflatoxins are the major class of mycotoxins, which are produced by 4 species, including *A. flavus*, *A. parasiticus*, *A. nominus*, and *A. pseudotamarii*.

Aspergillus flavus is the predominant species found on all commodities (Payne, 1992; Payne, 1998). The optimum temperature for growth of *A. flavus* is 37ºC, however the fungus can grow between 12 to 48ºC. The ability of *A. flavus* to grow more favorably than other fungi under drought conditions and high temperatures is due to its growth at water activities (a_w) as low as –35megapascals (Klich, et. al., 1994). Soil and crop debris are the major source of *A. flavus* inoculum, therefore the fungus is present in varying populations in crop planted fields, where it can easily colonize, proliferate, and cause infection.

2.20.1 Mycotoxins found in Botanical Plant Materials

Although there are hundreds of fungal metabolites that are toxic in experimental systems, there are only five that are of major health concerns for

humans and domestic animals: aflatoxins (AF), deoxynivalenol, fumonisins, ochratoxin A (OTA) and zearalenone (Miller, 1995). These toxins have a wide range of toxic effects, including carcinogenicity, neurotoxicity, immunotoxicity, and reproductive and developmental effects. AF and OTA have been associated with carcinogenic health problems in humans and animals.

Aflatoxins, a group of structurally related mycotoxins produced mainly by *A. flavus* and *A. parasiticus* are the most potentially mutagenic and carcinogenic substances known (JECFA, 1999). The major aflatoxins found in plants are B_1, B_2, G_1, and G_2. Of these, the most potent is aflatoxin B_1 (AFB_1). (The International Agency for Research on Cancer, IARC, 1993a). More than 35 types of food products in marketplaces have been reported to be contaminated with AF or AF metabolites. These include tree nuts, peanuts, figs, melon seed, pumpkin seed, sesame seed, sunflower seed, lotus seed, coix seed, red pepper, white pepper, nutmeg, paprika, mixed spice, rice, corn, mixed cereals, chilies, and copra (Wilson, 2002). AF has been found in herbs and spices at levels ranging from less than 10μg/g to 51μg/g (MacDonald & Castle, 1996).

Herbs, being plant-derived, may be heavily contaminated by viable fungi both in the field and during handling and storage. Medicinal plant materials have been examined by researchers to determine rate of occurrence of fungal species present and levels of mycotoxin contamination. Most of these studies have been focused on ginger and turmeric, which are herbals roots grown in the tropical and subtropical regions. These studies have found the presence of AF and OTA, often at high levels (Scott & Kennedy, 1975;Vrabcheva & Gareis, 2002; Seenappa & Kempton, 1980;

Horie, et. al, 1982; Flannigan & Llewellyn, 1992; Aziz, et. al., 1998; Baratine, & Tantaoui-Elaraki, 1997).

2.21 The botanical, ginseng

Ginseng is one of the most widely sold herbals in the US (Yat, et. al., 2002) and has been used for over 3000 years for the medicinal and adaptogenic properties of its roots. Until this 2003, three plant species were sold in products labeled as ginseng. These are: American ginseng (*Panax quinquefolius*), species native to and primarily grown in the United States; and Asian ginseng (*Panax ginseng*) more commonly grown in Korea, Japan, and China; and Siberian ginseng (*Eleutherococcus* senticosus) a native species of Russia. The active components of American and Asian ginseng are believed to be ginsenosides and the levels and forms of the constituent ginsenosides differ between species. These three species have been marketed worldwide as ginseng, although Siberian ginseng does not contain ginsenosides. However, since Siberian ginseng is not taxonomically similar to Asian or American ginseng, and furthermore not being in the genus *Panax*, legislation was passed in 2003 in the U.S., preventing products that do not contain *Panax* spp. to be labeled or sold as ginseng.

2.21.1 Harvesting and processing of ginseng roots

Ginseng roots are usually harvested in late summer or early fall. The procedures that growers and ginseng collectors employ for harvesting, drying, and storing ginseng are variable and there are no industry standards. The recommended post-production practices for American ginseng (*Panax quinquefolius*) involve

carefully digging the ginseng roots and removing the soil by gentle rinsing. The roots are then spread out on drying beds. Drying beds are often in heated sheds that speed drying by blowing heated air at temperatures from 16 – 27 degrees C over the roots (Pearsons, 1994; Court, 2000). Overheating while drying is avoided because it will spoil the root's color and texture and thus, reduce the crop's value. Depending on temperature and humidity, the drying of ginseng averages about two weeks. If the roots dry too rapidly, they will become moist inside, hard and dry outside (Duke, 1989). After drying, roots are usually stored in containers that are usually exposed to the surrounding air. Ginseng can remain suitable for sale if dried properly for 12-15 months. An alternate method for storage is commonly used for Asian ginseng (*P. ginseng*). After cleaning, Asian ginseng roots are often sterilized by steam between 120 and 130º C for 2 to 4 hours. This process caramelizes the sugars in the root and produces the red-brown coloration of this type of ginseng sold as "red ginseng". The quality of ginseng that has been steam sterilized should be retained for 2 – 3 years (Court, 2000). Fresh roots of both American and Asian ginseng are also marketed, which requires careful storage to prevent rodent damage, beetle infestation, as well as bacterial and mold growth. Due to the unregulated and variable nature of the cultivation, harvest, drying and storage processes associated with ginseng, fungi could grow on ginseng during any of these production phases. These fungi could present a health hazard if they are able to proliferate on the plant material and produce mycotoxins. High humidity and temperature offer the ideal growth conditions for *Aspergillus* and *Penicillium* species (MacDonald & Castle, 1996) and

the effect of environmental conditions on fungal contamination is not known for ginseng.

Although the current aim of quality assurance monitoring programs is to assess the herbals for the presence of adulterants, contaminants and plant toxins or poisons, there are currently no standardized protocols for investigating fungal mycotoxins and herbals (Chan, 1997). The mycotoxin studies cited in this proposal reported methods of analysis that have not evaluated or validated by other laboratories. However, it is essential to develop and validate a method for determining these toxins in dietary supplements that can be used to limiting the sale of mycotoxin-contaminated herbs. Regulatory limits could be developed and exposure to carcinogens in herbals minimized. Risks to human health from consumption of AF have been published for foods other than herbs. For most foods, the acceptable safe level for AF in the U.S. is 20ng/g and aflatoxins M_1 0.5 ng/g in milk.

To date, it is not known if there has been a survey conducted on Appalachian region ginseng, specifically roots cultivated in Maryland, Virginia and West Virginia. The goal of this study is to utilize the ginseng root as a model plant in order to develop a methodology for the detection of aflatoxins in botanical supplements. It is not expected that aflatoxins will be detected in root material that has been properly harvested, dried, processed and stored. Since *A. flavus* is typically a storage fungus, if ginseng is not properly processed and stored, then it is possible that it may become contaminated with *Aspergillus flavus* which may produce aflatoxins.

Chapter 3: The Use of Cobalt Irradiation and Electron Beam to Eliminate Fumonisin From Corn

3.1 Abstract

The purpose of this study was to determine the effects of gamma irradiation and electron beam irradiation on the levels of fumonisins in naturally contaminated field corn. Fumonisins are toxic metabolites produced by mold fungi and are toxic to humans and animals. Fumonisin (FB_1) is produced by the fungus *Fusarium verticillioides (moniliforme*) and is found frequently in corn kernels, some of which may be used as food or food based products. Fumonisin B_1 dissolved in aqueous solution at a concentration of 10.0 μg/mL was irradiated by placing 50.0 mL of the solution in glass tubes which were exposed to 0.5, 1.0, 1.5, 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 kGrays with both electron beam and cobalt irradiation. With the minimum dosage level (0.5kGray) fumonisin B_1 concentration was reduced drastically (99.7%) with both cobalt and electron beam irradiation. Further research was done to determine the effects of irradiation on the levels of toxigenic fungi and their mycotoxins in naturally contaminated whole and ground corn. When ground corn was exposed to gamma and electron beam irradiation of 10.0 kGray dosage, all the fungi, including *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. were killed. Whole kernel corn had no mold growth following a 10.0 mega-rad dosage treatment. In contrast, fumonisin B_1 present in both the ground and whole kernel corn was not significantly reduced after either irradiation treatment. This study demonstrated that although fumonisin B_1 was not significantly reduced or eliminated in corn, irradiation effectively eliminated the toxic molds present in corn.

3.2 Introduction

Mycotoxins are toxic metabolites produced by mold fungi in foods and feeds. These chemical substances are hazardous to both humans and animals and some are considered highly carcinogenic compounds (Sinha, 1993; Coulombe, 1993). To date, no effective means to completely eradicate mycotoxins have been found once they are present in a food product although they can be reduced. The most widespread is the mycotoxin fumonisin B_1, produced by the fungus *Fusarium verticillioides*. It has been reported that fumonisin can be found in any corn product we consume (Munkvold & Desjardins, 1997, Desjardins et al, 1998). Since food safety is of critical importance in today's society, the presence of harmful fumonisin toxins in corn and corn-based products creates a need for developing an effective means for their destruction. Of even more significance is that when aflatoxin B_1, produced by the fungus *Aspergillus flavus*, and considered a cancer promoter, is found in corn in conjunction with fumonisins, the potential for causing primary liver cancer is even greater (Ueno, et al, 1993;Visconte, et al, 1995). What is of concern is that once these toxins are present in the food or feed, it is very difficult to remove them, especially aflatoxins, using conventional cooking or processing methods such as boiling, baking or canning, (Bullerman, et al, 2002). It has been demonstrated that the effects of processing on fumonisin stability and concentration vary with the techniques used and the substance in which the fumonisins are found. Studies involving the bioprocessing and chemical processing effects on fumonisins are few. Of the chemical processes, nixtamalization (($Ca(OH)_2$) alone or in addition with $NaHCO_3$ + H_2O_2) caused a significant reduction in fumonisin levels however the resulting

hydrolyzed fumonisin products may still be toxic. Physical processing methods such as washing, cleaning, steeping also show a significant reduction in fumonisin levels in the uncooked product while dry milling used to produce grits used as a food, had lower levels of fumonisins. The thermal method called extrusion, uses very high heat (>150ºC) which combined with reducing sugars in the product, has shown a 93% reduction in fumonisin levels. It is also proposed that irradiation may be a method to eradicate fumonisins. One study involving the use of irradiation to eradicate aflatoxin B_1 in corn, wheat and soybeans, T-2 in wheat, deoxynivalenol in soybeans and zearalenone in corn was that of Hooshand, *et al*, 1995. They found that radiation doses as high as 20kGrays did not affect the levels of the mycotoxin aflatoxin B_1, yet the levels of the toxins deoxynivalenol and zearalenone were significantly reduced at 10 and 20 kGray treatment; T-2 was significantly reduced at 7.5 kGrays; fumonisin was not included in this study. Another investigation was conducted which surveyed the various physical methods of destroying mycotoxins in foods and feeds including visible and ultraviolet light, gamma rays and chemicals such as chlorine compounds, hydrogen peroxide, ozone, bisulphate, ammoniation, alkalis and acids (Santamarina, et al., 1995). The conclusion reached by the investigators was that no method alone is effective but that a combination of treatments involving gamma irradiation with other methods could possibly be utilized to eliminate mycotoxins. Peanut meal naturally contaminated with aflatoxins was exposed to gamma irradiation of 2.5 Mrad; however, the aflatoxins were not destroyed (Feuell, 1966). Aflatoxins exposed to electron irradiation and soft-X-rays in order to remove the mycotoxin, required doses so high that the commodity being irradiated would be destroyed as well (Frank &

Grunewald, 1970). None of the irradiation studies to date have investigated the effects of gamma irradiation or electron beam irradiation on fumonisins present in grains.

Studies on the effect of irradiation on the reduction and elimination of fungi in foods and feeds have been reported since the late 1950's. The first known study of the affect of gamma irradiation on toxigenic *A. flavus* was that of Frank, et al., 1971. The *A. flavus* isolates were irradiated in growth media in tubes with 1.6 to 2.4 kGrays of irradiation had only a 30% reduction in viability. In more recent studies, sterilization using gamma irradiation of botanical supplements using a dose of 8 kGray was sufficient to eliminate all microorganisms, including *Aspergillus*, from contaminated plant material (Castro Gamero, 1995). In studies looking at irradiating fungi in corn, *Aspergillus* spp. was eliminated from corn kernels at doses ranging from 1 to 2 kGray (Cuero, et al., 1986). *Fusarium verticillioides* in naturally contaminated corn kernels when irradiated at doses from 0.019 to 10.0 kGrays showed no loss in viability (Russell, et al., 1983).

The objectives of this study were to expose naturally contaminated ground and whole kernel corn with fumonisin B_1 to irradiation to determine if fumonisin B_1 could be eliminated or reduced in quantity. In some preliminary experiments, irradiation, either using cobalt or electron beam, appeared to be an effective means of eradicating toxigenic fungi from corn. Irradiation may also be an effective means of decontaminating foods that contain the mycotoxin fumonisin B_1. In this study the levels of toxic fungi in corn before and after irradiation were compared. The purpose of this experiment was to investigate the use of electron beam and cobalt irradiation

technology to eliminate toxigenic fungi and their toxic compounds from ground and whole kernel corn.

3.3 Materials and Methods

The cobalt irradiation and electron beam facilities within the Department of Nuclear Engineering at UMCP were used to irradiate samples; quantification of fumonisin B_1 was by HPLC (High Performance Liquid Chromatography), at the Food and Drug Administration, Washington, DC.

3.3.1 Preparation of Samples

3.3.1.1 Preparation of FB_1 test Solution

Fumonisin B_1 (2.1 mg), obtained from the FDA, was dissolved in 200.0 mL water by shaking for 1 hour; 47.6 mL of this solution was diluted to 50.0 mL to produce a fumonisin B_1 solution of 10.0μg/mL. Samples were prepared for irradiation by placing 50.0mLs of the fumonisin B_1 solution in 100 mL glass tubes, 2 for each dosage level.

3.3.1.2 Preparation of ground corn and whole kernel corn

Fumonisin B_1 naturally contaminated ground corn and whole kernel corn samples were obtained from the FDA. Random samples of 200 g at 12% moisture were selected from the ground corn and whole kernel corn and were placed into 0.5 L glass jars and irradiated (description to follow). After irradiation, samples were analyzed using A.O.A.C. Official Method 997.13 (Trucksess, 2000) for the presence of fumonisin B_1. In addition, for each treatment 0.25 mg samples of irradiated ground corn and 100 kernels of whole corn were plated on potato dextrose agar (PDA) and malt salt agar (MSA) and incubated at room temperature (21ºC) for 3

weeks to determine survivability of fungi in corn after irradiation. The MSA represented a low moisture environment, whereas the PDA represented a high moisture environment. Thus, the purpose for the two media was to see not only the dose resulting in a complete elimination of molds on nutrient rich PDA, but to see the dose resulting in a considerable reduction of molds on restrictive MSA media. There were only two replicates for each dose because the availability of the instrument was limited.

3.3. 2 Sample Extraction and Clean-Up

The Vicam Fumonitest Procedure for fumonisins was used (VICAM, 1999). A 50 g sample of either ground corn or whole kernel corn was placed in a blender jar and 100 mLs of the extract solvent, methanol + deionized water (80+20) plus 5 grams of sodium chloride were added and contents were blended at high speed for 3 minutes. The blended sample was poured into a fluted filter paper and 10 mL filtrate was collected and mixed with a 40 mLs of solution containing 2.5% sodium chloride, 0.5% sodium bicarbonate, 0.01% Tween-20 in water. The filtrate was filtered through a glass microfibre paper and 10 mLs of the filtrate was added to a Vicam Fumonitest Immunoaffinity column at 1 to 2 drops per second. The column was washed twice with 5 mL distilled water and the fumonisins were stripped from the column with 1.0 mL methanol. The eluted sample was derivatized with OPA reagent (see reagents and apparatus) and injected into HPLC for analysis.

3.3.3 Quantification of Fumonisin B_1 (FB_1)

1. Reagents and Apparatus

(a) LC mobile phase: acetonitrile-water-acetic acid (50 +50 + 1).

(b) *O*-phthaldiadehyde (OPA) reagent: 40.0 mg OPA was weighed out and dissolved in 1.0 mL methanol and diluted with 5.0 mL 0.1M sodium tetraborate. Fifty µL 2-mercaptoethanol was added. The OPA reagent was stored before use in the dark for a period not to exceed 1 week.

(c) FB_1 standard: 1.0 mg FB_1 was dissolved in 10.0 mL acetonitrile-water (1 + 1). Ten mL of fumonisin B_1 solution was transferred to a 100 mL volumetric flask and diluted with acetonitrile-water (1+1), to obtain a solution containing 10.0 µg/mL of FB_1.

(d) LC system: Model 510 pump and Millennium data standard station (Waters, Milford, MA 01757); RF-551 fluorescence detector and SIL-10A autoinjector (Shimadza, Columbia MD 21046); and YMC ODS-AQ, 3 micron particle, 4.6 x 150 mm column (YMC Company, Tokyo, Japan)

3.3.4 Derivation and LC Determination

1. The autoinjector was programmed to mix 50.0 µL standard or sample solution with 200.0 µL OPA reagent, and 50.0 µL injected immediately into the LC. The excitation was set at 335 nm, emission: 440 nm, flow rate: 1.0mL/min. FB_1 eluded as a sharp peak at 8.3 minutes. The amounts present were calculated by comparing peak areas of samples and standard. If peak area of sample fumonisin B_1 exceeded that of the standard, the extract was diluted and re-injected.

3.3.5 Radiation Facilities

1. 2-9.5 MeV linear accelerator (Liniac): The Liniac produces micro-second pulses with peak current up to 500 mA. It can operate at single pulse mode or it can deliver a continuous train of pulses up to 360 Hz.

2. **Gamma Test Facility:** It consisted of a 26,000-curie (9.6B114Bq) cobalt-60 source. The source consisted of ten pencil rods, 0.5" (1.27 cm) diameter and 12" (30.5 cm) long, in the form of a hollow cylinder with 4.4" (11.4cm) pitch. Dosimetry was performed in accordance with NIST traceable standards.
3. **Irradiation Dosage Levels:** 0.5, 1.0, 1.5, 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 kGrays for both electron beam and cobalt irradiation for fumonisin B_1 in aqueous solution were used. For ground corn the doses were 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 kGrays for both cobalt irradiation and electron beam; whereas, whole kernel corn the doses were 3.0, 5.0, and 10.0, mega-rads (10 x kGray) for cobalt and electron beam irradiation.

3.4 Results

The levels of fumonisin B_1 found in aqueous solution following both irradiation treatments are summarized in Table 3. The cobalt treatment showed a greater decrease in fumonisin B_1 initially from starting concentration of 13.0 µg/mL to 0.032 µg/mL, however at the highest dose, both treatments had effectively reduced fumonisin B_1 to 0.005-0.006 µg/mL. With the minimum dose (0.5 kGray), FB_1 concentration was reduced to less than 1% of the original concentration Irradiating fumonisin B_1 using electron beam caused the same results. For example, after the minimum dosage (0.5 kGray) the levels of fumonisin B_1 were reduced >99%, and increasing dosages caused only a slightly greater reduction (Table 3).

Table 4 shows the levels of fumonisin B_1 found in ground corn after irradiation treatments. Both cobalt and electron beam irradiation treatments caused very little reduction in fumonisin B_1 levels compared to the levels in the controls.

Table 3: Level of fumonisin B_1 in aqueous solution after treatment with cobalt or electron beam

	Dosage (kGrays)										
	Control	0.5	1	1.5	2	5	10	15	20	25	30
	----------µg/ml----------										
Treatment											
electron beam	13.0	0.082[a]	0.056	0.043	0.027	0.041	0.128	0.01	0.115	0.007	0.005
cobalt	13.0	0.032	0.034	0.026	0.01	0.019	0.01	0.033	0.013	0.014	0.006

[a]Average of 2 single analyses of 2 treated samples

Table 4: Fumonisin B_1 levels in naturally contaminated ground corn after treatment with cobalt or electron beam irradiation.

	Dose (kGray)					
	Control	0.1	0.3	1	3	10
	µg/g					
Treatment						
electron beam	11.7	11.1[a]	10.5	11.6	10.9	12.5
cobalt	12.8	12.8	13.5	13.1	12.9	13.1

[a]Average of 2 single analyses of 2 treated samples

With the highest dose of 30 kGray, the fumonisin B_1 levels were not reduced by either the cobalt or the electron beam treatments.

Whole kernel corn levels of fumonisin B_1 after both cobalt and electron beam treatments are summarized in Table 5. Although there seems to be an initial reduction in fumonisin B_1 levels at 3 mega-rads compared to the control, the level of fumonisin B_1 (7.2-12.0 μg/g) was slightly higher after treatment with 5 mega-rads and 10-mega rads. The results after cobalt treatment were the same except that overall the level of fumonisin B_1 was slightly higher at the 3 irradiation levels.

When ground corn was analyzed after irradiation for fungi present, doses at 10.0 kGray were effective at sterilizing the corn using MSA as the isolation medium (Table 6). There was a considerable difference in the number of kernels with mold growth between the two mediums used. The malt salt agar medium had considerably less mold growth especially at the higher irradiation dose levels. For example, following exposure to 3.0 kGrays on PDA, the plated ground corn had mold growth on all corn particles whereas on malt salt agar only 15% and 10% (electron beam vs. cobalt) of the particles had mold growth.

When whole kernel corn was analyzed for surviving fungi (Table 7), a dose of 10 mega-rads was also effective in killing all mold fungi present on the kernels. Even at the lower dosage level 3 mega-rads, only 10% of the kernels had mold growth compared to 100% in the control treatment. The isolation medium also influenced the levels of mold growth in the previous experiment. After exposure to 3 mega-rads there was 10 times the level of mold growth on PDA versus MSA.

Table 5: Fumonisin B_1 levels in naturally contaminated whole kernel corn after treatment with cobalt or electron beam irradiation

	Dose (mega-rads)			
	Control	3	5	10
	----------ug/g----------			
Treatment				
Electron Beam	7.2	6.8[a]	6.9	7.1
Cobalt	4.5	5.5	5.6	4.3

[a] Average of 2 single analyses of 2 treated samples

Table 6: Percent colonies of fungi present in naturally contaminated ground corn after treatment with cobalt or electron beam irradiation

	DOSE (kGrays)												
		(PDA)	(MSA)	(PDA)	(MSA)	(PDA)	(MSA)	(PDA)	(MSA)	(PDA)	(MSA)	(PDA)	(MSA)
	Control	0.1		0.3		1		3		10		30	
Treatment													
Electron Beam	100	100[a]	100	100	90	100	35	100	15	28	0	1	0
Cobalt	100	100	100	100	85	100	30	100	10	21	0	0	0

[a]0.2 mg ground corn was plated per each treatment on potato dextrose agar vs malt salt agar

Table 7: Fungi present in naturally contaminated whole kernel corn after treatment with cobalt or electron beam irradiation

	Dose (mega-rads)						
		(PDA)	(MSA)	(PDA)	(MSA)	(PDA)	(MSA)
	Control	3		5		10	
Treatment							
Electron Beam	100	10[a]	<1	3	<1	0	0
Cobalt	100	10	<1	3	<1	0	0

[a] percent kernels with mold growth on potato dextrose agar vs malt salt agar respec

3.5 Discussion

In the irradiation study to eliminate fumonisin B_1 in an aqueous solution what probably occurred is as follows. The radiation energy is absorbed by the water to produce: $^{\bullet}OH$, e_{aq}^-, $H^{\bullet}$, H_2O_2, H_3O^+ (Murano, 1995; Woods, 1994; AL-Sheikhly, 1999, Department of Material and Nuclear Engineering, UMD, personal communication). Of these fractions, $^{\bullet}OH$ and $H^{\bullet}$ atoms are the most reactive and abstract $H^{\bullet}$ atoms from the CH_2 chain of the fumonisin molecule causing it to breakdown. Thus the likely decomposition of fumonisin B_1 may result as in the following chemical reactions where FUM represents fumonisin B_1:

$$^{\bullet}OH + FUM \rightarrow FUM^{\bullet} + H_2O$$

$$H^{\bullet} + FUM \rightarrow FUM^{\bullet} + H_2$$

The radiolytically produced free radical $FUM^{\bullet}$ then reacts with dissolved molecular oxygen in water to produce peroxy radicals:

$$FUM\bullet + O2 \rightarrow FUM\ O2\bullet$$

The FUM O2• then undergoes further destruction reactions and the fumonisin B_1 molecule is broken down. Thus, irradiation could be a safe and effective means of decontaminating foods that contain fumonisin B_1. In addition, the level of irradiation needed to accomplish this should have no adverse effect on either the nutritive value of the corn or its organoleptic properties.

The next series of experiments involved exposing naturally fumonisin B_1 contaminated corn either ground and whole kernel to irradiation treatments in a non-

aqueous state to determine if fumonisin B_1 could be eliminated by irradiation. There were only two replicates for each dose because the availability of the instrument was limited. Results show that fumonisin B_1 was not as easily reduced in the corn matrix compared to water. Since it is water forming free radicals decompose the fumonisin molecule the absence of sufficient water in intact corn may be a plausible explanation for the results seen. Previous researchers found that irradiation doses that were less than the optimal dose used in this study did not affect the levels of aflatoxin B_1, in corn even though T-2, deoxynivalenol and zearalenone were reduced (Hooshand, et al, 1995). In previous experiments corn was spiked with the mycotoxins. However, in all of the previous studies looking at gamma irradiation, fumonisin was not used

Based on these experiments, although high doses of irradiation using ether cobalt or electron beam eradicated toxigenic fungi from corn irradiation did not remove their toxins. In these experiments, 2 types of media were used; nutrient rich PDA and a nutrient poor MSA which causes restrictive mold growth. The nutrient rich media allowed surviving fungi to grow after treatment even at increasing doses of irradiation. Whereas, MSA as the isolation medium, resulted in considerably less growth of the surviving fungi. This finding suggests that it is possible to combine low moisture storage with irradiation, as this may be effective in reducing toxic mold growth. A reduction in growth of ground corn plated on MSA was achieved at 0.3 kGrays, whereas on PDA, growth was diminished only after exposure to the higher dose of 10 kGray. No fungal growth occurred on ground corn exposed to 30.0 kGrays irradiation, suggesting that irradiation could be a cost effective means to reduce fungal growth on ground corn leading to a reduction in the toxins they

produce. Thus, total sterilization of a product by eliminating all fungi may not be necessary. Whole kernel corn showed a drastic reduction in fungal growth when plated on MSA after 3 mega-rads treatment (<0.1%) in contrast to whole kernel corn plated on PDA after exposure to 10 mega-rads irradiation. Thus, it may be feasible to control fumonisins in corn by first irradiating it (at a low dose) prior to storage under low moisture and or temperatures conditions. This was demonstrated in this experiment because MSA represented a low moisture media and thus a low moisture storage environment; whereas, PDA represented a high moisture environment.

Previous studies examining irradiation effects on toxic molds also reported that sensitivity to irradiation increasing energy levels. However, the energy doses in these previous studies were low compared to those in the current study and in addition, culture media was used as the growth substrate instead of whole or ground corn which appeared to provide some limited protection for the fungus against irradiation. These experiments also clearly demonstrate that *Aspergillus* spp. were more sensitive to low dose irradiation (1 to 2 kGrays) compared to *F. verticillioides* whose viability was not reduced unless a higher dose of 20 kGray was used. The fact that *A. flavus* is more of an external microbe on the kernel whereas *Fusarium* is found within the corn kernel is a likely explanation. In an earlier study, Trofa and Bean (1985) showed that *Fusarium* colonies could not be detected on growth medium unless the kernels were first surface disinfected to remove *Aspergillus* spp.

3.6 Conclusion

Although irradiation of fumonisin B_1 in solution required only a low dosage of irradiation for the molecule to be destroyed, irradiation did not affect the level of

fumonisin present in ground and whole kernel corn. To reduce levels of toxigenic fungi such as *Aspergillus* and *Penicillium* present in naturally contaminated corn required a lower dosage of irradiation compared to *Fusarium*. Ground corn was sterile as there was no mold growth on PDA at 10 kGrays, whereas the whole kernel corn was sterile at 10 mega-rads. The results of this study indicated that irradiation is a safe and effective means to remove toxic fungi from contaminated foods, however irradiation does not appear to be effective in eliminating toxins once they are produced. However, irradiation in conjunction with other treatments such as storage at low moisture after irradiation may be even more effective in reducing microbial toxins in foods and feeds during storage.

Chapter 4: Occurrence of Toxigenic Fungi and Mycotoxins in Transgenic Corn

4.1 Abstract

Fungal toxins are agents involved in both human cancers and acute animal illnesses; the fungi that produce toxins also contaminate 25% of the world's food crops yearly (FAO statistics). Current methods to prevent or eliminate fungal toxins from foods and feeds include altering the environmental conditions in the field to reduce mold growth, proper post-harvest storage, or to chemically treat the product after toxins are present. However, chemical treatments may not always be acceptable especially if they cause a potential health safety risk or change organoleptic properties of the food making it undesirable for human consumption. A number of studies have investigated the occurrence of toxigenic fungi in the recently developed Bt transgenic-field corn varieties (Munkvold, et al, 1999; Dowd, 2000; Dowd, 2001; Clements, et al., 2003, & Hammond, et al., 2004). Bt corn contains an insecticidal gene from the soil bacterium *Bacillus thuringiensis,* that protects the plant from Lepidopteran insects. Because high populations of Lepidoptera in corn increase in the levels of toxigenic fungi, Bt corn could have lower toxigenic levels of *Fusarium*/*Aspergillus* compared to non-Bt and as a result, lower toxin levels (Rice & Pilcher, 1997; Rice & Pilcher, 1998; Munkvold, et al, 1999). The focus of this study was to compare the development of the toxigenic fungi, *A. flavus* and *F. verticillioides* and toxins on Bt and non-Bt corn.. The fumonisin B_1 levels in 2000 of Maryland corn were 7.5 ppm in Bt and 7.4 ppm in Non-Bt (NBt) corn; in the 2001 crops the levels, were 5.6 ppm for Bt, and 3.3 ppm for Non-Bt corn. However, both the 2000 and 2001 corn showed fumonisin B_1 levels above the daily

tolerable intake level of 2ppm for degermed dry milled corn products in both Bt and NBt corn. Although there were not significantly lower levels of fumonisin B_1 in the 2000 Bt corn compared to NBt corn, there were higher fumonisin B_1 levels in 2001 Bt corn compared to NBt corn. The 2000 Bt field corn had significantly lower levels of aflatoxinB_1, with only two Bt replicates out of 48 replicates containing 6.5 to 5.0 ppb in 2000; no aflatoxins were detected in the 2001 crops. The data on differing levels of toxins in Bt versus non-Bt corn suggest that an interaction between *A. flavus* and *F. verticillioides* may be occurring which may have reduced or eliminated toxigenic fungi in corn. The interaction(s) of the two fungi under field conditions and in Bt corn varieties was evaluated.

4.2 Introduction

This study was an investigation of growth and toxin production by *Fusarium* and *Aspergillus* molds in Bt transgenic field corn. The Bt transgenic corn contains an insecticidal gene from the soil bacterium *Bacillus thuringiensis* (Bt). The gene codes for endotoxins, which protect the plant from *Lepidopteran* insects. These insects consume the crystalline protein protoxin, which is then modified in their midgut. The converted endotoxin binds to a specific epithelial receptor, which causes the cells in the gut to rupture. Paralysis of the gut occurs next, leading ultimately to death of the insect. The corn specific*Lepidopteran* insects, which are controlled by the crystalline protein, are the European corn borer, Southwestern corn borer and the corn earworm. It has long been known that the fungus *Fusarium*, and its toxic metabolite fumonisin, is omnipresent in corn within most kernels (Trofa & Bean, 1985). In contrast, *Aspergillus* and its toxic metabolite aflatoxin, is present in or on the exterior of the

kernel and toxic metabolites are produced when the corn is growing under stress conditions of unfavorable weather conditions or heavy insect infestation (Payne, et al., 1988). Thus if the incidence of insect damage to corn by the use of genetically modified (GM) corn can be prevented or reduced, it should affect the levels not only of aflatoxins but other toxic metabolites including those produced by *Fusarium* since both toxigenic fungi can occur simultaneously in corn. There have been only a few studies on the interaction of the toxigenic fungi *A. flavus* and *F. verticillioides* in Bt corn under field conditions. Dively, UMCP, observed in his field study of 1999, that under optimal conditions for corn ear rot, there was a high incidence of *Fusarium* infection and a lower incidence for infection by *Aspergillus* in Bt11 corn, which is not what was expected. These findings indicated a competitive inhibition by *Fusarium* against *Aspergillus*. The drought conditions of 1999 in addition to high insect infection levels should have created an environment favoring both high *Fusarium* and *Aspergillus* infection in the ears (Dively, 1999). The conclusions reached by his studies were that additional investigations of the interactions of *Fusarium* and *Aspergillus* are justified. Of primary importance is to know whether or not controlling one toxigenic fungus with Bt11-based transgenic corn i.e. *Aspergillus*, may result in increasing or decreasing levels of growth or toxin production by *Fusarium*. Although increasing cultivation of Bt11 corn may remove or reduce one problem, aflatoxins, it may theoretically cause an increase in another, the fumonisins. However, using Bt11 corn may also reduce both *Fusarium* and *Aspergillus* growth and toxin production which would be desired. This investigation addressed the issue of what if any interaction exists between *A. flavus* and *F. verticillioides* in Bt 11 corn,

and does this interaction result in changes in toxin production by the two fungi.

4.3 Materials and Methods

4.3.1 Field Plot Procedures

The experimental layout was a 2 X 6 randomized complete block split-plot design with four replications. The whole plot treatments were transgenic and non-transgenic corn hybrid isolines (N6800Bt and N6800). Subplot mold treatments randomized within the whole plots were:

1 = Fv = *Fusarium verticillioides* alone
2 = Af = *Aspergillus Flavus* alone
3 = Fv/Af = *F. verticillioides* first, followed 4 days later with *A. Flavus*
4 = Af/Fv = *A. Flavus* first, followed 4 days later with *F. verticillioides*
5 = Af+Fv = *A. Flavus* and *F. verticillioides* simultaneously
6 = C = Control

The corn was planted during the last two weeks of May of 2000 and 2001. Whole plots consisted of 6 rows 25' long seeded by hand at a rate of 1 seed per 8". Approximately 30 corn plants per row emerged. Mold treatments were applied to both N6800Bt and N6800 simultaneously. Seeds were treated with standard fungicidal protectants and planted at a rate to achieve 26,000 plants per acre (0.4 ha = 1 acre). The fertility regime included 30 and 15 lbs of N and P per acre respectively, as a starter fertilizer at planting, followed by a side-dress application of 100 lbs per acre of 30% nitrogen solution three weeks later. The herbicides, Gramoxone Extra at 1.5 pt/acre, was applied as a burn-down at planting to control existing weeds, in combination with Bicep II 5.9L (metolachlor + atrazine) at 3.0 qt/acre for residual action.

4.3.2 Inoculation Procedures

All treatments were applied to the primary ears of all plants in each row. The first set of inoculations occurred in the first week of August 2000 and 2001. The second inoculation occurred 4 days later. The silk tissue was manually inoculated with toxigenic strains of *Fusarium verticillioides* and *A. flavus* conidia (approximately 10^5/mL) suspended in sterile water. Gary A. Payne, North Carolina State, supplied the *F. verticillioides* isolate and the *A. flavus* isolate. *Fusarium verticillioides* and *A. flavus* inoculation of 240 ears each required approximately 1.5 L of a 10^5/mL-spore suspension. For a 1.5-liter suspension, approximately 15 PDA plates (60mm in diameter) were used. Spores were rinsed with sterile water from PDA cultures and the concentration was adjusted to 10^5/mL using a Bright Line Hemacytometer. Inoculation was performed using spray bottles calibrated to deliver 1.0 mL per spray.

4.3.3 Harvest and Insect Damage Evaluation

After plants reached maturity (kernel moisture was approximately 20%), 15 - 20 ears from each row were collected and husked during the last week of September 2000 and 2001. Each ear was visually rated for *F. verticillioides* and *A. flavus* ear rot severity according to the method of visual rating for the percentage of kernels with symptoms of *Fusarium* and *Aspergillus* ear rots. The number of kernels with symptoms of *Fusarium* and *Aspergillus* ear rot were recorded. Kernel insect damage was recorded as total damage in cm^2 of kernels consumed (kerdam). Percent of ears damaged was also calculated by dividing total cm^2 of insect damage on all ears per replicate by all samples in that replicate (ndam). The number of ears in each replicate

that were damaged out of all samples in that replicate was recorded (nears). The kernels were removed by hand or electric sheller prior to mycotoxin analysis. A 2-3-kg sample of the kernels from each replicated row were dried to 13% moisture in Dively lab, UMD and shipped to Romer labs for mycotoxin analysis. The fumonisin and aflatoxin data were then analyzed to determine the results of the interaction between *F. verticillioides* and *A. flavus* molds and the interaction effects of hybrids with and without the Bt insecticidal gene on both fungal and toxin levels exist.

4.3.4 Kernel Mold Plate Counts

To determine the levels of *Aspergillus* and *Fusarium* present in the various treatments, random samples of kernels were collected. Half of the kernels were surface disinfected with commercial bleach and the remaining half with sterile distilled water. One hundred kernels were placed on 10 malt salt agar medium plates (80 plates = 33.6 g Difco malt agar, 75.9 g NaCl, and 1.0 L deionized water) and after 10-14 days, colonies of *Aspergillus* and *Fusarium* were counted and averaged for each of the 4 replicates. *Aspergillus flavus* colonies found on non-surface disinfected kernels were transferred to rice and grown for 4 weeks to determine if they were aflatoxin producers. Rice was analyzed for aflatoxin production using AOAC procedures (TLC) (Trucksess, 2000a). Coconut agar was also used as a method to determine aflatoxin production. Davis, et al., (1987), developed a screening method using shredded coconut agar as an aflatoxin-promoting medium. Frozen, unsweetened Baker's coconut was purchased at a local grocery store and 100 g of shredded coconut was blended for 5 min with 300 mL of hot distilled water. The mixture was filtered through five layers of cheesecloth, and the pH of the filtrate was

adjusted to pH 7 with 2 N NaOH. Twenty grams/liter of agar was added, and the mixture was heated to boiling and cooled to about 50°C. The mixture was then autoclaved for 20 minutes, cooled to about 40 to 45°C, and poured while being stirred into petri plates. Aflatoxin production was verified using a UV detector for long-wave UV light (365 nm) for the presence of blue fluorescent areas on the reverse side of the inoculated coconut agar plate after 2 to 5 days growth; aflatoxins were then confirmed using TLC (Davis, et al., 1987; Trucksess, 2000a).

4.3.5 Statistical Analysis

The field plot data including mold counts, insect damage ratings and kernel ear rot assessments and mycotoxin levels were analyzed using Statistical Applications Software (SAS) Institute, v. 8.2 General Linear Model Procedure (GLM). The laboratory variables analyzed were *A. flavus* surface disinfected (afsd), *A. flavus* non-surface disinfected (afnsd), *A. niger* surface disinfected (ansd), *A. niger* non-surface disinfected (annsd), F. *verticillioides* surface disinfected (fvsd), *F. verticillioides* non-surface disinfected (fvnsd), A. *flavus* and *F. verticillioides* surface disinfected (affvsd), *A. flavus* and *F. verticillioides* non-surface disinfected (affvnsd), *Penicillium* spp. surface disinfected (psd), Penicillium spp. non-surface disinfected (pnsd). The average number of kernels damaged by insects per treatment (cm^2) (kerdam) was determined as well as the number of ears damaged by insects out of each replicate (ndam), aflatoxin B_1 (ab1), aflatoxin B_2 (ab2), aflatoxin G_1 (ag1), aflatoxin G_2 (ag2), fumonisin B_1 (fb1), fumonisin B_2 (fb2), fumonisin B_3 (fb3), total fumonisins (totalf). Treatment means were compared using a LSD procedure ($p= 0.05$) to determine differences between the treatment means.

4.4 Results

4.4.1 2000 Field Plot Study

It was expected that insect damage would be considerably higher in NBt corn compared to Bt corn. However, the average number of kernels damaged by insects per treatment (kerdam) in the NBt corn as measured by the cm^2/ear damaged was significantly different for only three of the six treatments. *A. flavus* alone (Af), *F. verticillioides* first, followed 4 days later with *A. flavus* (Fv/Af), *A. flavus* and *F. verticillioides* simultaneously (Af+Fv) and control (C) were significantly higher in NBt compared to Bt corn, but not in treatments *F. verticillioides* alone (Fv) and *A. flavus* first, followed 4 days later with *F. verticillioides* (Af/Fv) (Figure 8). The number of ears damaged per 20-ear sample (ndam) (Figure 9) was significantly higher in NBt corn in treatments Fv, Fv/Af, Af+Fv and C compared to Bt corn, but not in treatments Af and Af/Fv. Bt corn had significantly higher insect damage in the number of ears out of 20 (ndam) in treatment Af. The corn from the field plot study of 2000, when analyzed for mycotoxins, showed no aflatoxins in the NBt corn and only low levels of aflatoxins in the Bt corn (data not shown). The average level of aflatoxin B_1 in treatment Fv/Af was 1.6 ng/g, and in treatment Af/Fv it was 1.3 ng/g. The levels of fumonisin B_1 were higher in NBt corn in treatments Af, Af/Fv, and C, and higher in Bt corn in treatments Fv, Fv/Af, and Af+Fv (Figure 10). Mold growth is shown for the 6 treatments, both surface disinfected and non-surface disinfected kernels, to display relationships between inoculated mold and mold counts on the kernels of both the Bt and NBt corn. In the 2000 corn, there were no significant differences between Bt and NBt corn for any of the mold treatments except for fvsd.

Mold counts of surface disinfected and non-surface disinfected corn kernels showed *F. verticillioides, Penicillium, A. flavus, and A. niger* to be present (Table 8, Figures 11, 12, 13, 14). The NBt non-surface disinfected kernels from the C treatment had an average of 25 *A. flavus* mold colonies compared to an average of 20 *A. flavus* mold colonies in the Af treatment (Table 8).

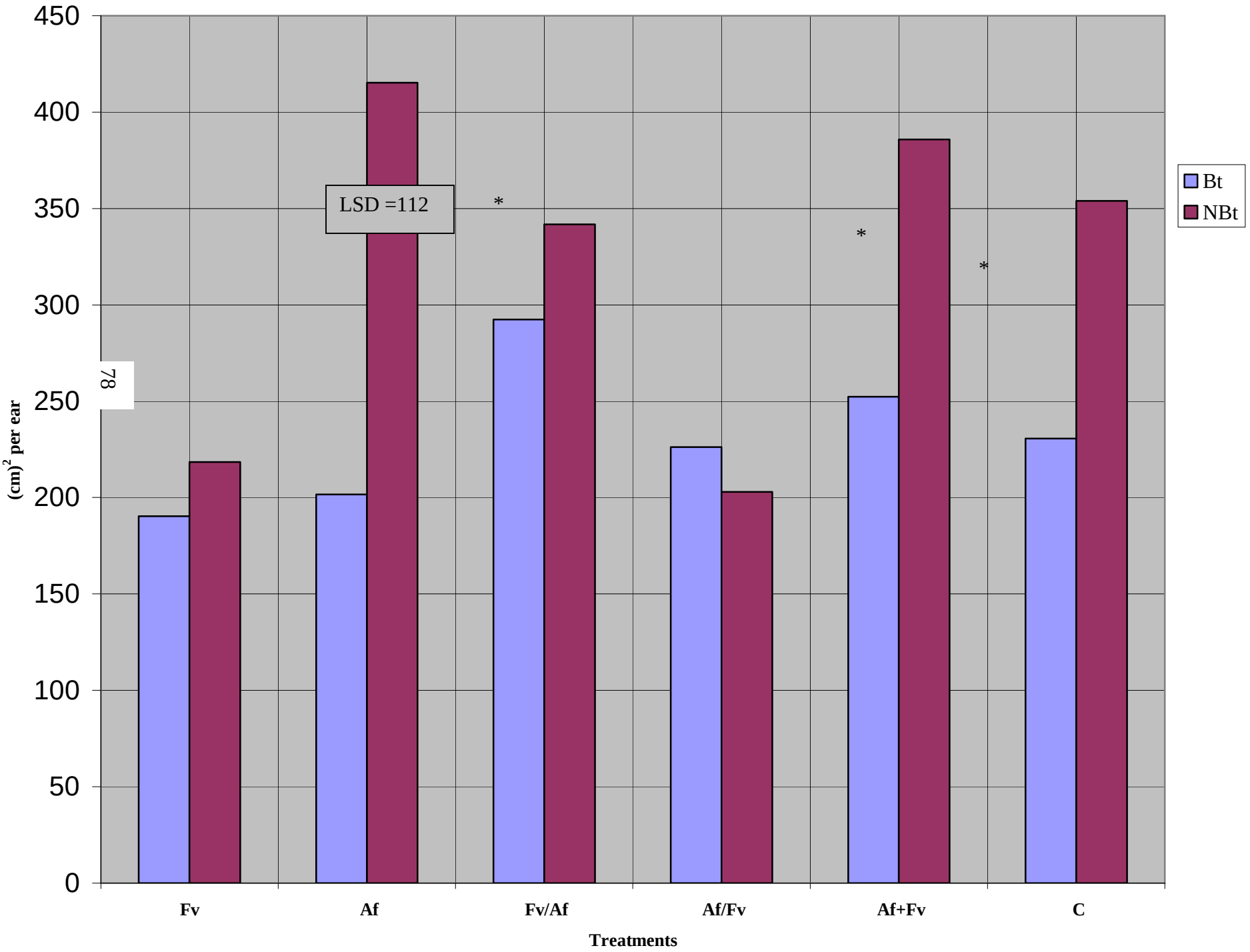


Figure 3: Insect damage in 2000 field corn study

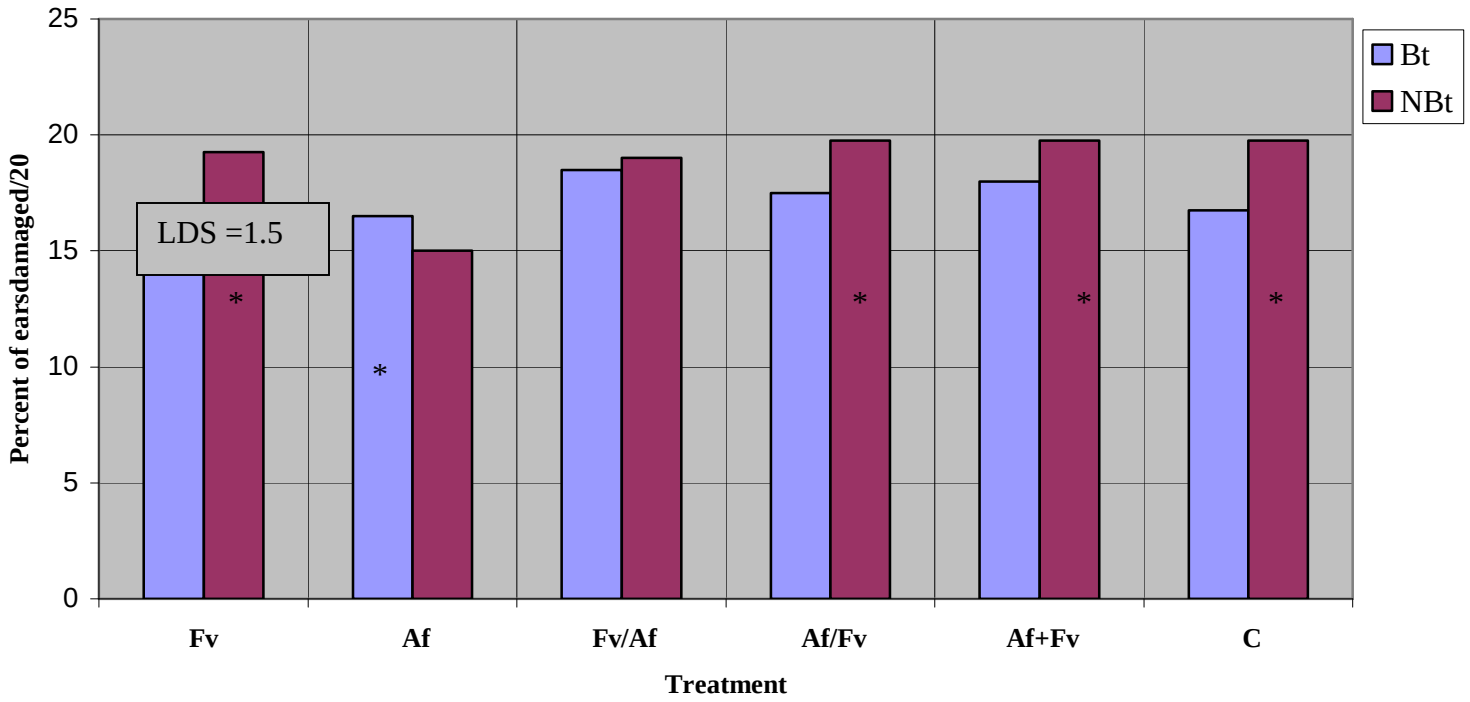


Figure 4: Corn ear damaged in 2000 field corn study

*Treatment means significantly different $p<0.05$ between Bt and NBt

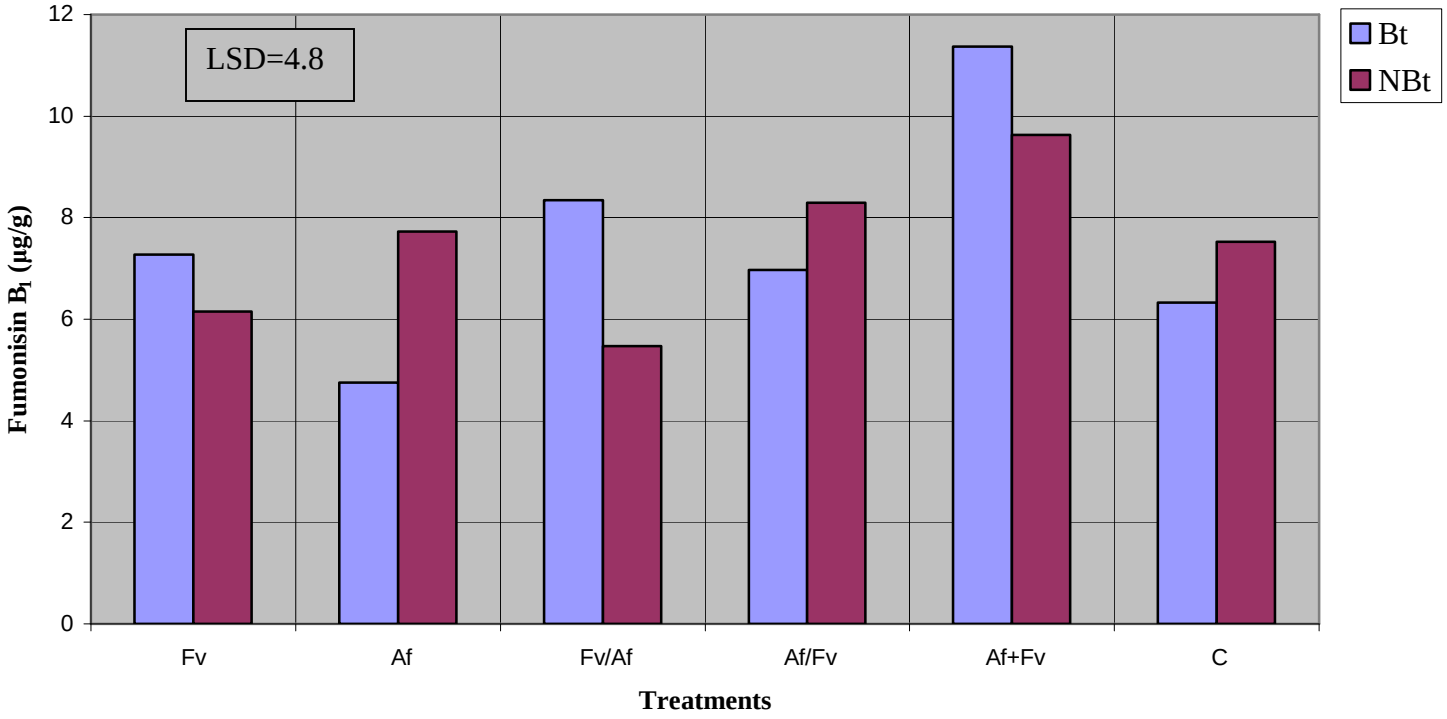


Figure 5: Fumonisin B_1 levels in 2000 field corn study

Table 8: Mean mold counts on surface disinfected and non-surface disinfected Bt and NBt corn kernels, 2000 field plot study.[a]

Treatment	Fv		Af		Fv/Af		Af/Fv		Af+Fv		C	
	Bt	NBt	Bt	NBt	Bt	NBt	Bt	NBt	Bt	NBt	Bt	NBt
Fungus Species	(SD)[b] (NSD)[c]	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)
A. flavus	(<1)(28)	(<1)(12)	(0)(18)	(0)(20)	(<1)(55)	(1)(15)	(1)(41)	(<1)(38)	(0)(15)	(0)(39)	(<1)(34)	(0)(25)
A. niger	(0)(10)	(0)(19)	(0)(25)	(0)(16)	(0)(16)	(0)(11)	(0)(23)	(0)(17)	(0)(11)	(0)(5)	(0)(37)	(0)(15)
F. verticillioids	(53)(100)	(67)(100)	(42)(100)	(68)(100)	(45)(100)	(71)(91)	(47)(100)	(59)(100)	(54)(100)	(64)(100)	(40)(100)	(56)(99)
A. flavus & F. verticillioids	(<1)(28)	(<1)(12)	(0)(18)	(0)(20)	(<1)(55)	(<1)(15)	(1)(41)	(<1)(38)	(0)(15)	(0)(39)	(<1)(34)	(0)(25)
Penicillium spp.	(1)(93)	(0) (98)	(0)(98)	(0)(92)	(0)(90)	(0)(83)	(1)(87)	(0)(89)	(<1)(99)	(<1)(83)	(<1)(96)	(0)(74)

[a]Treatment: Fv = *F. verticillioides* alone, Af = *A. flavus* alone, Fv/Af = *F. verticillioides 1st*, *A. flavus* 2nd (4 days later), *Af/Fv = A. flavus 1st*, *F. verticillioides* 2nd (4 days later), Af+Fv = *A. flavus* & *F. verticillioides* similtaneously, C = control

[b]SD = surface disinfected

[c]NSD = non-surface disinfected

In Bt non-surface disinfected corn, the treatment Fv/Af showed 55 average *A. flavus* mold colonies compared to 28 average *A. flavus* mold colonies in treatment AF (Table 8). In the non-surface disinfected NBt corn kernels, there were 100% *F. verticillioides and Penicillium,* 55% *A. flavus,* and 37% *A. niger* contaminated kernels (Figure 11). In surface disinfected NBt corn kernels there was 70% *F. verticillioides* colonies and less than 5% *A. flavus* mold colonies (Figure 12). *A. flavus* had fewer mold colonies compared to *F. verticillioides* in all treatments on non -surface disinfected NBt corn kernels. In non-surface disinfected Bt corn kernels there were 100% *F. verticillioides,* 100% *Penicillium* 55% *A. flavus* contaminated kernels (Figure 13) and in surface disinfected Bt kernels there were 55% *F. verticillioides* and less than 1% *Penicillium* and *A. flavus* contaminated kernels (Figure 14). *A. flavus* mold colonies were also lower in non-surface disinfected and surface disinfected Bt corn kernels compared to *F. verticillioides* in all Af inoculated treatments. Isolates of *A. flavus* from non-surface disinfected kernels were transferred to rice and then analyzed for aflatoxin production. The results showed that 86% of the *A. flavus* inoculated corn mold isolates were aflatoxin producers and 80% of the non-inoculated corn mold isolates were aflatoxin producers (Table 9).

Two-way analyses of variance using the GLM procedure were conducted for tests of significance between Bt and Non-Bt corn for to mold counts, insect damage ratings, kernel rot assessments and mycotoxin levels (Table 10). The only significant differences between Bt and Non-Bt were for fvsd and ndam (Table 10). The means and significant differences between Bt and Non-Bt corn and are shown in Table 11.

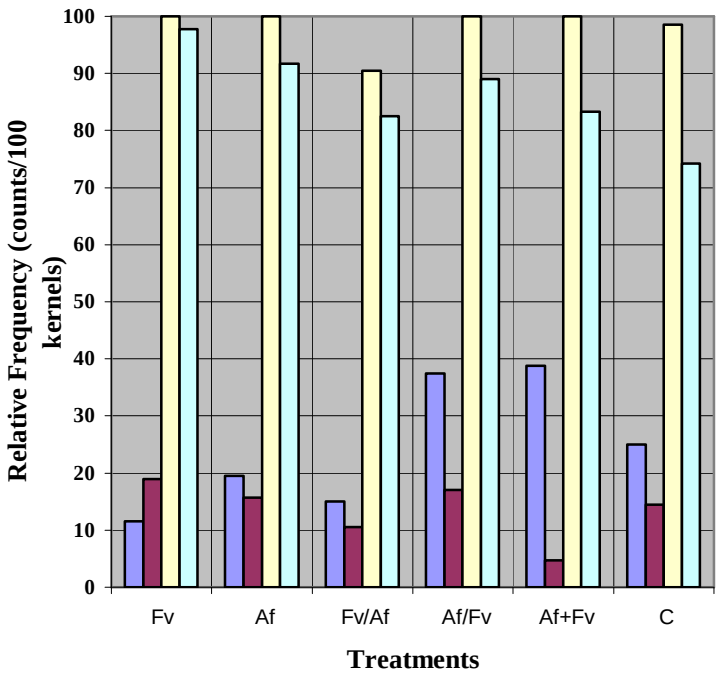
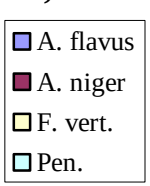


Figure 6: Mold growth on non-surface disinfected NBt corn kernels, 2000 field plot study



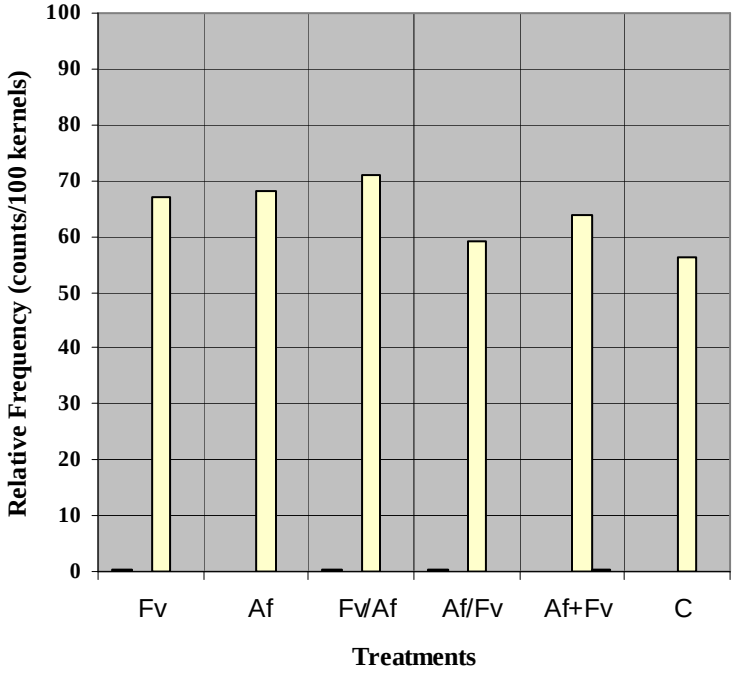
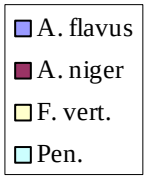


Figure 7: Mold growth on surface disinfected NBt corn kernels, 2000 field corn study



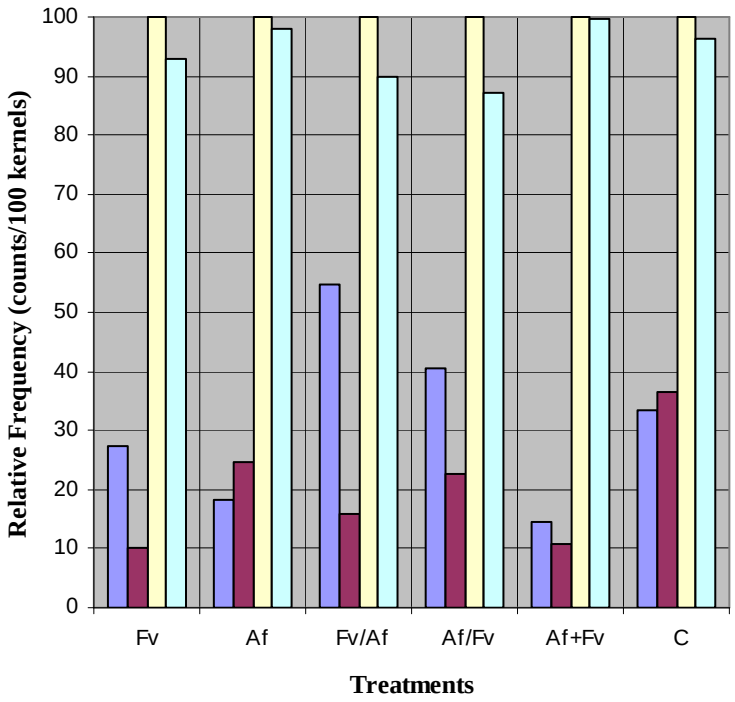


Figure 8: Mold growth on non-surface disinfected Bt corn kernels, 2000 field corn study

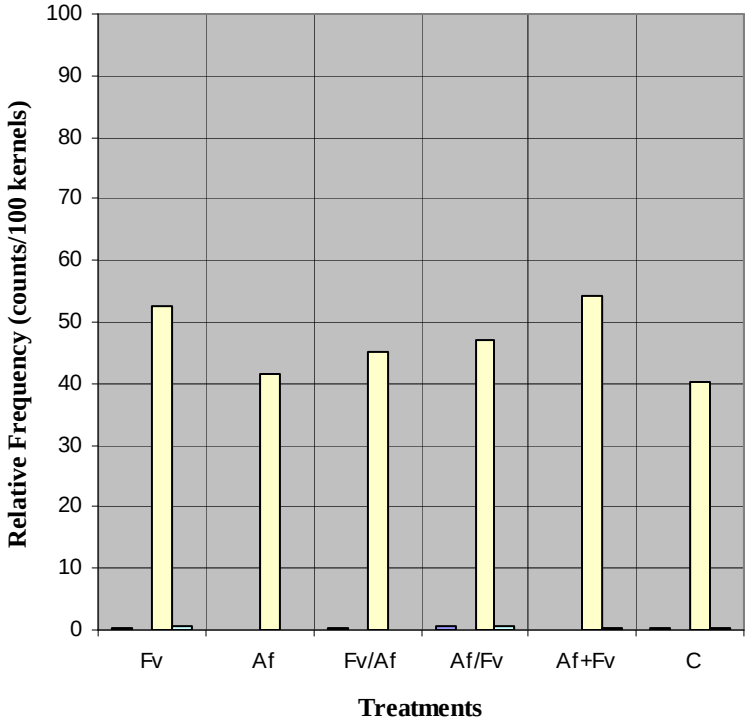


Figure 9: Mold growth on surface disinfected Bt corn kernels, 2000 field corn study

Table 9: Mean percent of aflatoxin producing *A. flavus* strains from Bt and NBt corn from 2000 season.[a]

Treatment	Inoc.[b]	Non-Inoc.[c]	Mean
	---------- % ----------		
Bt	75 (3)[d]	75 (3)	75
NBt	100 (3)	100 (1)	100
Mean	86	80	

[a]Treatments 2 & 6, Grown on Rice and Analyzed Using TLC

[b] Inoc. = Af = *A. flavus* alone

[c] Non-Inoc. = C = control

[d]Number of aflatoxin producers in 4 reps ( )

Table 10. ANOVA for tests of significance of mold counts, insect damage ratings and mycotoxin levels in Bt and Non Bt corn from 2000 field plot study.*

		F-Value																			
		af[a]		an[b]		fv[c]		affv[d]		p[e]											
Sources of Variation	DF	SD[a]	NSD[b]	SD	NSD	SD	NSD	SD	NSD	SD	NSD	kerdam	ndam	ab1	ab2	ag1	ag2	fb1	fb2	f b3	totalf
BT	1	0.1	0.2	-	0.6	17.9*	1.4	0.2	0.2	4.0	1.7	1.9	57.5***	1.0	-	-	-	0.0	0.4	0.1	0.0
Mold	5	1.0	0.7	-	0.7	1.2	1.0	1.1	0.7	0.9	0.4	3.7	2.0	1.0	-	-	-	1.6	1.6	1.4	1.6
Mold x Bt	5	0.3	1.1	-	0.4	0.8	1.0	0.2	1.1	0.9	0.5	2.7	2.3	1.0	-	-	-	0.8	0.9	1.4	0.9

*Significant at p<0.05 level, ***p<0.001 level.
[a]*A. flavus*, [b]*A. Niger*, [c]*F. verticillioides*, [d]*A. flavus and F. verticillioides*, [e]*Penicillium*, [f]Surface disinfected, [g]Non-surface disinfected

The insect damage measured in centimeters per ear of corn was 320cm^2/ear in Bt corn and 232cm^2/ear in NBT corn (Table 11). The mold count *F. verticillioides* surface disinfected (fvsd) had a mean of 64% for Bt and a mean of 47% for Non-Bt (Table 11). The statistical paired comparisons of the means among the 6 treatments for each mold treatment are presented in Table 12. The variable fb1 had a mean of 6.7 ppm for treatment Fv and a mean of 10.5 ppm for treatment Af+Fv and the two means were significantly different (Table 12).

Average rainfall per month during the planting, growing and harvesting period (May to September) over a thirty-year period for Beltsville, Maryland between 1971 through 2000 is shown in Table 13. Higher than average rainfall of 6.1 inches was observed in July of 2000, compared to the thirty-year average of 4.1 inches, and September 2000 with a with rainfall total of 5.9 inches compared to 4.4 inches for the thirty-year average. Drought conditions did not occur during the 2000 growing season (Maryland State Climatologist Office, 2004). Temperature data for the same thirty-year period during the planting, growing and harvesting period (May to September) is presented in Table 14. A higher than normal temperature of 73.3ºF was recorded in June 2000 compared to the thirty-year average of 61.4ºF. A temperature of 74.0ºF was recorded in August 2000, compared to 75.3ºF for the thirty-year average, slightly lower than normal (Maryland State Climatologist Office, 2004).

Table 11. Means of mold counts insect damage ratings and mycotoxin levels in Bt and Non Bt corn from 2000 field plot study. [1]

	Means																			
	af[a]		an[b]		fv[c]		affv[d]		p[e]											
Hybrid	SD[a]	NSD[b]	SD	NSD	SD	NSD	SD	NSD	SD	NSD	kerdam	ndam	ab1	ab2	ag1	ag2	fb1	fb2	f b3	totalf
Bt	0 a	32 a	0 a	20 a	64 b	100 a	0 a	32 a	0 a	94 a	320 b	20 b	1 a	0 a	0 a	0 a	8 a	2 a	1 a	11 a
NBt	1 a	25 a	0 a	14 a	47 a	98 a	0 a	25 a	0 a	86 a	232 a	17 a	0 a	0 a	0 a	0 a	8 a	2 a	1 a	10 a

[1]Means with the same letter are not significantly different.
[a]*A. flavus*, [b]*A. Niger*, [c]*F. verticillioides*, [d]*A. flavus and F. verticillioides*, [e]*Penicillium*, [f]Surface disinfected, [g]Non-surface disinfected

Table 12: Mold treatment means with significant differences for measured variable averaged over Bt and Non-Bt treatments for the 2000 field plot study.[1, 2]

Treatment	fvsd	Kerdam	ndam	fb1	fb2	fb3	totalf
Fv	59.8 a	204 c	17.4 b	6.7 b	2.1 b	0.7 a b	9.6 b
Af	54.8 a b	309 a	18.1 a b	6.0 b	1.9 b	0.8 a b	9.0 b
Fv/Af	58.3 a b	317 a	18.8 a	7.0 b	2.0 b	0.7 a b	9.6 b
Af/Fv	53.0 a b	215 b c	18.6 a	7.6 a b	2.3 a b	0.5 b	10.5 a b
Af+Fv	59.0 a b	319 a	18.9 a	10.5 a	2.3 a b	1.2 a	14.9 a
C	48.3 b	292 a b	18.3 a b	7.0 b	2.2 a b	0.8 a b	9.8 b

[1]Treatment: Fv = *'F. verticillioides* alone, Af = *A. flavus* alone, Fv/Af = *F. verticillioides1st, A. flavus* 2nd (4 days later), *Af/Fv = A. flavus 1st, F. verticillioides* 2nd (4 days later), Af+Fv = *A. flavus* & *F. verticillioides* similtaneously, C = control

[2]Means with the same letter within a column are not significantly different according to an LSD procedure (p=.05)

4.4.2 Field Plot of 2001

The 2001 insect damage measured as cm^2 per ear of corn affected (kerdam) was significantly higher in NBt corn in all treatments (Figure 15) and the percent of ears damaged per 20 ear sample (ndam) was also significantly higher in NBt corn in all treatments (Figure 16). No aflatoxins were found in the 2001field plot study. Fumonisin B_1 levels were significantly higher in Bt corn in treatment Af+Fv (Figure 17). Mold growth (% kernels with mold growth) is shown for the 6 treatments, both surface disinfected and non-surface disinfected kernels, to display relationships between inoculated mold and mold counts on the kernels of both the Bt and NBt corn. In the 2001 corn, there were no significant differences between Bt and NBt corn for any of the mold treatments.

Table13 : Average monthly precipitation (in.), Beltsville, Maryland.

	Precipitation		
	1971-2000	2000	2001
Month	---------- **Inches**----------		
May	4.5	2.4	5.1
June	3.6	5.0	5.3
July	4.1	6.1	7.2
August	3.7	3.7	4.1
September	4.4	5.9	3.4

Table 14: Average monthly temperature (ºF), Beltsville Maryland.

	Temperature		
	1971-2000	2000	2001
Month	---------- **Fahrenheit**----------		
May	63	65	63.8
June	61.4	73.3	72.6
July	76.9	72.9	72.8
August	75.3	74	75.5
September	68.4	67	66.4

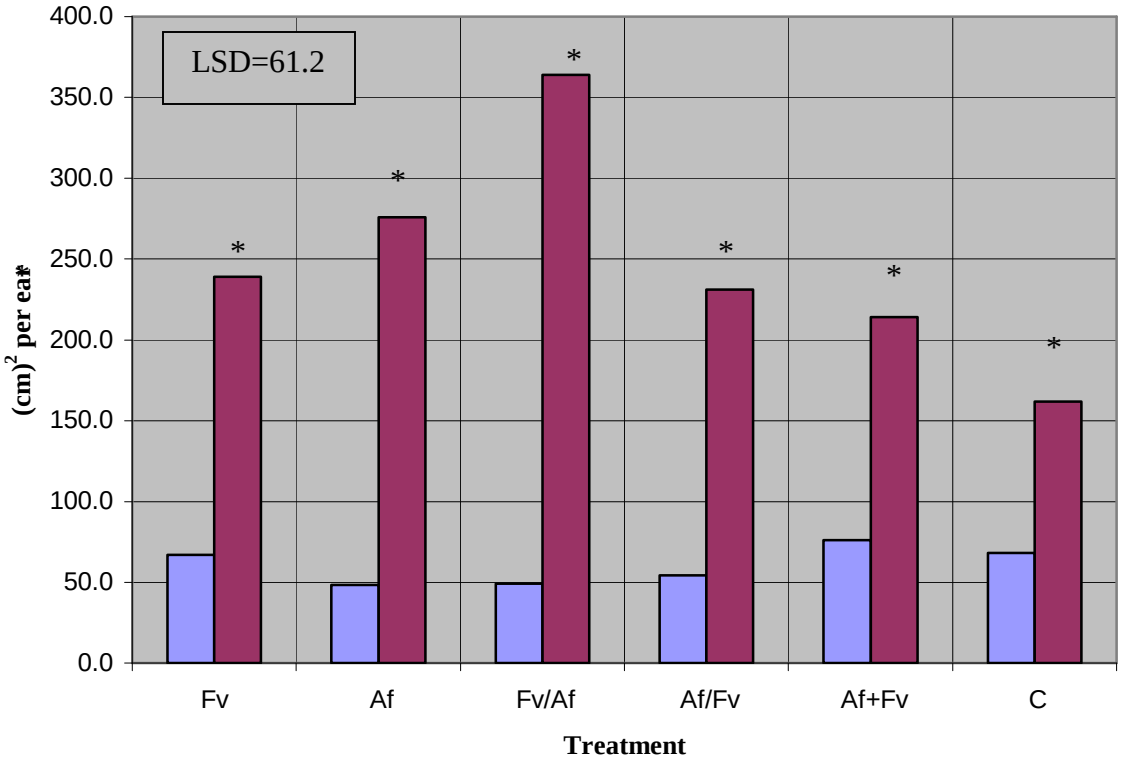


Figure 10: Insect damage in 2001 field corn study

*Treatment means significantly different $p<0.05$ between Bt and NBt

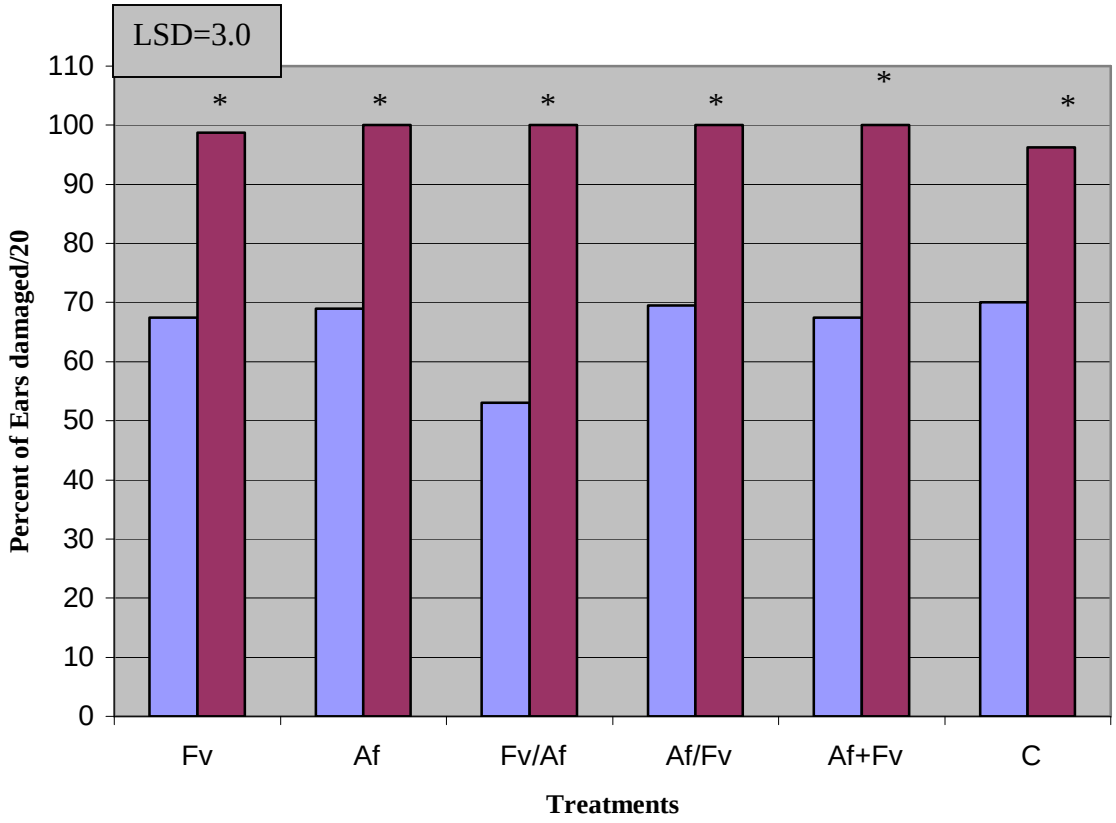


Figure 11: Corn ear damaged in 2001 field plot study

*Treatment means significantly different $p<0.05$ between Bt and NBt

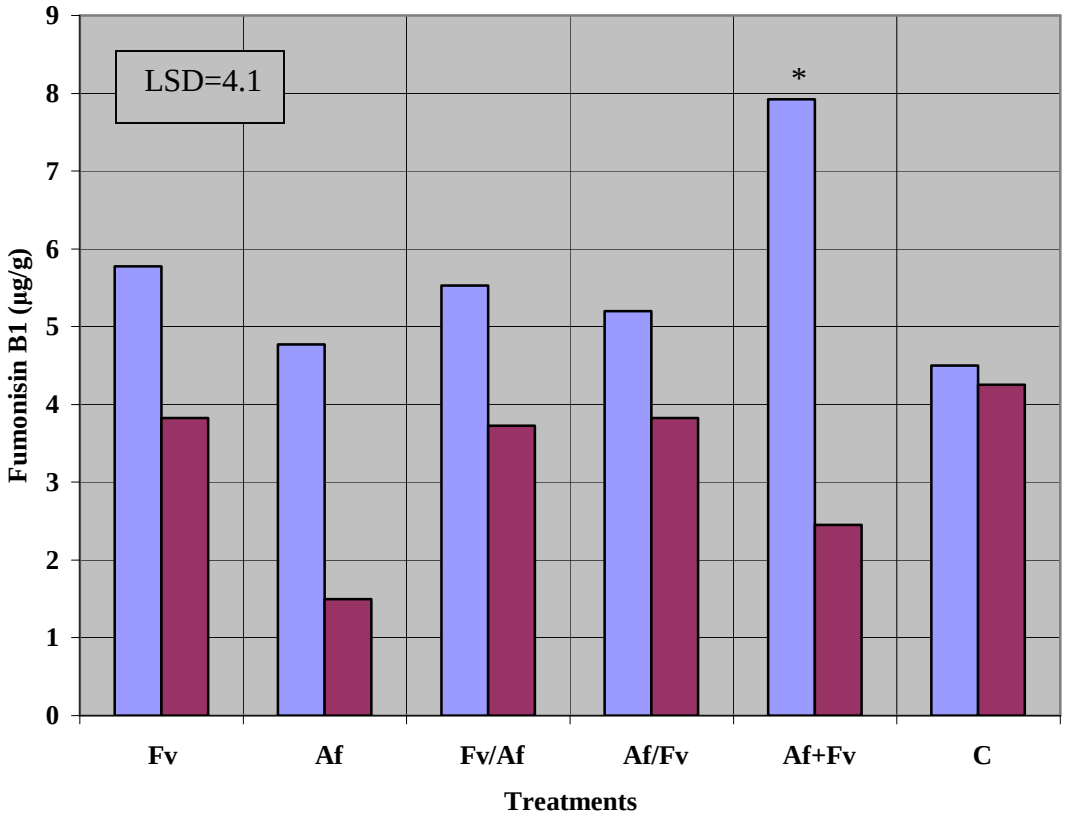


Figure 12: Fumonisin B_1 levels in 2001 field corn study

*Treatment means significantly different $p<0.05$ between Bt and NBt

Mold counts of surface disinfected and non-surface disinfected corn kernels showed only *F. verticillioides, Penicillium, A. flavus, and A. niger* (Table 15, Figures 18, 19, 20, 21). The NBt surface disinfected kernels from treatment Af had an average of 2.0 *A. flavus* mold colonies compared to an average of 6.0 *A. flavus* mold colonies in the Af+Fv treatment (Table 15). *A. flavus* had significantly lower mold colonies compared to *F. verticillioides* in all treatments on non-surface disinfected NBt corn kernels. In Bt surface disinfected corn, treatment Af /Fv resulted in an average of 9.0 *A. flavus* mold colonies compared to an average of 1.0 *A. flavus* colony in treatment Af (Table 15). In non-surface disinfected NBt corn kernels, there were 100% *F. verticillioides and Penicillium* colonies, 6.0% *A. flavus,* and less than 1% *A. niger* contaminated kernels (Figure 18). In surface disinfected NBt corn kernels 100% *F. verticillioides*, 24% *Penicillium* and 6% *A. flavus* contaminated kernels were observed (Figure 19). *Aspergillus flavus* colonies were also lower in number in non-surface disinfected and surface disinfected Bt corn kernels compared to *F. verticillioides* in all treatments. In non-surface disinfected Bt corn kernels there were 100% *F. verticillioides* and *Penicillium* colonies, 26.5% *A. flavus* and less than 1% *A. niger* (Figure 20). In surface disinfected Bt kernels there were 100% *F. verticillioides*, 15% *Penicillium* and 8.5% *A. flavus* colonies (Figure 21). In both surface and non-surface disinfected Bt corn, *Aspergillus flavus* colonies were higher in numbers in Af /Fv than in all other treatments.

Table 15: Mean mold counts on surface disinfected Bt and NBt corn kernels, 2001 field plot study.[a]

Treatment	Fv		Af		Fv/Af		Af/Fv		Af+Fv		C	
	Bt	NBt	Bt	NBt	Bt	NBt	Bt	NBt	Bt	NBt	Bt	NBt
Fungus Species:	(SD)[b] (NSD)[c]	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)	(SD)(NSD)
A. flavus	(1) (3)	(1)(2)	(1)(2)	(3)(2)	(0)(1)	(0)(7)	(9)(32)	(0)(6)	(1)(1)	(6)(3)	(0)(1)	(<1)(4)
A. niger	(<1)(<1)	(0)(0)	(0)(<1)	(<1)(1)	(<1)(<1)	(0)(1)	(0)(0)	(0)(0)	(0)(0)	(0)(0)	(0)(1)	(0)(<1)
F. verticillioides	(99.8)(100)	(100)(100)	(99.8)(100)	(100)(100)	(100)(100)	(100)(100)	(100)(100)	(100)(100)	(100)(100)	(99.9)(100)	(100)(100)	(100)(100)
A. flavus & ***F. verticillioides***	(1)(3)	(1)(1)	(<1)(2)	(2)(<1)	(0)(1)	(0)(6)	(9)(32)	(0)(6)	(1)(1)	(6)(1)	(0)(1)	(<1)(4)
Penicillium spp.	(15)(100)	(9)(100)	(15)(100)	(16)(100)	(10)(100)	(13)(100)	(8)(100)	(12)(100)	(8)(100)	(24)(98)	(12)(100)	(16)(100)

[a]Treatment: Fv = *'F. verticillioides* alone, Af = *A. flavus* alone, Fv/Af = *F. verticillioides1st, A. flavus* 2nd (4 days later), Af/Fv = *A. flavus* 1st, *F. verticillioides* 2nd (4 days later), Af+Fv = *A. flavus* & *F. verticillioides* similtaneously, C = control

[b]SD = surface disinfected

[c]NSD = non-surface disinfected

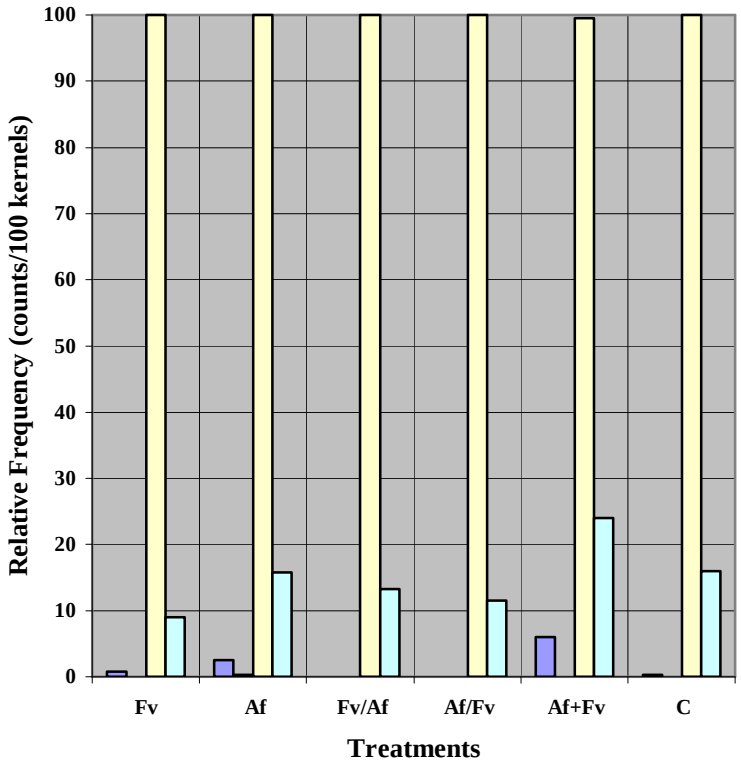
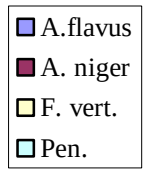


Figure 14: Mold growth on surface disinfected NBt corn kernels, 2001 field plot study



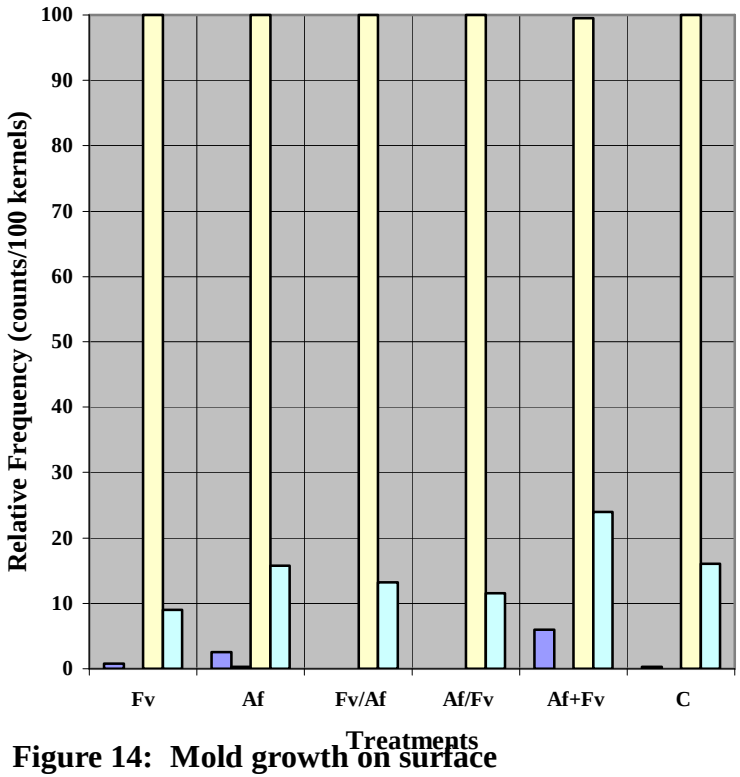
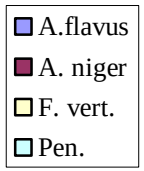


Figure 14: Mold growth on surface disinfected NBt corn kernels, 2001 field plot study



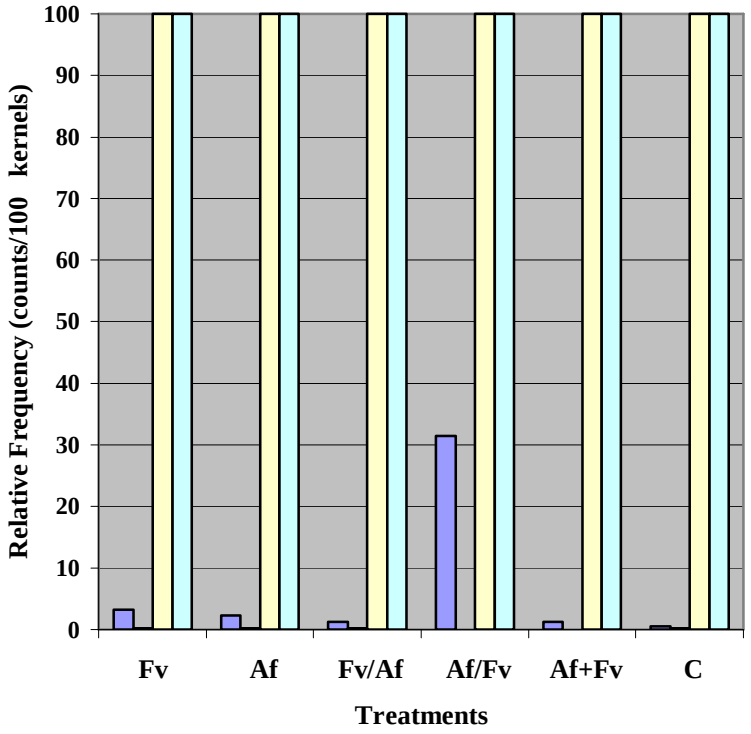


Figure 15: Mold growth on non-surface disinfected Bt corn kernels, 2001 field plot study

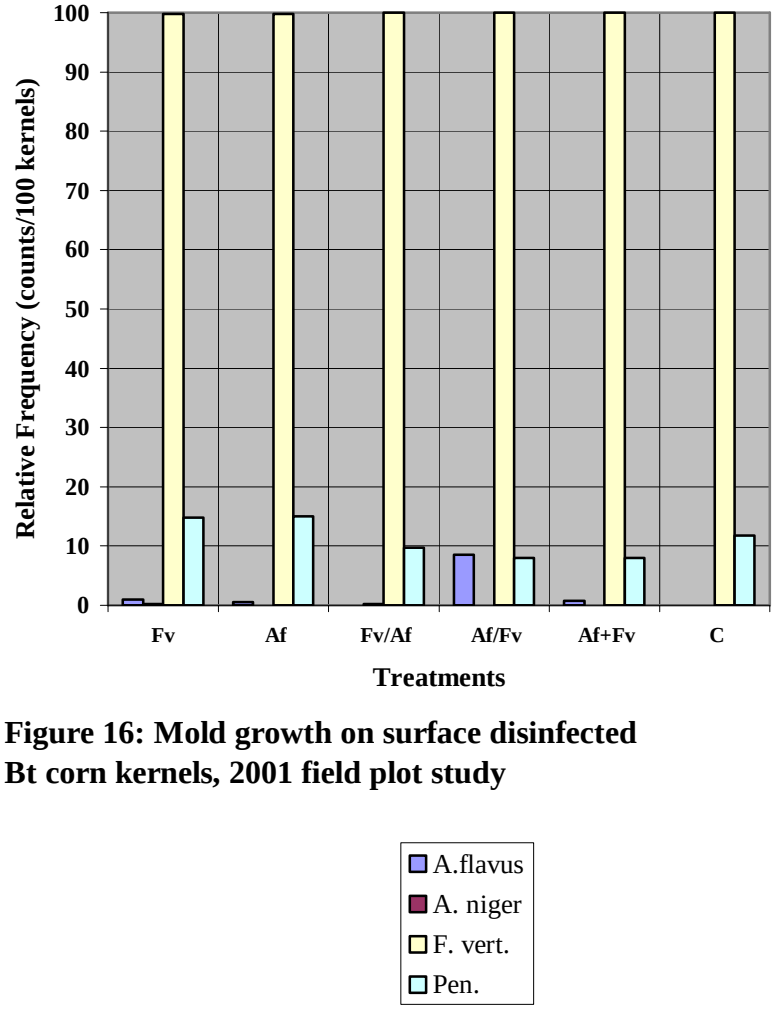


Figure 16: Mold growth on surface disinfected Bt corn kernels, 2001 field plot study

To test for aflatoxin production, *Aspergillus flavus* colonies from surface disinfected and non-surface disinfected kernels were transferred to PDA and incubated at room temperature for one week. A small plug of *A. flavus* was then placed on coconut agar and incubated for 24 to 48 hours at room temperature. The results showed that 100% of all *A. flavus* isolates from corn kernels in the *A. flavus* alone (Af) treatment and control (C) treatment were aflatoxin producers.

Two-way analysis of variance using the GLM procedure was done for tests of significance between Bt and Non-Bt corn with regards to mold counts, insect damage ratings and kernel rot assessments and mycotoxin levels on the data from both the field and lab (Table 16). There were significant differences with respect to kerdam 234 cm^2/ear and ndam 158 ears/20 (number of insect damaged ears out of 20) between Bt and Non-Bt with a $p<0.001$ and $p<0.01$ respectively. Means of each data set analyzed were reported for both Bt and Non-Bt replicates (Table 17). For fb1, there was a significant difference between the hybrid means with 5.6 ppm fb1 for Bt, and 3.3 ppm fb1 for NBt (Table 17). The variable (ndam) had a mean of 20 for Bt and of 12 for Non-Bt. The statistical pared comparisons of the means among the 6 treatments for each corn replicate are shown in Table 18. The variable afnsd mean for treatment Fv was 2.4, which was significantly different from the mean of 18.8 for treatment Af/Fv. There was also a significant difference between the Bt and NBt corn means with respect to fb2, fb3, and totalf. In Table 18, pared comparisons of the means among the 6 treatments for each replicate are presented.

Table 16. ANOVA tests of significant differences in mold counts, insect damage ratings and mycotoxin levels in Bt and Non Bt
corn from 2001 field plot study.

		F-Value																			
		af[a]		an[b]		fv[c]		affv[d]		p[e]											
Sources of Variation	DF	SD[a]	NSD[b]	SD	NSD	SD	NSD	SD	NSD	SD	NSD	kerdam	ndam	ab1	ab2	ag1	ag2	fb1	fb2	fb3	total f
BT	1	0.0	0.8	0.3	0.0	0.0	-	0.0	1.2	0.5	3.0	234***	158**	-	-	-	-	5.4	8.3	6.3	5.8
Mold	5	0.7	2.7	0.6	1.1	.07	-	0.7	2.8	0.6	3.0	4.1	1.2	-	-	-	-	0.5	0.5	0.1	0.4
Mold x Bt	5	1.2	2.0	1.1	0.3	1.3	-	1.2	1.9	1.2	3.0	6.6	1.8	-	-	-	-	0.8	0.9	0.8	0.8

**Significant at p<0.01 level, ***p<0.001 level.
[a]*A. flavus*, [b]*A. Niger*, [c]*F. verticillioides*, [d]*A. flavus and F. verticillioides*, [e]*Penicillium*, [f]Surface disinfected, [g]Non-surface disinfected

Table 17. Means of mold counts insect damage ratings and mycotoxin levels in Bt and Non Bt corn from 2001 field plot study.[1]

	Means																				
	af[a]		an[b]		fv[c]		affv[d]		p[e]												
Means	SD[a]	NSD[b]	SD	NSD	SD	NSD	SD	NSD	SD	NSD	kerdam	ndam	ab1	ab2	ag1	ag2	fb1	fb2	f b3	totalf
Bt	1.8 a	6.7 a	0.1 a	0.2 a	100 a	100 a	1.8 a	6.7 a	15 a	100 a	248 b	20 b	0 a	0 a	0 a	0 a	5.6 b	2.7 b	0.4 b	8.8 b
NBt	1.6 a	3.7 a	0.5 a	0.2 a	100 a	100 a	1.6 a	3.0 a	11 a	100 a	60 a	12 a	0 a	0 a	0 a	0 a	3.3 a	1.5 a	0.2 a	4.9 a

[1]Means with the same letter are not significantly different.
[a]*A. flavus*, [b]*A. Niger*, [c]*F. verticillioides*, [d]*A. flavus and F. verticillioides*, [e]*Penicillium*, [f]Surface disinfected, [g]Non-surface disinfected

Table 18: Mold treatment means with significant differences for measured variables averaged over Bt and Non-Bt treatments for the 2001 field plot study.[1, 2]

Treatment	afnsd[a]		affvnds[b]		pnsd[c]		kerndam[d]			ndam[e]		
Fv	2.4	b	2.2	b	100	a	152.9	b	c	16.6	a	b
Af	1.9	b	1.8	b	100	a	162.1	b		15.9	a	b
Fv/Af	3.9	b	3.6	b	100	a	206.5	a		14.6		b
Af/Fv	18.8	a	18.6	a	100	a	142.8	b	c	16.3	a	b
Af+Fv	2.3	b	1.0	b	99	b	145.1	b	c	16.8	a	
C	2.0	b	2.0	b	100	a	114.9		c	16.3	a	b

[1]Treatment: Fv =*F. verticillioides* alone, Af =*A. flavus* alone, Fv/Af =*F. verticillioides* 1st, *A. flavus* 2nd (4 days later), Af/Fv =*A. flavus* 1st, *F. verticillioides* 2nd (4 days later), Af+Fv =*A. flavus* & *F. verticillioides* similtaneously, *C*= control

[2]Means with the same letter within a column are not significantly different according to an LSD procedure (p=.05).

[a] *A. flavus* surface disinfected, [b]*A. flavus* & *F. verticillioides* non-surface disinfected, [c]*Penicillium* non-surface disinfected, [d]cm^2/ear kernel damage, [e]number ears/20 damaged

The variable representing the number of ears damaged (ndam) showed that there was a significant difference between treatment Fv/Af and treatment Af+Fv means.

The 2001 field plot year averages for rainfall per month during the planting, growing and harvesting period (May to September), are included with the thirty-year data for Beltsville, Maryland between 1971 to 2000 (Table 13). Higher than average rainfalls of 7.2 inches and 4.1inches were recorded for July and August 2001 respectively compared to the thirty-year average. Drought conditions did not occur during the 2001growing season (Maryland State Climatologist Office, 2004). 2001 temperature data are included with the thirty-year period during the planting, growing and harvesting period (May to September) and is presented in Table 14. Higher than normal temperature of 72.6 ºF was recorded in June 2001 compared to the average thirty-year normal temperature of 61.4ºF. A lower than normal temperature of 72.8ºF was seen in July 2001 compared to 76.9ºF for the thirty-year average (Maryland State Climatologist Office, 2004).

4.5 Discussion

This study of comparing *A. flavus* and *F. verticillioides* in Bt corn yielded a number of interesting results. Bt corn did not always have less insect damage than NBt corn. In 2000, insect damage (kerdam) in NBt corn was significantly higher in only 3 of the treatments, Af, Fv/Af, Af+Fv and C compared to Bt corn. It was not significantly different in treatments Fv and Af/Fv. This may have been due to weather conditions and hybrid performance. The number of ears damaged per 20-ear sample (ndam) was significantly higher in NBt corn in 3 of the 6 treatments; however, Bt corn had significantly higher insect damage (ndam) in treatment Af. In

2001, insect damage (kerdam) in NBT corn was significantly higher in all treatments. The number of ears damaged per 20-ear sample (ndam) was also significantly higher in all NBt corn treatments. These results are unlike other transgenic corn field plot studies where insect damage in Bt corn was significantly lower than non-Bt corn (Pilcher, et al., 1997; Rice, et al., 1997, & Rice, et al., 1998). However, one study established that when corn was damaged by corn earworm, there were no significant differences between Bt and non-Bt corn (Clements, et al., 2003). The levels of aflatoxins were significantly lower in the Bt corn in the 2000 field plot study, and no aflatoxins were present in the in 2001 study. In addition, aflatoxins were well below the action level of 20 ppb. Interestingly, the one Bt replicate with higher levels of insect damage than NBt, Af/Fv was one of two replicates that also contained aflatoxin B_1 in the 2000 field plot study. However, there were not higher levels of fumonisins in that replicate. These results were similar to studies conducted in Illinois and Iowa where very low levels of aflatoxins were found in Bt corn (Munkvold & Hellmich, 2000). The levels of fumonisins in 2000 in Bt corn were not significantly lower than NBt corn. However, all replicates in the 2000 corn showed fumonisin B_1 levels above the daily tolerable intake level of 2ppm for degermed dry milled corn products, ranging from 6.0 to 10.5 ppm (Table 12). In 2001, Bt corn showed significantly higher levels of fumonisins compared to NBt corn in replicate Af+Fv. In addition, the mean level of fumonisin B_1 in Bt corn was 5.6ppm, compared to 3.3 ppm found in NBt, both above the daily tolerable intake level for corn of 2ppm. These results are not consistent with most other studies done on Bt corn where significantly lower levels of fumonisin were shown in Bt compared to NBt corn (Munkvold, 1999;

Dowd, 2000; Dowd, 2001). One study with similar results showed that with corn earworm damage, fumonisin levels in Bt corn did not show significant differences compared to NBt corn in one of three study years (Clements, et al., 2003). Reduced insect damage is critical for reducing fumonisin levels in Bt corn and the specific insect infesting the corn seems to also be important. However, the occurrence of *Fusarium* in corn plants is also through the roots, therefore it may bet hat the source of the *F. verticillioides* and higher levels of fumonisins in the Bt corn was from *Fusarium* that entered the corn through the roots (Riley, et al., 1993). Finally, weather is a critical factor in fumonisin accumulation in corn. When corn plants are under stress, either by drought or over watering, the fumonisin synthesis is induced by these conditions (Bacon, et al., 2001). Weather conditions in 2000/2001 were wetter than normal which may have contributed to higher fumonisin accumulation in this study (Tables 13 and 14). The isolates of *A. flavus* obtained from the Bt and NBt corn kernels when analyzed for aflatoxin production showed that more than 80% were producers of the isolates in 2000, and 100% were producers from the 2001 study. Even though the potential of *A. flavus* to produce aflatoxins exists, field conditions did not allow *A. flavus* to either grow or produce aflatoxins. It may also be that the low insect damage during those years coupled with high levels of *F. verticillioides* and *Penicillium* fungi created an environment that was unfavorable for *A. flavus* growth and aflatoxin production. *A. flavus* prefers an environment with high heat, high humidity and drought conditions (Payne, 1992). Weather conditions for the 2000 and 2001 growing seasons were not favorable for *A. flavus* growth and aflatoxin production due to higher than average rain and average to low temperatures

(Tables 13 and 14). The levels of *F. verticillioides* and *Penicillium* were much greater in the Bt corn than the levels of *Aspergillus flavus*. In non-surface disinfected kernels, nearly 100% of kernels were contaminated with *F. verticillioides* and *Penicillium*, compared to 55% by *A. flavus*. Similarly, it has been determined that it is not unusual to find 100 percent internal kernel infection by *F. verticillioides* (Marasas et al., 1984b). It is very likely that the reduced insect damage resulted in a different fungal ecology on the corn affecting *A. flavus* growth. Interestingly, *Penicillium* levels were as high as that of *F. verticillioides*, suggesting that *Penicillium* mycotoxins should be examined in Bt as well as levels of *Fusarium*/*Aspergillus* toxins. The use of Bt corn to reduce levels of aflatoxins seems to be effective, however the levels of other molds in Bt corn may indicate a change in the ecology of Bt corn resulting in differences in levels of other fungi and the toxins that they produce. Similar results were seen in Bt corn in Illinois in 2002 showing low *A. flavus*, 100% *F. verticillioides* and 100% *Penicillium* spp. (Kendra, D. F., 2002, personal communication).

The results of the various inoculated mold treatments were not significant except for fvsd. It was hypothesized that by inoculating one mold in advance of another, its earlier establishment would cause it to out compete the second inoculated mold for substrate. For example, in treatment Af/Fv it was thought that *A. flavus* should have higher counts that *F. verticillioides* and even produce greater levels of aflatoxins, however this was not the case. In fact, these results indicate that *F. verticillioides* was always the dominant fungi in all treatments and both Bt and NBt corn. The variable fvsd (*F. verticillioides* surface disinfected) did show a result in the

2000 corn with significant difference at a $p<0.05$ level (Table 10). It is known that *Fusarium* species are the most encountered toxigenic contaminant in human foods including cereal grains, beans and oil seeds and that in corn, *Fusarium* species are both plant pathogens as well as soil saprophyte (Chelkowski, 1989a and Chelkowski, 1989b). In addition, *F. verticillioides* is the most common soil-borne pathogen found in corn in all regions of the world (Marasas et al., 1984b). These factors may explain why *F. verticillioides* was the dominant fungi in this study.

In this study there was a significant difference between treatments Fv and C, which demonstrates that the inoculated *F. verticillioides* may have been able to establish itself in the Bt and NBt corn.

Fumonisin levels were not significantly lower in Bt 2000 corn compared to NBt 2000 corn. These results were expected since there was significantly lower insect damage in Bt corn compared to NBt corn. Reduced insect damage in earlier studies resulted in lowered fumonisin levels in Bt corn (Munkvold, et al, 1999; Dowd, 2000; Dowd, 2001 & Hammond, et al., 2004). In the 2001 study, there were significantly higher levels of fumonisins in the Bt corn compared to NBt, however there was significantly greater insect damage in NBt compared to Bt. A similar result found in a 2000 and 2001 Illinois study that Bt corn damaged by corn earworm did not show reduced fumonisin levels compared to non-Bt corn (Clements, et al., 2003). It is possible that the increased insect damage in this study was due to corn earworm infestation leading to higher fumonisin levels in Bt corn. Another explanation is that the hybrid used in this study had been developed under non-Maryland climatic conditions. Thus, growing the out of its area of adaptation, could

lead to increased stress on the plant resulting in higher levels of *F. verticillioides* infection and increased toxin production.

Temperature during 2000 and 2001 could also be playing a role in these studies. For example, temperature for the 2000 growing season was slightly higher than normal in May and June and precipitation was lower than average in May, and higher than average in June, July and September. The temperature for the 2001 growing season was slightly higher than normal in May and June however precipitation was higher than average in May, June, July and August. Since *A. flavus* prefers an environment with very high temperatures and drought like conditions (Payne, 1992; Smart, 1990), which did not exist in either 2000/2001, it is likely that *A. flavus* was not able to effectively colonize and produce aflatoxins in corn during these years. Whereas *F. verticillioides* produces fumonisins in the corn plant under stress conditions, higher than normal rain may have created conditions that induced fumonisin accumulation (Bacon, et al., 2001).

The results from this field plot study suggest that it may be prudent to develop hybrids that are adapted to a specific growing region so as to lower the inherent stresses on the plant that might occur which could lead to increased susceptibility to toxigenic fungi (Marasas, W.F.O, personal communication, June 12, 2003). It may also valuable to do similar at co-inoculation studies with *A. flavus* and *F. verticillioides* on hybrids developed for specific regions worldwide. Another issue is the high levels of *Penicillium* in the Bt and NBt corn. The Bt corn hybrids may be affecting levels of *Fusarium*/*A. flavus* as well as *Penicillium* and the toxins this genus produces. Thus future studies should monitor *Penicillium* toxins as well.

4.6 Conclusion

It is known that Bt corn can reduce insect damage which theoretically should reduce the incidence of aflatoxins. However the problem may be more complex. These studies show that the ecology of Bt corn is such, that all naturally occurring fungi, including *Fusarium*, *Aspergillus* and *Penicillium*, have been affected by Bt corn such that the levels of these respective microorganisms are different in Bt corn; the levels of the toxins they produce may also be different as well. Future studies should monitor levels of both *Penicillium* and its toxic metabolites as well as *F. verticillioides*/*A. flavus* and their toxins.

Chapter 5: The Presence of Toxigenic Fungi and Mycotoxins in Irradiated Dietary Supplements

5.1 Abstract

In 2001, the sales of vitamin and herbal supplements in the U.S. exceeded 13 billion dollars (Blumenthal, 2002). Between the years of 1991-2000, U.S. average imports of medicinal plants from third world countries totaled 135.5 million dollars 49.6 thousand tons of imported goods (Vasisht, K. 2004). In 2003 imports of medicinal plant material rose to between 350-400 million dollars. More than 90% of herbs sold on the domestic market are imported into the U.S. with less than 50% irradiated to reduce insect and fungal contamination (Betz, J., personal communication, July, 2001). These are concerns to companies that formulate and sell herbal products of the possibility of adulteration of the herbal with non-active or possibly toxic ingredients, pesticide contamination and of increasing concern, contamination with pathogenic microbes and/or their metabolites. Especially alarming is the fact that much of the herbal plant material is obtained from third world countries where there are inadequate quality control practices. In this study, irradiated and non-irradiated botanical supplements were obtained from a producer in Peru for toxigenic fungi and/or toxins analysis. The microbe populations of irradiated and non-irradiated samples were examined and the levels, of aflatoxins present in the samples were determined. There were no fungi in the irradiated samples; however, the non-irradiated samples contained *Rhizopus, Penicillium* spp., as well as *A. niger*, and *A. flavus*. When the *A. flavus* isolates were grown on rice and analyzed for aflatoxins all produced aflatoxin B_1. Mold-free irradiated samples were also analyzed

for aflatoxins, 24 of 25 samples were positive for aflatoxins and the levels varied from trace to 450 ng/g.

5.2 Introduction

Although irradiation is done to eliminate molds from spices (McCormick Spice Company, personal communication) currently, there are no federal restrictions or regulations that require irradiation of herbals which frequently originate from third world countries where quality control is often lacking. Toxigenic molds such as *A. flavus, A. parasiticus, A. ochraceus, Rhizopus, Penicillium* spp., and *Alternaria* have been reported to be the predominant fungi detected in herb tea and medicinal plants (Efuntoye, 1999; Halt, 1998). Some of the previously reported molds are capable of producing toxins including aflatoxins, ochratoxin A and patulin. In one study, aflatoxins were found in herbs and spices at levels ranging from less than 10 μg/kg to 51 μg/kg. (MacDonald, & Castle, 1996). Currently, FDA regulations that assure the effectiveness and safety of over the counter and prescription drugs do not apply to botanicals imported from third world countries since they are considered dietary supplements. The 3 most important concerns of consumers who use botanicals should be: 1) is the product beneficial to one's health, 2) has the product been adulterated with potentially harmful additives and, 3) is the product contaminated in any way due to pesticides or pathogenic organisms including toxigenic fungi. While irradiated botanical supplements could be free of toxigenic fungi especially if they have been irradiated, mycotoxins they produce during shipping/processing could still be present and undetectable in the product. In this study of irradiated Peruvian medicinal herbals, the presence of toxigenic fungi and/or their toxic metabolites was

investigated. Both irradiated and non-irradiated samples were examined. The microorganisms present and their levels in the non-irradiated herbals were determined and compared to irradiated herbals; irradiated and non-irradiated herbals were also examined for the presence of aflatoxins

5.3 Materials and Methods

5.3. 1 Medicinal Herbals Examined from Peru

- Uña De Gato - Cat's Claw (*Uncaria tomentosa*). The inner bark is used. For rheumatic afflictions, reinforces natural defenses and immune system depression. Dose: 1-2 capsules with meals.

- Pasuchaca - (*Geranium dielsiannum*). Leaves and stem are used. Indicated for the treatment of diabetes, for its hypoglycemic action and favors pancreatic function. Dose: 3- 6 capsules daily.

- Hercampuri - (*Gentianella nitida*). Leaves, flowers and stems are used. Hepatic tonic and depurative with a weight reducing action. Dose: 1-2 capsules with meals.

- Herbal Pros - It is a combination of Huamanpinta (*Chuquiraga spinosa*) and Cat's Claw. The leaves and inner bark are used. It is recommended for its anti-inflammatory properties in the urogenital system and prostatic adenoma. Doses: 1-2 capsules with meals.

- Sen - (*Cassia angustifolia*). Leaves and fruit are used. Natural laxative in case of constipation.

- Maca – (*Lepidium meyenii, Walp*). Bulbs, leaves, flowers and stems are used. Excellent dietary supplement, improves physical and mental capacity, and invigorates reproductive functions.

5.3.2 Isolation/growth of *A. flavus*

Ground irradiated and non-irradiated plant material was transferred to Difco potato dextrose agar in petri dishes, and examined for mold growth 5-7 days after incubation. The *A. flavus* isolates that occurred were then grown on sterilized rice for

two weeks and the rice extracted for the presence of aflatoxins. Aflatoxins present in the rice cultures were determined using "VIACAM Aflatest Method" and the results were confirmed by thin layer chromatography using approved AOAC methodology.

5.4 Results

Whereas the irradiated herbal products contained no detectable microorganisms, the non-irradiated products were contaminated with *Aspergillus* spp., *Penicillium* spp., and *Rhizopus*, and all 3 fungi occurred simultaneously in all the samples showing mold growth. Only 1 of the 6 non-irradiated products,

Table 19: Mold occurrence on irradiated and non-irradiated Peruvian herbal products.[a]

	Treatment	
Product	Irradiated	Non-Irradiated
Herbal Pros-A	Yeast	*Penicillium, Aspergillus, Rhizopus*
Herbal Pros-B	-	*Penicillium, Aspergillus, Rhizopus*
Pasuchaca-A	-	-
Pasuchaca-B	-	-
'Uña De Gato-A	-	*Penicillium, Aspergillus*
Uña De Gato-B	Bacteria	*Penicillium, Aspergillus*
Hercampuri -A	-	*Penicillium, Aspergillus, Rhizopus*
Hercampuri -B	-	*Penicillium, Aspergillus, Rhizopus*

[a]PDA Plated Samples

Pasuchaca A, was mold free (Table 19). In all the irradiated samples only yeast or bacteria were detected. When the *Aspergillus* isolates from the contaminated products were grown on sterilized rice and analyzed for aflatoxins after two weeks, all of the isolates of *A. flavus* produced aflatoxin B_1 (AFB_1). A total of 25 irradiated herbal samples were analyzed for total aflatoxins. The levels of aflatoxins ranged from trace levels to 450ng/g (450ppb). (Table 20) and only aflatoxin B_1 was detected using TLC.

Table 20:Aflatoxins in irradiated Peruvian herbals.[a]

Product/Lot Number	Grams Extracted	Aflatoxins (ng/g)
Herbal Pros		
Unknown	23.5	280.0
005030 (A)	25.0	5.0
005030 (B)	25.0	5.4
912029 (A)	21.5	4.5
912029 (B)	22.3	6.0
Hercampuri		
Unknown	21.5	2.5
001010 (A)	25.0	2.5
001010 (B)	25.0	4.0
005060 (A)	22.8	1.0
005060 (B)	23.0	0.5
909049 (A)	25.0	0.9
909049 (B)	25.0	0.1
Maca		
002010 (A)	25.0	1.0
Pasuchaca		
Unknown	25.0	110.0
005050 (A)	25.0	360.0
005050 (B)	25.0	450.0
Sen		
005040 (A)	25.0	2.0
005040 (B)	25.0	0.0
905029 (A)	21.0	1.5
905029 (B)	21.0	0.5
909029 (A)	24.0	2.0
909029 (B)	23.0	3.5
Uña de Gato		
Unknown	25.0	7.5
006010 (A)	25.0	1.5
006010 (B)	25.0	3.0

[a]Aflatoxin B_1 was confirmed using TLC (AOAC)

5.5 Discussion

Irradiation of herbal plant material is an effective means of eliminating mold fungi. Whereas the irradiated samples in this study were mold free, the non-irradiated samples contained both *Aspergillus* and *Penicillium* plus *Rhizopus*. Since most of the herbal products entering the U.S. originate from developing countries where inadequate storage facilities exist, there appears to be the potential that herbal products have been contaminated with mycotoxins prior to their being imported Although irradiation eliminated toxigenic fungi in these samples, it had no effect on the level of aflatoxin B_1 already present in the products sampled. Failure of irradiation to destroy aflatoxins has been confirmed by earlier studies; irradiating fungal cultures, foods or feeds has no effect on the levels of AB_1 present (Frank & Grunewald, 1970; Frank, et al., 1971; Hooshmand & Klopfenstein, 1995). Thus, better monitoring of herbal products entering the U.S. especially from third world countries, should be done to determine the extent of microbial contamination in order to protect the consumer against the likelihood that microbial toxins may be present in the products they are using to help improve their health.

5.6 Conclusion

Although irradiation may provide an effective and safe means of removing some mycotoxins from as fumonisins, this study shows that it does not remove aflatoxins that are already present in a product. Irradiation may eradicate the fumonisin molecule since the molecule can easily be destroyed by the free radicals produced as a result of the irradiation process since the molecule is a long carbonyl

chain and easily broken down. In contrast, the aflatoxin molecule is a three pentane ring and two-hexane ring structure, and the central hexane ring is a benzene ring which makes the molecule very stable and resistant to irradiation treatment. These studies suggest that imported herbal plant material can be a health hazard especially if the product has not been stored properly.

Chapter 6: Determination of Aflatoxins in Ginseng Roots

6.1 Abstract

Ginseng, one of the most widely used herbals, is designated by the FDA to be a dietary supplement. Unfortunately, the FDA cannot regulate dietary supplements in the same manner as foods or drugs due to this products designation. Manufacturers are thus responsible to the consumer that their herbal products are safe, properly labeled and do not contain any untruthful or misleading information. The FDA can only act on an herbal product after it reaches the market. For example, Ephedra (Ma huang) was banned by the FDA after a number of deaths and, the safety of dietary supplements is increasingly being questioned. Since it is the manufacturers that are responsible for monitoring dietary supplements, it is important to determine if they have done so and the product has not been adulterated or contaminated with pesticides or mold, especially with toxigenic fungi. As the previous study demonstrated, herbal plant material, especially from third world countries, can be either contaminated with molds including toxigenic fungi and/or their toxic metabolites.

The analysis of ginseng has focused primarily on measuring ginsenosides in the ginseng roots since these are considered to be the compounds responsible for ginseng's medicinal activity (Yat, 2002). However, since ginseng is a root, care should be taken to ensure that during growth, harvest and storage of ginseng, infection by molds, especially, toxigenic root infecting microbes, be avoided. The most notorious of the toxigenic soil inhabiting microbes most likely to be present is either *Fusarium* or *Aspergillus*. In this study, an analytical method for the

determination of aflatoxins, a group of structurally similar mycotoxins, in ginseng root tissue first had to be developed. This method was then used in a survey of ginseng root for the presence of aflatoxins.

Recovery studies of aflatoxins using this method were done by adding a solution of aflatoxins (AFB_1: AFB_2: AFG_1: AFG_2: = 4: 1: 2: 1) to toxin-free 4 to 5 year old dried sliced Wisconsin ginseng, to equal 2, 4, 8 and 16 ng/g (dried weight basis). Extraction of spiked root tissue was done using methanol and water (80:20). The toxins were then filtered and isolated with a VICAM Aflatest immunoaffinity column packed with antibodies specific for aflatoxins. The samples were then derivatized with water, trifluoroacetic acid and acetic acid. Using reverse phase liquid chromatography, equipped with a fluorescence detector, the levels of aflatoxins were determined by pre-column derivatization and confirmed with UV irradiation derivatization. Mean recoveries ranged from 77% to 92 %, and mean coefficient of variation ranged from 2 to 8%.

This extraction method was then used to detect aflatoxins in wild simulated and cultivated ginseng roots. The roots were harvested from Appalachia areas of Maryland and West Virginia in August of 2002 and 2003. The weather conditions in this area were very dry (-12.35 inches normal) in 2002 and very wet (+ 25.95 inches normal) (Maryland State Climatologist Office, 2004) in 2003 so that the roots were subjected to moisture stresses in both years, a factor known to increase plant susceptibility especially to *Aspergillus* infection. The first type of root, wild simulated, was harvested after six years growth. Roots were dried, ground and encapsulated using 400mg pure ginseng, harvested and processed under strict quality

control standards. One hundred capsules containing approximately 400mg ginseng were emptied and the material mixed before analysis. Three 8-year-old wild simulated root samples were also obtained, a clean root, a visibly moldy root and a powdered root, and were also analyzed using this method. Since 2002 was a drought year for the Maryland cultivated root field plots, not many plants germinated in 2003, two additional one ounce amounts of wild simulated roots harvested after the 2002 growing season were obtained from growers in Maryland and West Virginia for comparison. The second type or "cultivated root" was maintained under controlled growing conditions of fertilizing and watering. These roots were harvested in the summer of 2003, and 2004. Only one cultivated site in Garrett County, Maryland, produced ginseng in 2003 because of the drought in 2002, however all three sites produced ginseng in 2004.

6.2 Introduction

Ginseng is one of the most commonly used dietary supplements in the U.S (Herbalgram, 2001). Ginseng is a popular ingredient in herbal teas and cosmetics, it is available in a variety of dosage forms, and is consumed to improve overall health and well-being. Presently, very little data are available on the contamination of botanical supplements, especially root tissue, with toxigenic fungi such as *Aspergillus flavus* which is a root-inhabiting microbe. Since ginseng is a root-derived herbal, the possibility exists that the product could be contaminated with mycotoxins, especially aflatoxins. Most of the studies on ginseng have focused on the pharmacological effects on humans and animals, and no studies have looked at the possible occurrence of toxigenic mold and mycotoxins in ginseng root tissue. This study seeks to

determine whether or not aflatoxins are present in naturally grown ginseng using a previous analytical method developed for detection of aflatoxin in ginseng root tissue. This project addresses the potential that botanical supplements (i.e. root tissue) could contain harmful carcinogenic contaminants originating from toxic soil fungi. If this were so, then regulatory limits on mold toxins in root herbal products should be developed to prevent exposure of individuals to toxic herbals materials.

In 1994, herbals were legally defined as dietary supplements by the Dietary Supplement Health and Education Act (DSHEA). The DSHEA exempted manufacturers of dietary supplements from having to prove safety or efficacy before marketing and placed the burden of proof on the FDA to show that a supplement is unsafe as well as ineffective. In March 2003, the FDA proposed labeling standards for dietary supplements. This rule would establish federal standards to ensure that dietary supplements are not adulterated with contaminants or contain other harmful substances and are labeled to accurately reflect the ingredients present in the product.

Many herbals are imported from countries where quality control may be deficient or else proper storage conditions are not available which in warmer climates ultimately leads to mold contamination problems. Some of these molds such as *A. flavus* are known to produce carcinogenic secondary metabolites, such as the aflatoxins. Aflatoxins are a group of structurally related mycotoxins produced mainly by *A. flavus* and *A. paraciticus* and are considered the most mutagenic and carcinogenic naturally occurring compounds known (JECFA, 1999). The International Agency for Research on Cancer (IARC) has classified aflatoxin as a probable human carcinogen (IARC, 1993a). The FDA action level for aflatoxins in

foods is 20ppb (Table 1). Aflatoxins have been found in herbs and spices at levels ranging from less than 10 μg/g to 51μg/g (MacDonald and Castle, 1996). Recent studies of botanical supplements indicate that aflatoxins may be present in plant root material (MacDonald and Castle, 1996, Patel, et. al., 1996, Reddy, et. al., 2001).

Therefore, the quality and/or purity of our most popular herbal medicines are often not adequately assured because the methods of harvesting and storage are often not regulated. In addition the sources of these herbals are often from developing countries where adequate facilities for drying and storage are improper or not available. All of these factors could ultimately result in herbal products being sold in the U.S. that are contaminated with toxigenic fungi and toxic fungal metabolites, thus posing a problem to the consumer who consumes them to improve health. Herbals, being plant-derived, may be heavily contaminated by viable fungi both in the field and during handling and storage. Medicinal plant materials have been examined by researchers to determine rate of occurrence of fungal species present and levels of mycotoxin contamination. Most of these studies have been focused on herbals roots grown in the tropical and subtropical regions. These studies have found the presence of aflatoxins, often at high levels (Scott and Kennedy, 1975; Seenappa et al., 1980; Horie et al., 1982; Flannigan and Llewellyn, 1992; Aziz et al., 1998; Baratine and Tantaoui-Elaraki, 1997).

Ginseng root is grown under both wild simulated and cultivated conditions. Wild cultivated ginseng root growing environments are usually not maintained nor subjected to controlled conditions such as watering, fertilizing and the application of pesticides including fungicides. Under these conditions, environmental stresses are

more likely to occur which often leads to increasing infection by opportunistic fungi such as *A. flavus* the notorious aflatoxin producing fungus. In contrast, cultivated ginseng roots are under more strict growing conditions and thus should not be subjected to stress conditions and fungal invasion. Since ginseng is among the top botanical supplements consumed to improve health and it is a root crop, it would be the ideal botanical to study for the presence of aflatoxins of all the botanical root materials sold on the market. Thus, the objective of the study was to assay field grown ginseng roots for the presence of aflatoxins using a previously developed aflatoxin extraction procedure.

The most recently developed generally used mycotoxin purification and isolation method is an Official AOAC International Immunoaffinity Columns (IAC) method (991.31). It was developed for the presence of aflatoxins in corn, peanut and peanut butter (Trucksess, M.W., 2000a). This method was selected for its applicability for the analysis of aflatoxins in ginseng root tissue. After slight modifications the method was used to detect the presence of aflatoxins in wild simulated and cultivated root tissue collected from the wild for mycotoxins.

In this study, 23 samples ranging from 1g -100 g each from field, storage, processing, and retail area were obtained. Unprocessed roots were lyophilized and then ground, mixed, and subsampled for aflatoxins analysis. The analytical method focused on extraction, purification, separation, quantitation and confirmation of aflatoxins found. Aflatoxins are easily extracted into various mixtures of water and organic solvents including chloroform methanol, acetonitrile and acetone (Wilson, et. al., 1998). Solid phase extraction (SPE), immunoaffinity column (IAC), liquid-liquid

partition, and precipitation are commonly used for purification. The preliminary results of this study using the Aflatest IAC (Scott and Trucksess, 1997), which is a single column to purify botanical extracts, indicated that the botanical components produced non-specific bounding with the toxin specific antibodies. Recoveries of the toxins in this study were poor (<20%). In the ginseng study, the sample was extracted with aqueous methanol, the extract was filtered and centrifuged for clean up on a SPE. The eluate should contain aflatoxins which would be diluted with water and applied to an Aflatest IAC. The toxins were then eluted with methanol, the solvent was evaporated and the residue was be redissolved in appropriate solvent before injection into the HPLC.

6.3 Materials and Methods

6.3.1 Herbal Plant Material

Dried ginseng was obtained from commercial Wisconsin ginseng growers. Wild simulated ginseng roots were obtained from Maryland and West Virginia farms harvested during the 2002 and 2003 growing seasons. In addition, field plot ginseng root was also collected from one site in Maryland in the summer of 2003, and from three sites in Maryland during the summer of 2004. The roots were dried and stored under the recommended conditions (see below).

6.3.1.1 Cultivated ginseng

a. Wisconsin ginseng was grown in small areas in the shade. In October the shade structure was removed and the remaining straw and foliage was scraped from the field and the roots harvested. The roots were then placed in boxes and placed into a refrigerated building at 40°F for 3 weeks. This

is a process that only a limited number of growers will do but it appears to result in the highest quality ginseng available. During this cooling process the sugars and starches and their levels in the roots change the same as would occur in the ground as winter approaches. These changes produce a root that is more wrinkled and the inside is very white and "chalky". This allows for a root that is much easier to slice for use by the consumer. The roots are then removed from the cooler after 3 weeks washed and placed in a kiln dryer where the humidity and temperature are precisely controlled to produce the "perfect dry". The temperature is maintained at approximately 98°F and the humidity is monitored closely to insure that the level drops consistently from 100% to 2% or lower. The drying process takes approximately 10 to 14 days depending on the size of the roots. Once the roots are dried they are inspected and any dirt or damaged roots are removed, then they are packaged and shipped. The roots purchased for this study ranged in age from four to five years. They were obtained dried and sliced and were ground and 5g obtained for analysis.

6.3.1.2 Non-cultivated “wild simulated” ginseng

a. The Maryland ginseng crop was grown from wild seed and maintained in the woods under the canopy of hardwood forests located in the Appalachian Mountains of Western Maryland near the town of Friendsville. The ginseng is valued for the wild ginseng characteristics of taste, color, texture plus very high concentration of ginsenosides, which is considered the medicinally active ingredient. After the roots are

harvested, they are placed in boxes and stored in a refrigerated building at 40°F for 3 weeks. Ginseng root is then dried in forced air electric dryers at 98º F with exhaust with constant tumbling to insure an even dryness throughout the root. Roots are usually dried in 3-4 days. One hundred grams of six-year-old dried, encapsulated ground root were removed from the capsules and mixed and 5 g amounts were taken for analysis. Additional samples of 28 g of 6 eight-year-old small rootlets, 28 g of 8-year-old powered roots, and one 4g 4-year-old moldy root were analyzed in 1.0 g amounts.

b. The American Ginseng (*Panax quinquefolium*) samples had been cultivated in raised beds and grown under wild simulated conditions. The location of the beds was in a forested area on the eastern slope of the Catoctin Mountains in Frederick County, MD. No pesticides or synthetic fertilizers were used. The rootlets were cultivated under a hardwood canopy in ancient, mountain forest soil. After the roots were harvested, they were cleaned and spread on screen racks in the shade for drying. They were turned frequently and provided adequate aeration. Drying time varied with root size, and the root was exposed to the open air. No attempt was made to prevent mold growth during the drying. One ounce (28 g) of approximately 4 to 5 six-year-old roots harvested in 2002 were collected for analysis. Four replicates of the sample root material was analyzed in 5 g amounts.

c. West Virginia native wild ginseng root tissue was also obtained. The location of the West Virginia farm was in the northeast corner of West Virginia close to the borders of Pennsylvania and Maryland. Plants grew in a partially wooded northern exposure hillside with adequate moisture. After harvest, the roots were placed in boxes and stored in a refrigerated building for 3 weeks at 40°F. Root was then dried in kiln dryers in wooden boxes at 98º F in 3-4 days. One ounce (28 g) of 4 to 5 six-year-old roots harvested in 2002 were analyzed. Four replicates of the sample root material was analyzed in five-gram amounts.

d. Fresh ginseng root was also purchased from a local Asian market, identified as Midwestern American Ginseng (*Panax quinquefolium*). The sample was dried and ground and a five-gram sample was analyzed for aflatoxins using post column bromination derivatization in a Kobra cell.

6.3.1.3 Cultivated experimental ginseng

University of Maryland grown ginseng in field plots was maintained under controlled conditions. One and two year old fresh root was obtained and analyzed in 0.5 g samples. The roots were lyophilized and ground before analysis.

a. Samples harvested summer 2003:

    1. A sample was obtained from M. McIntosh, UMCP, from forested field plots in Garrett County, Maryland. It was harvested after the severe drought year of 2002, so samples available were only available from one of the three field plot sites. Nine 0.5 g dried and ground samples were analyzed.

c. Samples harvested in the summer of 2004:

1. Sample one was grown in Clarksville, Maryland in a wooded area. It was harvested on the 14th and 17th of June. Nineteen 2-year-old rootlets were obtained, cleaned, freeze-dried and ground. The wet weight of the rootlets was 7.8 g and dry weighed 2.1 g. Samples were analyzed in three 0.5 g amounts.
2. Sample 2 was obtained from the Keetysville Maryland Experiment station located in a forested area. Samples were harvested on June 23rd. Nineteen two-year-old rootlets were collected, cleaned, freeze-dried and ground. Wet weight of the rootlets was 8.9 g and dry weighed 2.5 g. Samples were analyzed from 0.5 g amounts. There were 3 replications for each sample.
3. The third sample was harvested on August 4th from field plots at the Wye Plantation on the eastern shore of Maryland; this was also a wooded area. Sixteen two-year- old rootlets were obtained, cleaned, freeze-dried and ground. The wet weight of the rootlets was 18.3 g and dry weighed 7.1g. Samples were analyzed from 0.5 g amounts. There were 3 replications.

6.3.2 Aflatoxin spiked ginseng root tissue

Preliminary experiments were done to determine the most effective experimental design, sample size, and treatment factors for technique development for maximum recovery levels. Analysis of variance and planned comparisons were

also used to test relevant hypotheses related to the prevalence of mycotoxins for various species of ginseng studied.

After the analytical method for aflatoxins recovery was developed and validated, ginseng representative of the common forms marketed in the U.S. were obtained for toxin analysis. These included wild simulated and cultivated samples and included materials that differed in origin, cultivation, and delivery system.

These quantitative analytical methods were then used to survey field samples of ginseng for aflatoxins. Mycotoxin analytical methods consisted of sampling, sample preparation and analysis. The methods used in this study were as follows:

6.3.3 Sample Preparation

Five grams of powdered ginseng plus 1.0 g NaCl was added to a 50 mL centrifuge tube. Twenty-five mL MeOH: H_2O (80:20) was added, and the sample was mixed and then shaken for 30 minutes in a VWR Orbital Shaker # DS500E at room temperature at 300 rpm. The sample was centrifuged for 10 min at 7000 rpm and 10^{o}C using a Fisher Scientific Marathon 21000R refrigerated centrifuge. Seven mL of the filtered supernatant was pipetted into a 50 mL tube, 28 mL of water was added, and the sample mixed. The mixture was filtered through a Whatman glass microfibre filter (Cat#: 1821090) into a 25-mL graduated cylinder.

For smaller samples, 1.0 g of powdered ginseng was weighed in a 50-mL centrifuge tube and 0.2 g NaCl (0.1g NaCl for 0.5 g ginseng) was added. Eight mL of MeOH: H_2O (80:20) was added and mixed as above. Seven mL or whatever adjusted volume of supernatant recovered was pipetted into a 50-mL centrifuge tube and diluted with 28 mL water to make the total volume 35-mL. The entire sample was

filtered through a Whatman glass microfibre filter (Cat#: 1821090) and into another 50-mL centrifuge tube.

6.3.3.1 Immunoaffinity column (IAC) cleanup

Twenty-five mL of filtrate or whatever adjusted volume of filtered supernatant recovered was added onto the IAC (VICAM Aflat-test P Immunoaffinity Column). A flow rate of 1-2 drops per second was maintained until all of the eluate had passed through the column. The column was rinsed twice with 10 mL of water and the column was allowed to run dry. Air was forced into the column to remove excess water from the column.

Aflatoxins were eluted through the IAC column into a 4 mL vial using 3 rinses with 1.0 mL acetonitrile. The column was allowed to drain 1 minute between each 1 mL acetonitrile wash. A syringe was used to apply air to the column. The eluate was evaporated to dryness under a stream of nitrogen in a 50^{o} C water bath.

6.3.3.2 Pre-Column Trifluoroacetic Acid (TFA) Derivatization

A C18 bonded silica gel column was used for pre-column quantitation of aflatoxins. Two hundred μL of CH_3CN was added to the dry sample film. The sample was mixed by votexing and 300 μL of derivatization reagent [TFA: HOAc: H_2O (20:10:70)], was added and mixed. Vials were placed in a 65^{o} C heating block for 9 minutes, cooled, and diluted to appropriate volume with water before LC analysis.

6.3.3.3 Post Column Bromination Derivitation

Bromination derivitation using a Kobra cell (Romer Labs, 2003) was used for post column quantitation of aflatoxins. Samples were placed in LC vials directly after running through the IAC. Approximately 1.5 mL aliquots were immediately

analyzed by LC. The final volume after derivatization was 0.5 mL and there was a 1.0 g sample in each vial. Test samples were diluted in the following manner:

aflatoxin level	derivatized test sample (μL)	Water (μL)
2 ng/g	100	100
4 ng/g	100	300
6 ng/g	100	500
8 ng/g	100	700

6.3.2.5 HPLC Analysis

Standard stock solutions were prepared from aflatoxin B_1, B_2, G_1 and G_2 standards using a spectrophotometer to determine weight in grams. Standards were diluted with acetonitrile to achieve a final standard concentration of B_1: B_2: G_1: G_2 of 2.0: 0.5: 1.0: 0.5 μg/mL. The spiking solution were then prepared by adding 1.0mL of the standard stock solution to a 10 mL volumetric flask and diluted to the mark with acetonitrile. Five dilutions of the spiking solution were made and analyzed on the HPLC system to construct the standard curve for the each aflatoxin. The dilutions prepared were 0.5 ng/g, 1.0 ng/g, 2.0 ng/g, 4.0 ng/g and 10 ng/g. Linear regression analysis was used to establish a straight-line best fit on the data plot for each concentration.

For pre-column TFA derivatization**,** the LC mobile phase was a mixture of MeOH: CH_3CN: H_2O in a ratio of 25: 15: 60. Degassing was done with a sonicator. The fluorescence detector was set at Ex 360, Em 440. The elution order of the four aflatoxins was G_2, B_2, G_{2a} (G_1 derivative), B_{2a} (B_1 derivative). For post-column Bromination derivitation, The LC mobile phase was: methanol: acetonitrile: water (22: 16: 62); each L contains 120 mg KBr and 350 μL 4N nitric acid. The

fluorescence detector was set at Excitation 362, and Emission 455. Elution order of the four aflatoxins was G_2, G_{2a} (G_1 derivative), B_2, B_{2a} (B_1 derivative). The limit of detection (LOD) is 0.2 ng/g for aflatoxin B_1, therefore any data <0.2 is reported as ND (non-detected).

6.4 Results

Results from the recovery study are presented in Table 21. The spiking levels of total aflatoxins used were 2 ng/g, 4 ng/g, 8 ng/g, and 16 ng/g. Mean percent recoveries ranged from 77% to 92%. The coefficient of variance (CV) ranged from 2% to 8%. These data show that the method performance was acceptable. Figure 17 shows the HPLC chromatogram aflatoxin standards and Figure 18 shows the HPLC chromatogram aflatoxin spiked ginseng root tissue.

A total of 11 wild simulated and 12 cultivated ginseng root samples were analyzed for the presence of aflatoxins using the previous method. Of the wild simulated samples, 5 were 2002 roots and 5 were 2003 roots. All of the Harding 2003 wild simulated encapsulated roots were found to contain no aflatoxin except for a single sample which showed a presumptive aflatoxin B_1 peak (Table 22, Figure 19).

Table 21: Percent recovery of total aflatoxins added to ginseng.[a]

Level Added	$AFLB_1$	$AFLB_2$	$AFLG_1$	$AFLG_2$	Total AFL	[b]Mean AFL	Mean Rec	[c]SD	[d]CV
----------ng/g----------							%	ng/g	%
16.0	7.3	1.7	3.7	0.8	13.6				
16.0	7.1	1.7	3.5	0.7	13.0				
16.0	7.3	1.8	3.5	0.6	13.2				
16.0	7.4	1.8	3.4	0.6	13.2	13.2	82.8	0.3	2.0
8.0	3.9	1.3	2.0	0.4	7.5				
8.0	3.9	1.3	2.0	0.4	7.6				
8.0	3.5	1.1	1.5	0.3	6.5				
8.0	3.9	1.2	1.9	0.4	7.5	7.3	90.9	0.5	6.9
4.0	1.7	0.5	0.8	0.2	3.1				
4.0	1.8	0.5	0.9	0.2	3.3				
4.0	1.5	0.4	0.7	ND	2.7				
4.0	1.8	0.4	0.9	0.2	3.2	3.1	77.4	0.2	8.0
2.0	1.0	0.3	0.5	ND	1.9				
2.0	1.0	0.2	0.6	ND	1.9				
2.0	0.9	0.2	0.5	ND	1.8				
2.0	1.0	0.2	0.5	ND	1.8	1.8	91.5	0.1	3.6

[a]Spiking levels of total aflatoxins were 2 ng/g, 4 ng/g, 8 ng/g, and 16 ng/g.

[b]Average of the 4 replicates at the spiking level

[c]Standard Deviation, n=4, [d]Coefficient of Variance

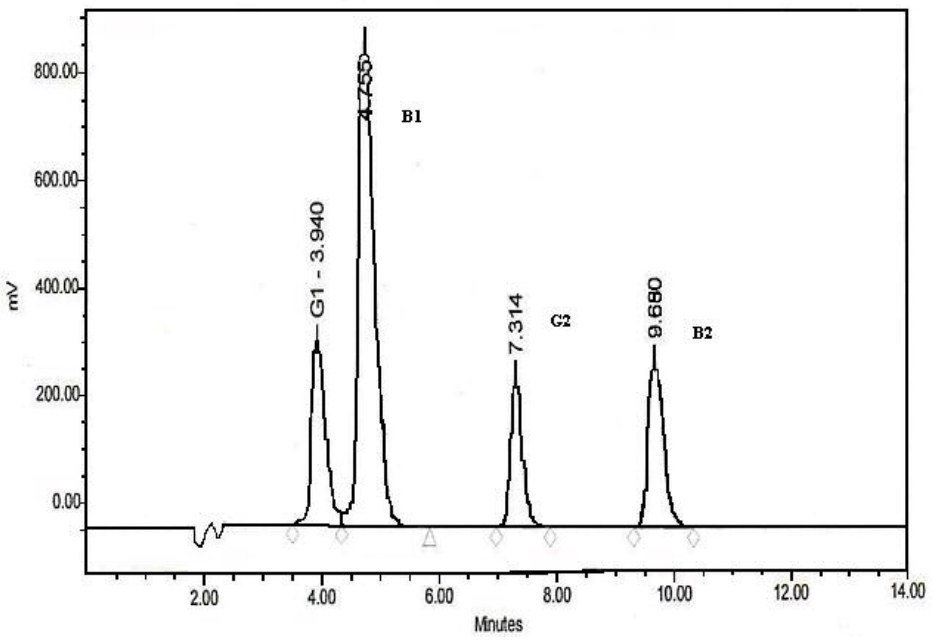


Figure 17: HPLC chromatogram of aflatoxin standards, 5 ng/g added
Column: YMC ODS-AQ S-3 120A 4.6 x 150mm. Mobile Phase: acetonitrile: methanol: water (25+12+63)

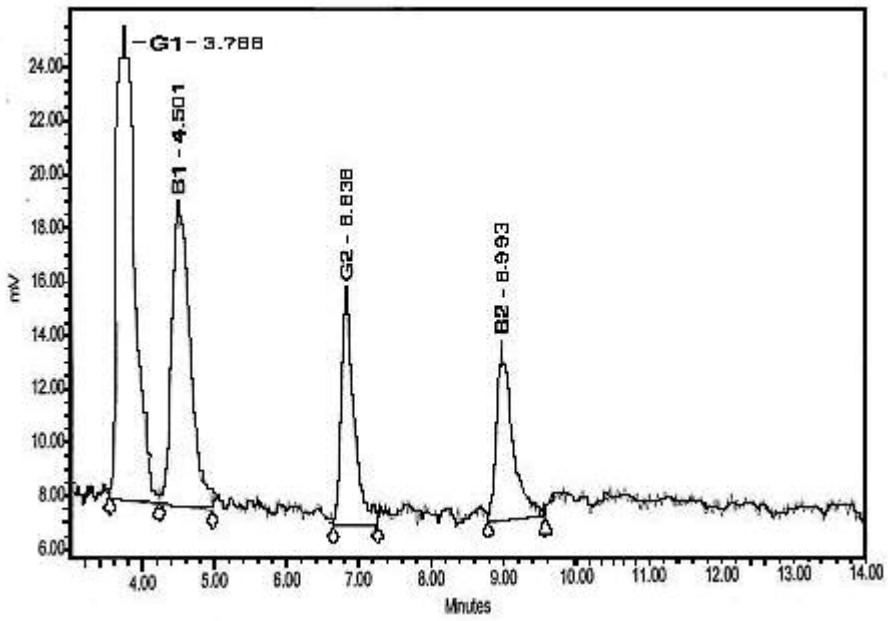


Figure 18: HPLC chromatogram of aflatoxin spiked ginseng root tissue (Spiking levels of total aflatoxins were 2 ng/g, 4 ng/g, 8 ng/g, and 16 ng/g)
Column: YMC ODS-AQ S-3 120A 4.6 x 150mm. Mobile Phase: acetonitrile: methanol: water (25+12+63)

Table 22: Aflatoxins in wild cultivated ground encapsulated Ginseng roots.[a]

Sample:	$AFLB_1$	$AFLB_2$	Total AFL	[b]Mean AFL
Wild	ng/g	ng/g	ng/g	ng/g
1	ND	ND	ND	0.3
1	ND	ND	ND	
2	ND	ND	ND	
2	0.2	ND	0.2	
3	0.2	ND	0.2	
3	0.2	ND	0.2	
4	1.5	0.4	1.9	
4	0.4	ND	0.4	
5	ND	ND	ND	
5	0.2	ND	0.2	

[a] n=2

[b]Average of 5 samples with 2 repetitions per sample; each sample is one bottle from the same lot

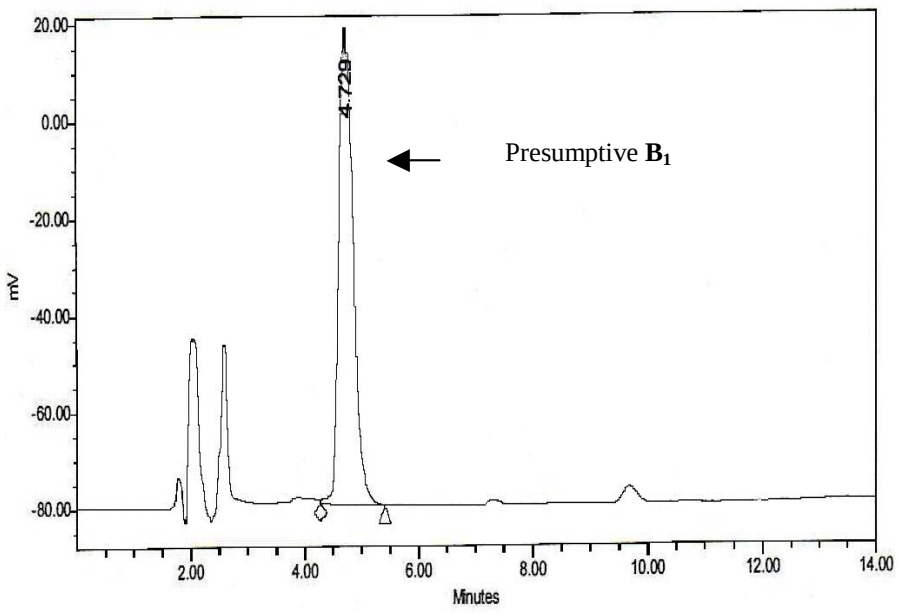


Figure 19: HPLC chromatogram of wild simulated ginseng root tissue, 5 g sample
Column: YMC ODS-AQ S-3 120A 4.6 x 150mm. Mobile Phase: acetonitrile: methanol: water (25+12+63)

The Harding 8 year old dry clean root sample and 8-year-old powered root sample were aflatoxin free, however the Harding moldy root sample had 0.49 ng/g of aflatoxins, whereas the fresh retail Mid-western root had 16.0 ng/g (Table 23, Figure 20). The Slagle wild simulated ginseng contained no aflatoxins however the Galloway wild simulated root sample contained aflatoxins in levels ranging from 5.5 ng/g to 16.9 ng/g (Table 23, Figure 21).

Of the cultivated samples, there were 9 samples from 2002 from Garrett County Md. There was no aflatoxin found at the level of detection of 0.2ng/g in the cultivated ginseng from 2002 (Figure 22). There were 3 larger samples from 2003 from three UMD field plot sites, with 3 replicates of each sample analyzed. The 2003 cultivated ginseng was completely aflatoxin free.

Table 23: Aflatoxins in fresh and wild simulated ginseng roots from Maryland and West Virginia.[a]

Sample:	AFL B_1	AFL B_2	Total AFL	[b]Mean AFL
Root	(ng/g)	(ng/g)	(ng/g)	(ng/g)
[c]1	5.5	ND	5.5	15.1
1	31.8	0.4	32.2	
1	5.3	ND	5.3	
1	16.7	0.7	17.3	
[d]2	0.3	ND	0.3	0.2
2	0.2	ND	0.2	
2	ND	ND	ND	
2	ND	ND	ND	
[e]3	14.8	0.4	15.2	15.2

[a] n=4, except fresh root of n=1

[b]Average of 4 analyses except for fresh root and moldy root, of 1 analysis

[c]Galloway 8 year old dried clean root harvested in 2002

[d]4 year old moldy root

[e]Fresh Midwestern Ginseng Root

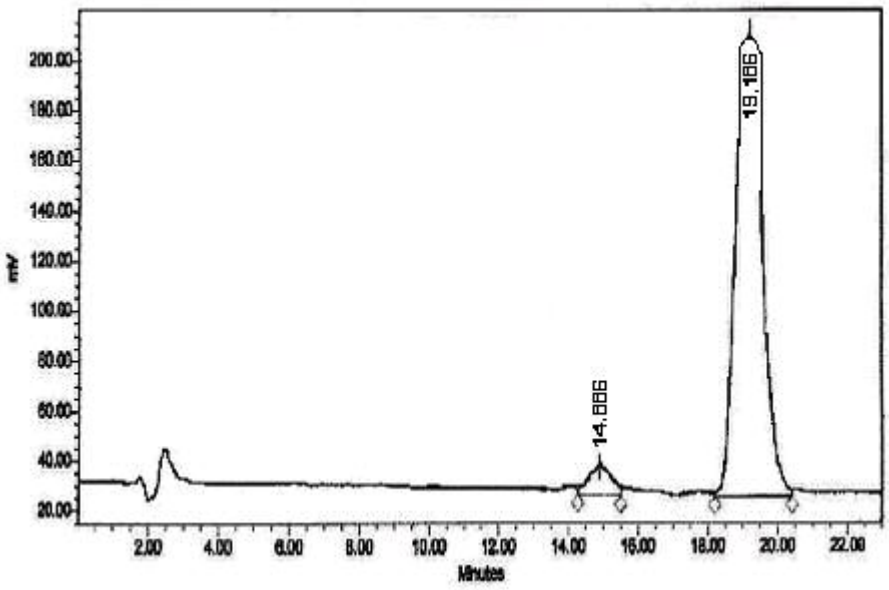


Figure 20: HPLC chromatogram of fresh retail ginseng root tissue
Column: YMC ODS-AQ S-3 120A 4.6 x 150mm. Mobile Phase: methanol: acetonitrile: water (22: 16: 62); each L contains 120 mg KBr and 350 μL 4N nitric acid.

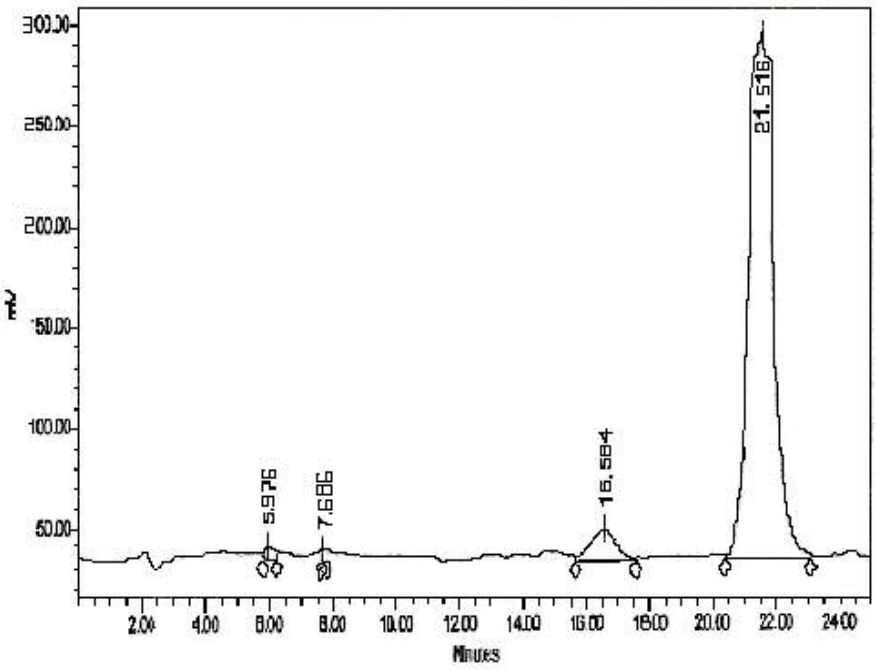


Figure 21: HPLC chromatogram of 2002 wild simulated Appalachian ginseng root tissue
Column: YMC ODS-AQ S-3 120A 4.6 x 150mm. Mobile Phase: methanol: acetonitrile: water (22: 16: 62); each L contains 120 mg KBr and 350 µL 4N nitric acid.

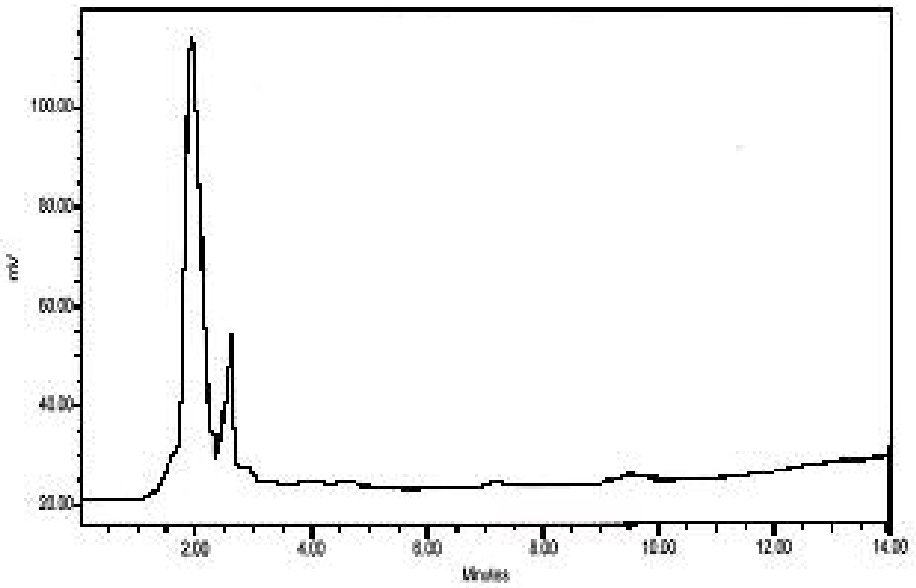


Figure 22: HPLC chromatogram of cultivated ginseng root tissue
Column: YMC ODS-AQ S-3 120A 4.6 x 150mm. Mobile Phase: methanol: acetonitrile: water (22: 16: 62); each L contains 120 mg KBr and 350 µL 4N nitric acid.

6.5 Discussion

A method for detection of aflatoxins in ginseng was developed. The method was a modification of AOAC Official Method 991.31. A mixture of 4 aflatoxins was added to toxin free ginseng. The total levels of aflatoxins added (B_1: B_2: G_1: AG_2: = 4: 1: 2: 1) were 2, 4, 8, and 16 ng/g. The official method was modified by adding a larger volume of methanol and water to the dried root tissue. The sample to solvent ratio was 1 part root tissue and 5 parts solvent. The sample solvent solution was then centrifuged for 10 minutes at 7000 RPM at 10°C. After immunoaffinity column clean up aflatoxins were eluted with acetonitrile in three, 1-mL aliquots instead of methanol to facilitate the evaporation of water in the filtrate.

In this study the recovery rate ranged from 77 to 92% of total aflatoxins. The method was shown to be repeatable with the ginseng samples during continuous toxin extraction. The method was then applied to a survey of root samples for the natural occurrence of aflatoxin in 2002/2003-ginseng root material.

Wild simulated ginseng roots from the 2002-growing season showed the highest levels of aflatoxins. This may have been due to the stress on the plant because of a lack of sufficient water during 2002 which encouraged infection by root fungi, especially toxigenic *A. flavus*. Wild simulated ginseng is not fertilized nor watered which may also increase its susceptibility to fungal infection compared to ginseng produced under cultivated conditions. Note that aflatoxins were not found in the cultivated 2002 ginseng samples. In addition, the drying process for ginseng may be responsible for fungal infection. Many growers of ginseng dry their root in forced air electric dryers (98º F) and the tissue is tumbled to insure an even dryness

throughout the root. The root is usually dried in 3-4 days. This method of "flash drying" is preferable by the grower because it prevents mold contamination. The Galloway ginseng root which was contaminated with aflatoxins was not flash dried. In this case, the clean roots are spread on screen racks in the shade. The roots were turned frequently and provided adequate aeration to facilitate drying. With this method, drying time varied with root size from 4 to 6 weeks during which time the fungi present on the root surface such as *A. flavus* could grow and release aflatoxins into the root tissue. Aflatoxins found in the Galloway root ranges from 5.5 to 31.8ppb, and given that the FDA action level for foods of 20ppb, the sample range went above the action level limit thus posing a problem area of great concern in the drying and storage of ginseng. It is important that ginseng growers be informed of this problem area so as to avoid aflatoxin accumulation above the action level of 20ppb. Therefore, proper and rapid drying and storage of ginseng root tissue after harvest is critical to mold growth and possibly mycotoxin contamination. It is known that spices are common substrates for *A. flavus*, and subsequent aflatoxin production in these foodstuffs is almost always due to poor drying, handling, or storage (Arim, 1995; Dhavan and Choudary, 1995). Aflatoxin levels are normally higher where drying and storage facilities are limited and there is high heat and humidity (Dhavan and Choudary, 1995; Lubulwa & Davis, 1994).

The wild simulated 2003 ginseng was aflatoxin free. The higher than usual rain may have prevented stress to the roots, thus protecting them against *A. flavus* growth and toxin contamination. The Midwestern ginseng that was analyzed was freshly, harvested material but sealed under plastic wrap. Thus the conditions under

which the root was harvested and stored under high moisture stimulated mold growth even during 3 days of refrigerator storage; ideal conditions for aflatoxin occurrence. This is another problem area in ginseng storage therefore growers and distributors should be made aware of the potential of *A. flavus* contamination and aflatoxin accumulation during shipping and storage, especially of fresh roots sealed in plastic.

The Harding moldy root was improperly dried and kept outside under the sun. According to the Western Maryland ginseng growers, the best quality ginseng roots are those that are dried at a specific temperature of 95-100˚F in a tumbling dryer, and then sealed and stored at a low humidity to prevent mold contaminated. Harding also stated that the poor quality ginseng root was sold to him from a native "Sanger" who may have harvested the root illegally, failed to properly dry the roots which often results in poor quality molded roots. Thus, the conditions, which could result in mycotoxin contaminated ginseng roots, are during periods of drought or poor drying and storage of fresh and dried roots. The conditions most favorable for maximum growth and aflatoxin production by *A. flavus* are temperatures greater than 30ºC, maximum relative humidity of greater than 85%, and water activity of 0.98 to 0.99 (Payne, et al, 1988). Thus, *A flavus* can infect of almost any stored product with proper moisture/temperature conditions during storage (Payne, 1992).

Due to the drought conditions of 2002, only a few ginseng roots produced shoots and leaves. The only ginseng roots recovered from the UMD sites, were those in Garret County. The roots that were analyzed contained no detectable aflatoxins. In contrast, the 2003 season was much wetter, and roots produced shoots and leaves so that all plots were harvested at the UMD sites and when analyzed were devoid of

aflatoxins. Since the cultivated roots are fertilized and watered regularly, it is possible to avoid one possible source of contamination by *A. flavus* and production of aflatoxins. In addition, the 2003 roots had been harvested, dried and stored according to proper methods, thus the conditions for fungal contamination did not occur and thus the roots were mold and toxin free. In this survey aflatoxins were found in drought year roots and improperly processed roots so it may be possible to predict the occurrence of aflatoxins especially after a drought year, or when they have been improperly dried and/or stored. This developed modified AOAC official method for detection of aflatoxins in ginseng roots might be applicable to studies of other botanical root supplements as well.

6.6 Conclusion

A method for detection of aflatoxins in ginseng was developed and applied to the study of different ginseng root samples. Aflatoxins appeared not to be a problem in the cultivated ginseng samples that were analyzed, however a number of wild simulated samples were positive for aflatoxins. The methods of growing wild simulated ginseng may produce environmental conditions especially during drought years where stress on the root may lead to *A. flavus* infection and aflatoxin accumulation. Post harvest conditions may also lead to the occurrence of aflatoxins in ginseng. Thus, proper drying and storage are necessary to avoid mold contamination, growth and aflatoxin production in ginseng root.

Bibliography

1. Aibara K, & Miyaki, K. 1970. Aflatoxin and its radiosensitivity. *In*: Panel on the Radiation Sensitivity of Toxins and Animal Poisons, Proceedings of the IAEA, Bangkok, Thailand, 41-62. International Atomic Energy Agency, Publ., Vienna

2. Al-Hilli, A.L., and Smith, J.E. 1992. Influence of propionic acid on growth and aflatoxin production by *Aspergillus flavus* in liquid submerged and solid substrate conditions, J. Environ. Pathol. Toxicol. Oncol. 11(2):121-124.

3. Allcroft, R. and Carnaghan, R.B.A. 1963. Toxic products in groundnuts-biological effects. Chem. Ind. 1963:50-53.

4. Alsberg, C.L., and Black, O.F. 1913. Contributions to the study of maize deterioration: Biochemical and toxicological investigations of *Penicillium puberulum* and *Penicillium stoloniferum*. Bull. Bur. Anim. Ind. U.S. Dept. Agric. 270:1-47.

5. Anonymous. 2004. Corn Consumption. National Corn Growers Association. http://www.ncga.com/WorldOfCorn/main/consumptionData.htm.

6. Arim, R.H. 1995. Present status of the aflatoxin situation in the Philippines. Food Addit. Contam. 12:291-296.

7. Asao, T., Buchi, G., Abdel-Kader, M.M., Chang, S.B., Wick, E.L., and Wogan, G.N. 1963. Aflatoxins B and G. J. Amer. Chem. Soc. 85:1706-1707.

8. Ashworth, L.J., McMeans, J.L., and Brown, C. M. 1969. Infection of cotton by *Aspergillus flavus*; Regulatory aspects of the S and L problem. Mycol. Soc. Amer. Newslett. 42:5.

9. Austwick, P.K.C., and Ayerst, G. 1963. Groundnut microflora and toxicity. Chem. Ind. 1963:55-61.

10. Aziz, N.H, Y.A. Youssef, M. Z. El-Fouly and Moussa, L.A. 1998. Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. Botanical Bull. Academia Sinica. 39:279-284.

11. Bacon, C.W., Hinton, D.M., and Richardson, M.D. 1994. A corn seedling assay for resistance to *Fusarium moniliforme*. Pl. Dis. 78(3):302-305.

12. Bacon, C.W. and Hinton, D.M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. Can J. Bot. 74:1195-1202.

13. Bacon, C.W., Yates, I.E., Hinton, D.M, and Filmore, M. 2001. Biological control of *Fusarium moniliforme* in maize. Environmental Health Perspectives 109 (5):325-343. Suppl. 2.

14. Baratine, H. and Tantaoui-Elaraki, A. 1997. Growth and toxigenesis of *Aspergillus flavus* isolates on selected spices. J. Environmental Pathology, Toxicology and Oncology 16(1):61-65.

15. Barry, D., Widstrom, N.W., Darrah, L.L., McMillian, W.W., Riley, T.J., Scott, G.E., and Lillehoj, E.B. 1991. Maize ear damage by insects in relation to genotype and aflatoxin contamination in pre-harvest maize grain. J. Econ. Entomol. 85(6):2492-2495.

16. Bennett, G.A. and Anderson, R.A. 1978. Distribution of aflatoxin and/or zearalenone in wet-milled corn products: A review. J. Agric. Food Chem. 26:1055-1060.

17. Bennett, G.A. and Richard, J.L. 1996. Influence of processing on *Fusarium* mycotoxins in contaminated grains. Food Tech. 50: 235-238.

18. Betz, J. 2001. Personal communication to author.

19. 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. Comm. 743-745.

20. Blount, W.P. 1961. Turkey "X" disease. J. Brit. Turkey Feed. 9:52-54.

21. Blumenthal, M. 2002. Herb sales down in mainstream market, up in natural food stores. HerbalGram 55:60-63.

22. Brown, R.L., Cotty, P.J., Cleveland, T.E., and Widstrom, N.W. 1993. Living maize embryo influences accumulation of aflatoxin in maize kernels. J. Food Protection 56(11):967-971.

23. Brown, R.L., Cleveland, T.E., Woloshuk, C.P., Payne, G.A., and Bhatnagar, D. 2001. Growth inhibition of a *Fusarium verticillioides* GUS strain in corn kernels of aflatoxin-resistant genotypes. Appl. Microbiol. Biotechnol. 57:708-711.

24. Bullerman. L.B. 1986. Mycotoxins and Food Safety. A Scientific status summary by the Institute of Food Technologist's expert panel on food safety and nutrition. Institute of Food Technologists, Chicago, IL.

25. Bullerman, L.B., 1987. Methods for detecting mycotoxins in foods and beverages. *In:* Food and Beverage Mycology, 2nd ed., ed. L.R.Beuchat, 571-598. Van Nostrand Reinhold Company Inc., Publ., New York, NY.

26. Bullerman, 2001. Fusaria and toxigenic molds other than Aspergilli and Penicillia. *In* Food Microbiology: Fundamentals and Frontiers, eds. M.P. Doyle, L.R. Beuchat, and T. J., Montville, 481-497. Amer. Soc. Micb. Press, Publ., Washington D.C.

27. Bullerman, L.B., Dojin, R., and Jackson, L.S. 2002. Stability of fumonisins in food processing. *In* Mycotoxins and Food Safety, eds. J.W. DeVries, M.W. Trucksess, and L.S. Jackson, 195-204. Kluwer Academic Plenum Publ., New York, NY.

28. Burgess, L.W., Dodman, R.L., Pont, W. and Mayers. 1991. *Fusarium* diseases of wheat, maize and grain sorghum in Eastern Australia. *In Fusarium*: Diseases Biology and Taxonomy, eds. P E. Nelson, T.A. Tousson and R.J. Cook, 64-76. Pennsylvania State U. Press, Publ., U. Park, PA.

29. Butler, T. 1902. Notes on a feeding experiment to produce leucoencephalitis in a horse, with positive results. Am. Vet. Rev. 26:748-751.

30. Campbell, K.W., and White, D.G. 1995a. Evaluation of corn genotypes for resistance to *Aspergillus* ear rot, kernel infection, and aflatoxin production. Pl. Dis. 79:1039-1045.

31. Campbell, K.W., and White, D.G. 1995b. Inheritance of resistance to *Aspergillus* ear rot and aflatoxin in corn genotypes. Phytopathol. 85:886-896.

32. CAST. 2003. Mycotoxins: Risks in Plant, Animal, and Human Systems. *In:* CAST Report 139, 4-29. CAST, Publ., Ames, IA.

33. Castegnaro, M., and Wild, C.P. 1995. IARC activities in mycotoxin research. Natural Toxins 3:327-331

34. Castro Gamero, E. 1995. Investigative Study. Irradiation of cat's claw (*Unicaria tormentosa*) in Peru. (personal correspondence).

35. Chan, T.Y.K. 1997. Monitoring the safety of herbal medicines. Drug Safety 17(4):209-215.

36. Chelkowski, J. 1989a. Formation of mycotoxins produced by fusaria in heads of wheat triticale and rye. *In: Fusarium.* Mycotoxins, Taxonomy and Pathogenicity, ed. J. Chelkowski, 63-84. Elsevier Science Publ. Company, New York, NY.

37. Chelkowski, J. 1989b. Mycotoxins associated with corncob fusariosis. *In: Fusarium.* Mycotoxins, Taxonomy and Pathogenicity, ed. J. Chelkowski, 53-62. Elsevier Science Publ. Company, New York, NY.

38. Chen, Z-Y., Brown, R.L., Lax, A.R., Guo, B.Z., Cleveland, T.E., and Russin, J.S. 1998. Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. Phytopathol. 88:276-281.

39. Chen, Z.-Y., Brown, R.L., Lax, A.R., Guo, B.Z., Cleveland, T.E., and Russin, J.S. 1999. Inhibition of plant-pathogenic fungi by a corn trypsin inhibitor over expressed in *Escherichia coli*. Appl. Environ. Microbiol. 65(3):1320-1324.

40. Chen, Z-Y., Brown, R.L., Cleveland, T.E., Damann, K.E., and Russin, J.S. 2001. Comparison of Constitutive and Inducible Maize Kernel Proteins of Genotypes Resistant or Susceptible to Aflatoxin Production. J. Food Protect. 64(11): 1785-1792.

41. Chen, Z-Y., Brown, R.L., Damann, K.E., and Cleveland, T.E. 2002. Identification of unique or elevated levels of kernel proteins in aflatoxin-resistant maize genotypes through proteome analysis. Phytopathol. 92:1084-1094.

42. Chourasia, H.K., and R.K. Sinha. 1994. Potential of the biological control of aflatoxin contamination in developing peanut (*Arachis hypogaea* L.) by atoxigenic strains of *Aspergillus flavus*. J. Food Sci. Technol. Mysore 31:362-366.

43. Christensen, C.M. 1974. Storage of Cereal Grains and Their Products, 2nd Ed. American Assoc. of Cereal Chemists, Publ., St. Paul, MN.

44. Christensen, C.M., Mirocha, C.I., and Meronuck, R.A. 1977. Mold, mycotoxin and mycotoxicosis. *In:* Agriculture Exp. Sta. Rpt. 142, Publ., St. Paul, U. of Min.

45. Chu, F.S., Chang, C.C., Ashoor, S.H., and Prentice, N. 1975. Stability of aflatoxin B1 and ochratoxin A in brewing. Appl. Microbiol. 29(3):313-316.

46. Clarkm T.J., Foster, J.E., Kamble, S.T., and Heinrichs, E.A. 1999. Comparison of Bt (*Bacillus thuringiensis* Berliner) Maize and Conventional Measures for Control of the European Corn Borer (Lepidoptera;Crambidae). J. Entomol. Sci. 35(2):118-128.

47. Clements, M.J., Campbell, K.W., Pilcher, C., Headrick, J.M., Pataky, J.K., and White, D.G. 2003. Influence of cry1Ab protein and hybrid genotype on Fumonisin contamination and Fusarium ear rot of corn. Crop Sci. 43: 1283-1293.

48. Clements, M.J., Maragos, C.M., Pataky, J.K., and White, D.G. 2004. Sources or Resistance to Fumonisin Accumulation in Grain and Fusarium Ear and Kernel Rot of Corn. Phytopathol. 94(3):251-260.

49. Cole, R.J., and R.H. Cox. 1981. Handbook of toxic fungal metabolites. Academic Press, Publ., New York, N.Y.

50. Constable, P.D., Smith, G.W., Rottinghaus, G.E. & Haschek, W.M. (2000) Ingestion of fumonisin B1-containing culture material decreases cardiac contractility and mechanical efficiency in swine. Toxicol. Appl. Pharmacol. 162: 151–160.

51. Cotton, T.K. and Munkvold, G.P. 1998. Survival and seed transmission of *Fusarium moniliforme, Fusarium proliferatum* and *Fusarium subglutinans* in maize stalk residue. Phytopathol. 88:550-555.

52. Cotty, P.J. 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on aflatoxin content of cottonseed. Phytopathol. 84:1270-1277.

53. Cotty, P.J., P. Bayman, D.S. Engle, and K.S. Elias. 1994. Agriculture, aflatoxins and *Aspergillus*. *In:* The Genus *Aspergillus:* From Taxonomy and Genetics to Industrial Application, eds. K.A. Powell, A. Renwick, and J.F. Peberdy, 1-27. Plenum Press, Publ., New York, NY.

54. Coulombe, R.A. Jr., 1993. Symposium: biological action of mycotoxins. J. Dairy Sci. 76:880-891.

55. Court, W.E. 2000. Ginseng: The Genus Panax. Harwood Academic Publ., Singapore.

56. Chu, F.S., and Li, G.Y., 1994. Simultaneous occurrence of fumonisin B_1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. Appl. Envir. Microbiol. 60:847-852

57. Cuero, R.G., Smith, J.E., and Lacey, J. 1986. The influence of gamma irradiation and sodium hypochlorite sterilization on maize seed microflora and germination. Food Microbiol. 3:107-113.

58. Daigle, D.J., and P.J. Cotty. 1995. Formulating atoxigenic *Aspergillus flavus* for field release. Biocontrol Sci. Tech. 5:175-184.

59. Davis, N.D., Iyer, S.K., and Diener, U.L. 1987. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53:(7): 1593-1595.

60. Dawlatana, M., Coker, R., Nagler, M.J., Blunden, G. 1995. A normal phase HPTLC method for the quantitative determination of fumonisin B_1 in rice. Chromatographia. 41(3/4):187-190.

61. De la Riva, G.A., Gonzáles-Cabrera, J., Vázquez-Padrón, R., and Ayra-Pardo, C. 1998. *Agrobacterium tumefaciens*: a natural tool for plant transformation. Mol. Biol. Gen. 1(3):118-133.

62. Desjardins, A.E., Plattner, R.D., Lu, M. and Claflin, L.W. 1998. Distribution of fumonisins in maize ears infected with strains of *Fusarium moniliforme* that differ in fumonisin production. Pl. Dis. 82:953-958.

63. Dhavan, A.S. and M. R. Choudary. 1995. Incidence of aflatoxins in animal feedstuff: a decade's scenario in India. JAOAC Int. 78:693-698.

64. Dickens, W.J., and Whitaker, T.B. 1975. Efficacy of electronic color sorting and hand picking to remove aflatoxin contaminated kernels from commercial lots of shelled peanuts. Peanut Sci. 2:45-50.

65. Dively, G. P. 1999. Efficacy of Bt11 based single and stacked gene events of transgenic field corn against key lepidopteran pests and associated *Fusarium* infection. Unpublished data, G.P. Dively, U. of Maryland 1999 Report.

66. Doko, M.B. and Visconte, A. 1994. Occurrence of fumonisin B_1 and B_2 in corn and corn-based foodstuffs in Italy. Food Addit. Contam. 11:433-439.

67. Dollear, F.G., R.J. Cole and Wicklow, D.W. 1999. Aflatoxin reduction in corn through field application of competitive fungi. J. Food Prot. 62:650-656.

68. Dowd, P.F. 2000. Indirect reduction of ear molds and associated mycotoxins in Bacillus thuringiensis corn under controlled and open field conditions: utility and limitations. J. Econ. Entomol. 93(6):1669-1679.

69. Dowd, P.F. 2001. Biotic and abiotic factors limiting efficacy of Bt corn in indirectly reducing mycotoxin levels in commercial fields. J. Econ. Entomol. 94(5): 1067-1074.

70. Dowd, P.F. and White, D.G. 2002. Corn Earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae) and other insect associated resistance in maize inbred Tex6. J. Econ. Entom. 95(3):628-34.

71. Duke, J.A. 1989. Ginseng: A Concise Handbook. Research Publications, Inc. Algonac, MI

72. Efuntoye, M.O. (1999) Mycotoxins of fungal strains from stored herbal plants and mycotoxin contents of Nigerian crude herbal drugs, Mycopathol. 147(1):43-48.

73. Farag, R.S., Rashed, M.M., Hussein, A. A., and Abo-Hager, A. 1995. Effect of gamma irradiation on the infected yellow corn and peanuts by *Aspergillus flavus*. Chem. Mikrobiol. Tech. Der Lebensmitten 17(3/4):93-98.

74. Farag, R.S., Rashed, M.M., and Abo-Hager, A. 1996. Aflatoxin destruction by microwave heating. Intern. J. Food Sciences Nutr. 47:197-208.

75. Feuell, A.J. 1966. Aflatoxin in groundnuts. IV. Problems of detoxification, Trop. Sci. 8:61.

76. Flannigan, B and Llewellyn, G.C. 1992. The microbiology of spices: A review. *In:* International Biodeter. and Biodegrad. 29(2):111-121. Elsevier Science, Limited. Oxford, U.K

77. Flannigan B., and A.R. Pearce. 1994. Aspergillus spoilage: Spoilage of cereals and cereal products by the hazardous species *A. clavatus*. *In*: The Genus Aspergillus: From Taxonomy and Genetics to Industrial Application, eds. K.A. Powell, A. Renwick, and J.F. Peberdy, 115-127. Plenum Press, Publ., New York, NY.

78. Food and Drug Administration Industry Activities Staff Booklet. 2000. http://www.cfsan.fda.gov/~lrd/fdaact.htmL.

79. Food and Drug Administration. 2004. FDA Announces Major Initiatives for Dietary Supplements. http://www.fda.gov/bbs/topics/news/2004/NEW01130.htmL.

80. Foley, D.C. 1962. Systematic infection of corn by *Fusarium moniliforme*. Phytopathol. 52:870-872.

81. Frank, H.K., and Grunewald, T., 1970. Radiation resistance of aflatoxins. Food Irradiat. 11:15.

82. Frank, H.K., Munzner, R., and Diehl, J.F. 1971. Response of toxigenic and non-toxigenic strains of *Aspergillus flavus* to irradiation. Saboraudia. 9:21-26.

83. Gams, W., M. Christensen, A.H.S. Onions, J.I. Pitt, and Samson, R.A. 1985. *Intrageneric taxa of Aspergillus*. *In*: Advances in *Penicillium* and *Aspergillus* Systematics, eds, R.A. Samson, and J.I. Pitt, 55-62. Plenum Press, Publ., New York, NY.

84. Gatch, E.W. and Munkvold, G.P. 2002. Fungal species composition in maize stalks in relation to European corn borer injury and transgenic insect protection. Pl. Dis. 86(10):1156-1162.

85. Gelderblom, W.C. A., Jaskiewicz, K., Marasas, W.F.O., Theil, P.G., Horak, R.M., Vleggaar, R., and Kriek, N.P. 1988. Fumonisins: novel mycotoxins with cancer- promoting activity produced by *Fusarium moniliforme*. Appl. Envir. Microbiol. 54:1806-1811.

86. Gelderblom, W.C. A., Marasas, W.F.O., Vleggaar, R., Theil, P.G., and Cawood, M.W. 1992. Fumonisins: isolation, chemical characterization and biological effects. Mycopathol. 117:11-16.

87. Groves, F.D., Zhang, L., Chang, Y.S., Ross, P.F., Casper, H., Norred, W.P., You, W.C., Fraumeni, J.F. Jr. 1999. Fusarium mycotoxins in corn and corn products in a high-risk area for gastric cancer in Shandong Province, China. J. AOAC International. 82 (3): 657-662.

88. Guo, B.Z., Russin, J.S., Cleveland, T.E., Brown, R.L., and Widstrom, N.W. 1994. Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. J. Food Prot. 58(3):296-300.

89. Guo, B.Z., Chen, Z.-Y., Brown, R.L., Lax, A.R., Cleveland, T.E., Russin, J.S., Mehta, A.D., Selitrennikoff, C.P., and Widstrom, N.W. 1997. Germination induced accumulation of specific proteins and antifungal activities in corn kernels. Phytopathol. 87(11):1174-1178.

90. Halt, M. 1998). Moulds and mycotoxins in herb tea and medicinal plants, European J. of Epidemiol. 13(3):269-274.

91. Hammond, B. G., Campbell, K. W., Pilcher, C. D., Degooyer, T. A., Robinson, A. E., McMillen, B. L., Spangler, S. M., Riordan, S. G., Rice, L. G. and Richard, J. L. 2004. Lower Fumonisin Mycotoxin Levels in the Grain of Bt Corn Grown in the United States in 2000-2002. J. Agricul. Food Chem. 52:1390 - 1397

92. Harris Poll. 2001. Exploring Consumer Attitudes About Dietary Supplement. Barometer Survey, An Executive Summary. [press release]. Dietary Supplement Education Alliance, Harris Poll pp4.

93. Headrick, J.M., and Pataky, J.K. 1991. Maternal Influence on the resistance of sweet corn lines to kernel infection by *Fusarium moniliforme*. Phytopathol. 81:268-274.

94. Henry K.and Lovell, R. 2001. Fumonisin Levels in Human Foods and Animal Feeds, Final guidance. Center for Food Safety and Applied Nutrition (CFSAN). http://www.cfsan.fda.gov/~dms/fumongu2.htmL.

95. Herbalgram. 2001. Ginseng Evaluation Program. American Botanical Council. http://www.herbalgram.org/default.asp?c=ginseng_eval.

96. Hocking, A.D., 2001. Toxigenic *Aspergillus* Species. *In*: Food Microbiology: Fundamentals and Frontiers, eds. M.P. Doyle, L. R. Beuchat and T. J. Montville, 451-465. Amer. Soc. Micb. Press, Publ., Washington D.C.

97. Hooshand, H., Klopfenstein, C.F. 1995. Effects of gamma irradiation on mycotoxin disappearance and amino acid contents of corn, wheat and soybean, with different moisture contents. Plant Food Human Nutr. 47:337.

98. Horie, Y., Yamazaki, M., and Mitake, S. 1982. Toxigenic fungi contaminating herbal drugs. Trans. Mycol. Soc. of Japan 23(4):435-447.

99. Howard, P. C., Eppley, R. M., Stack, M. E., Warbritton, A., Voss, K. A., Kovach, R. M., Lorentzen, R. J. and Bucci, T. J. 2001. Fumonisin B_1 carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. Environ Health Perspect 109 Suppl 2 277-82

100. Huang, Z., White, D.G., and Payne, G.A. 1997. Corn seed proteins inhibitory to *Aspergillus flavus* and aflatoxin biosynthesis. Phytopathol. 87:622-627.

101. The International Agency for Research on Cancer (IARC). 1993a. IARC Monographs on the evaluation of carcinogenic risks to humans: heterocyclic amines and mycotoxins, 56, IARC Lyon, France.

102. The International Agency for Research on Cancer (IARC). 1993b. Toxins derived from *Fusarium moniliforme* fumonisins B_1 and B_2, and fusarian C. IARC Monogr. Eval. Carcinog. Risk Hum 56:445-466.

103. JECFA 1999. Evaluation of certain food additives and contaminants. Aflatoxins and recommendations. WHO Technical Report Series, 884, 69-76, 80.

104. Kane, A., Diop, N.B.A., and Diack, T.S. 1996. Removal of aflatoxin from crude peanut oil. IX Int. IUPAC Symposium on Mycotoxins and Phycotoxins, Rome, Italy. 179 (Abst).

105. Kedera, C.J., Leslie, J.F. and Claflin, L.E. 1994. Genetic diversity of *Fusarium* section Liseola (*Giberella fujikuroi*) in individual maize stalks. Phytopathol. 84:603-607.

106. Kendra, D. F. 2002. Personal communication with the author.

107. Klich, M.A., Tiffany, L.H., and Knaphus, G. 1994. Ecology of the Aspergilli of soils and litter. *In*: *Aspergillus* Biology and Industrial Applications, eds. M.A. Klich, and J.W. Bennett, 329-353. Butterworth-Heineman, Publ., Boston.

108. Kozakiewicz, Z. 1994. *Aspergillus*. *In*: Food Borne Disease Handbook, Vol. 2, eds. Y.H. Hui., J.R. Gorham, K.D. Murrell and D.O. Cliver, 575-616. Marcel Dekker, Publ., New York, NY.

109. Lacey, J. 1989. Pre- and post-harvest ecology of fungi causing spoilage of foods and other stored products. J. Appl. Bacter. Symp. Suppl. 1989, 11S-25S.

110. Lashof, J. C., Margen S. and Swartberg, J.E. 2002. Regulating Natural Health Products. Sci. 2 96(5565):46-47.

111. Lebars, J., Lebars, P., Dupey, J., Boudra, H. and Cassini R. 1994. Biotic and abiotic factors in fumonisin B_1 production and stability. JAOAC Int. 77:517.

112. Lew, H., Adler, A. and Edinger, W. 1991. Moniliformin and the European corn borer (*Ostrinia nubilalis*). Mycotox. Res. 7:77-76.

113. Lillehoj, E.B., Kwolek, W.F., Guthrie, W.D., Barry, D., McMillian, W.W., and Widstrom, N.W. 1982. Aflatoxin accumulation in pre-harvest maize (*Zea mays* L.) kernels, interaction of 3 fungal species European corn borer *Ostrinia nubilalis* and 2 hybrids. Pl. Soil 65:95-102.

114. Lubulwa, A.S.G., and J.S. Davis. 1994. Estimating the social costs of the impacts of fungi and aflatoxins. *In:* Stored Product Protection. Proceedings of the 6th International Working Conference on Stored-Product Protection, eds. E. Highley, E. J. Wright, H. J. Banks and B.R. Champ, 1017-1042. CAB International, Wallingford, Publ., Oxford, United Kingdom.

115. Luter, L., Wyslouzil, W. and Kashyap, S.C. 1982. The destruction of aflatoxins in peanuts by microwave roasting. Can. Inst. Food Sci. Technol. J. 15:236-238.

116. MacDonald, S. and Castle, L. 1996. A UK retail survey of aflatoxins in herbs and spices and their fate during cooking. Food Addit. Contam. 13(1):121-128.

117. Marasas, W.F.O., Kriek, N.P.J., Fincham, J.E. and van Rensburg, S.J. 1984a. Primary liver cancer and oesophageal basal cell hyperplasia in rats caused by *Fusarium moniliforme*. Int. J. Cancer 34:383-387.

118. Marasas, W.F.O., Nelson, P.E., and Tousson, T.A. 1984b. Toxigenic *Fusarium* Species. Pennsylvania State U. Press. University Park, PA.

119. Marasas, W.F.O. 1995. Fumonisins: Their implications for human and animal health. Natural Toxins, 3:193-198.

120. Marasas, W.F.O. 2003. Personal communication with author.

121. Marín, S., Sanchis, V., Arnau, F., Ramos, A.J., and Magan, N. 1998. Colonization and competitiveness of Aspergillus and Penicillium species on maize grain in the presence of *Fusarium moniliforme* and *Fusarium proliferatum*. Intl. J. of Food Microbiol. 45:107-117.

122. Marín, S., Sanchis, V., Arnau, F., Ramos, A.J., Vinas, I., and Magan, N. 1998. Environmental factors, in vitro interactions, and niche overlap between *Fusarium moniliforme*, *F. proliferatum*, and *F. graminearum*, Aspergillus and Penicillium species from maize grain. Mycol. Res. 102(7):831-837.

123. Marsh, S.F. and Payne, G.A. 1984. Preharvest infection of corn silks and kernels by *Aspergillus flavus*. Phytopathol. 74: 1284.

124. Maryland State Climatologist Office. 2004. University of Maryland, College Park. http://www.atmos.umd.edu/~climate/beltsville.htmL.

125. Maupin, L.M., Clements, M.J., and White, D.G. 2003. Evaluation of the MI82 corn lines as a source of resistance to aflatoxin accumulation in grain and use of BGYF as a selection tool. Pl. Dis. 87:1059-1066.

126. McMillian, W.W. 1983. Role of anthropods in field contamination. *In* Aflatoxin and *Aspergillus flavus* in corn, eds. U. L. Diener, R.L. Asquith and J.W. Dickens, 20-22. Alabama Agr. Exp. Sta., Auburn U., Auburn.

127. Miller, J.D. 1995. Fungi and mycotoxins in stored grains: implications for stored product research. J. Stored Prod. Res., 31: 1-16.

128. Miller, J.D. 2001. Factors that affect the occurrence of Fumonisin. Environmental Health Perspectives. Suppl. 2. 109: 321-325.

129. Mirocha, C.J., and Christensen, C.M. 1982. Mycotoxins, Fungi, cereal grains, foodstuffs, feed. *In:* Storage of cereal grains and their products, 3rd ed., ed. C. M. Christensen, 241-280. American Association of Cereal Chemists, Publ., St. Paul, MN.

130. Miyake, I., H. Naito, and H. Sumeda. 1940. Replicate. Res. Inst. Rice Improvement 1:1. (In Japanese).

131. Munkvold, G.P., Hellmich, R.L., and Rice, L.G. 1999. Comparison of Fumonisin Concentrations in Kernels of Transgenic Bt maize hybrids and nontransgenic hybrids. Pl. Dis. 83: (2): 130-138.

132. Munkvold, G.P. and Hellmich, R.L. 2000. Genetically modified insect resistant maize: implications for management of ear and stalk diseases. Pl. Health Progress. Plant Health Reviews - Accession On-line DOI:10.1094/PHP-2000-0912-01-RV

133. Munkvold, G.P., and Desjardins, A.E. 1997. Fumonisins in Maize. Can we stop their occurrence? Pl. Dis. 81(6):557-565.

134. Murano, P.S. 1995. Quality of Irradiated foods. *In:* Food Irradiation: A source book, ed. E.A. Murano, 67. Iowa State U. Press, Publ., Ames, IO.

135. Murphy, P. A., Rice, L.G., and Ross, P.F. 1993. Fumonisin B_1 and B_2 and B_3 content of Iowa, Wisconsin and Illinois corn and corn screenings. J. Food Chem. 41:263-266.

136. Musser, S.M., Eppley, R.M., and Trucksess, M.W. 2002. Electrospray mass spectrometry for fumonisin detection and method validation. *In*: Mycotoxins and Food Safety, eds. J.W. DeVries, M.W. Trucksess and L.S. Jackson, 95-116. Kluwer Academic/Plenum Publ., New York, NY.

137. Naidoo, G., Forbes, A.M., Paul, C., White, D.G., and Rocheford, T.R. 2002. Resistance to Aspergillus ear rot and aflatoxin accumulation in maize F_1 hybrids. Crop Sci. 42:360-364.

138. Norred, W.P., Voss, K.A., Bacon, C.W., and Riley, R.T. 1991. Effectiveness of ammonia treatment in detoxification of fumonisin-contaminated corn. Food Chem. Toxicol. 29:815-819.

139. Ominski, K.H., Marquardt, R.R., Sinha, R.N. and Abramson, D. 1994. Ecological aspects of growth and mycotoxin production by storage fungi. *In:* Mycotoxins in Grains: Compounds other than Aflatoxin, eds. J.D. Miller and H.L. Trentholm, 287-312. Eagen Press Publ., St. Paul. MN.

140. Park, D.L., Lopez-Garcia, R., Trujillo-Preciado, S., Price, R.L. 1996. Reduction of risks associated with fumonisin contamination in corn. *In*: Fumonisin in Food, eds. L.S. Jackson, J.W. Devries and L.B. Bullerman, 335-344. Plenum Press Publ., New York, NY.

141. Pascale, M., Visconte, A., Proñczuk, M., Wiśniewska, H., and Chelkowski, J. 1997. Accumulation of fumonisins in maize hybrids inoculated under field conditions with *Fusarium moniliforme*, Sheldon. J. Food Agric. 74:1-6.

142. Patel, S., Hazel, C.M., Winterton, A.G.M., and Mortby, E. 1996. Survey of ethnic foods for mycotoxins. Food Addit. Contamin. 13:833-841.

143. Payne, G.A. 1992. Aflatoxin in maize. Crit. Rev. Plant Sci. 10:423-440.

144. Payne, G.A., Thompson, D.L., Lillehoj, E.B., Zuber, M.S., and Adkins, C.R. 1988. Effect of temperature on the preharvest infection of maize kernels by *Aspergillus flavus*. Phytopathol. 78: 1376-1380.

145. Peers, F G. 1975. Aflatoxin contamination and its heat stability in Indian peanut cooking oils. Trop Sci. 17(4):229-232.

146. Persons, .W. S. 1994. American Ginseng: Green Gold. Bright Mountain Books, Inc. Asheville, NC.

147. Phillips, T.D., Bashir Sarr, A, and Grant, P.G., 1995. Selective Chemisorption and Detoxification of Aflatoxins by Phyllosilicate Clay. Natural Toxins 3: 204-213.

148. Picco, M. Nesci, A., Barros, G., Cavaglieri, L., and Etcheverry, M. 1999. Aflatoxin B_1 and fumonisin B_1 in mixed cultures of *Aspergillus flavus* and *Fusarium proliferatum* on maize. Natural Toxins. 7:331-336.

149. Pilcher, C.D., Rice, M.E., Obrycki, J.J., and Lewis, L.C. 1997. Field and laboratory evaluations of transgenic Bacillus thuringiensis corn on secondary Lepidopteran pests (Lepidoptera: Noctuidae). J. Econ. Entomol. 90:669-678.

150. Pittet, A. 2002. Natural occurrence of mycotoxins in foods and feeds: a decade in review. *In:* Mycotoxins and Phytotoxins in Perspective at the Turn of the Millennium, eds. W.J. de Koe, R.A. Samspm, H.P.van Egmond, J. Gilbert and M. Sabino, 153-172. Ponsen & Looyen, Wageningen Publ., the Netherlands.

151. Powell. D., 1999. Backgrounder: genetically engineered Bt-containing field corn. Dept. of plant agriculture of Guelph, Ontario. Technical Report 11.

152. Reddy, S.V., Mayi, D.K., Reddy, M.U., Thirumala-Devi, K. and Reddy, D.V.R. 2001. Aflatoxin B1 in different grades of chillies (Capsicum annum L.) in India as determined by indirect competitive-ELISA. Food Addit. Contamin. 18: 553–558.

153. Rice, M.E., and Pilcher, C.D. 1997. Perceptions and performance of Bt corn. *In*: Proc. 52nd annual Corn & Sorghum Research Conf., p.144-156, Chicago, IL.

154. Rice, M.E. and Pilcher, C.D. 1998. Potential benefits and limitations of transgenic Bt corn for management of the European corn borer (Lepidoptera: Crimbidae) Am. Entomol. 75-78

155. Richard, J.L, Bennett, G.A., Ross, P.F., and Nelson, P.E. 1993. Analysis of naturally occurring mycotoxins in feedstuffs and foods. J. Anim. Sci. 71:2563-2574.

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

157. Romer Labs. 2003. Aflatoxin Standard Method # afl-1c-01-00.1. http://www.romerlabs.com.

158. Russell, J.A., Roy, M.K, and Sanford, J.C. 1992. Major Improvements in Biolistic Transformation of Suspension-cultured Tobacco cells. In Vitro Cell. Dev. Biol. 28P: 97-105.

159. Russell, G.H., and Berjak, P. 1983. Some attempted control measures against *Fusarium verticillioides* in stored maize seeds. Seed Sci. & Technol, 11:441-448.

160. Santamarina, F.J., Gimenez, S.J., Sabater, C., Sanchis, V. 1995. Measure to reduce and eliminate mycotoxins in food and feeds. Revista Iberoamericana de Micologia. 12:52-59.

161. Sargent, K., Carnaghan, R.B.A., and Allcroft, R. 1963. Toxic products in groundnuts-chemistry and origin. Chem. Ind. 1963:53-55.

162. Schwarte, L.H., Biester, H.E., and Murray, C. 1937. A disease of horses caused by feeding moldy corn. J. Am. Vet. Assoc. 90:76-85

163. Scott, P.M., and Kennedy, B.P.C. 1975. The Analysis of spices and herbs for aflatoxins. Can. Instit. Food Sci. and Tech. J. 8(2):124-125.

164. Scott, P.M. 1993. Fumonisins: mini review. Int. J. Food Microbiol. 18(4):257-270.

165. Scott, P.M. 1994. Penicillium and Aspergillus Toxins. *In:* Mycotoxins in Grain. Compounds other than aflatoxin, eds. J.D. Miller and H.L. Trenholm, 261-285. Eagan Press Publ., St. Paul MN.

166. Scott, P.M. and Trucksess, M.W. 1997. Application of immunoaffinity columns to mycotoxin analysis. JAOAC Int. 80:941-949.

167. Seenappa, M., & Kempton, A.G. 1980. Application of a multicolumn detection method for screening spices for aflatoxin. J. Environm. Sci. Health 15:219-231.

168. Sharma, A. and Nair, P.M. 1994. Food Irradiation. Encycl. Agric. Sci. 2:293.

169. Shelby, R.A., White, D.G., and Burke, E.M. 1994. Differential fumonisin production in maize hybrids. Pl. Dis. 78:582-584.

170. Shephard, G.S., Theil, P.G., Stockenström, S., and Sydenham, E.W. 1996. Worldwide survey of fumonisin contamination of corn and corn-based products. JAOAC Int. 79:671-687.

171. Sinha, K.K. 1993. Mycotoxins. Association of Southeast Asian Nations Food Journal (ASEAN) 8:87-93.

172. Sloderbeck, P., Higgins, R.A., Buschman, L., and Bowling, R., 1995. Corn borer management using *Bt* corn. Cooperative Ext. Serv., Kansas State U., Manhattan. 129-131.

173. Smart, M.G., Wicklow, D.T., and Caldwell, R.W. 1990. Pathogenesis in Aspergillus ear rot of maize. Light microscopy of fungal spread from worms. Phytopathol. 80:1287-1294

174. Smith, G.W., Constable, P.D., Tumbleson, M.E., Rottinghaus, G.E. & Haschek, W.M. 1999. Sequence of cardiovascular changes leading to pulmonary edema in swine fed fumonisin-containing culture material. Am. J. Vet. Res., 60: 1292–1300.

175. Smith, G.W., Constable, P.D., Eppley, R.M., Tumbleson, M.E., Gumprecht, L.A. & Haschek-Hock, W.M. 2000. Purified fumonisin B1 decreases cardiovascular function but does not alter pulmonary capillary permeability in swine. Toxicol. Sci., 56:240–249.

176. Smith, J.E. 1997. Aflatoxins. *In:* Handbook of Plant and Fungal Toxicants, ed. J.P. Felix D'Mello, 269-285. CRC Press Inc. Publ., Boca Raton, FL.

177. Stack, M.E. 1997. Analysis for fumonisin B_1 in tortillas and tortilla chips using immunoaffinity column and liquid chromatography. AOAC Int. Ann. Mtg., San Diego, CA. (Abstr)

178. Stephenson, L.W., and Russell, T.E. 1974. The association of *Aspergillus flavus* with hemipterous and other insects infesting cotton bracts and foliage. Phytopathol. 64:1502.

179. Steyn, P.S., Thiel, P.G., and Trinder, D.W. 1991. Detection and quantification of mycotoxins by chemical analysis. *In:* Mycotoxins in animal foods, eds. J.E. Smith, and R.S. Hendershot, 165-221. CRC Press Publ., Inc. Boca Raton, FL.

180. Sydenham, E.C., Shephard, G.S., Thiel, P.G., Marasas, W.F.O., and Strokenström, S. 1991. Fumonisin contamination of commercial corn-based human foodstuffs. J. Agric. Food Chem. 39:2014-2018.

181. Theil, P.G., Shephard, G.S, Sydenham, E.W, Marasas, W.F.O., Nelson, P.E., and Wilson, T.M. 1991. Levels of fumonisins B_1 and B_2 in feed associated with confirmed cases of equine leukoencephalomalacia. J. Agric. Food Chem. 39:109-111.

182. Theil, P.G., Marasas, W.F.O., Sydenham, E.W., Shephard, G.S., and Gelderblom, W.C. A., 1992. The implications of naturally occurring levels of fumonisins in corn for human and animal health. Mycopathol. 117:3-9.

183. Trenholm, H.L., Prelusky, D.B., Young, J.C., and Miller, J.D. 1989. A practical guide to prevention of Fusarium mycotoxins in grains and animal feedstuffs. Arch. Environ. Toxicol. 18:443-451.

184. Trofa, S and Bean G.A. 1985. The level of toxigenic fungi in 1985 Maryland corn before and during storage. *In:* Biodeterioration Research 1, ed. G.C. Llewellyn and C. E. O'Rear, 119-126. Plenum Press Publ, New York NY.

185. Troxell, T.C. 1996. Regulatory aspects of fumonisins in the United States. *In:* Fumonisins in Food, eds. L.S. Jackson, J. W. Devries and L.B. Bullerman, 355-362. Plenum Press Publ., New York, NY.

186. Trucksess, M.W., Stack, M.E., Nesheim, S., Page, S.W., Albert, R.H. 1991. Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivatization for determination of aflatoxins in corn, peanuts, and peanut butter: collaborative study, JAOAC. 74:81-88.

187. Trucksess, M.W. 1998. Mycotoxins. JAOAC Int. 81:128-137.

188. Trucksess, M.W. 2000a. Natural Toxins. *In*: Official Methods of Analysis of AOAC International, ed. W. Horwitz, 17th edition. (49):1-64. AOAC Intl., Gaithersburg, MD.

189. Trucksess, M. W. 2000b. Rapid analysis (thin layer chromatographic and immunochemical methods) for mycotoxins in foods and feeds. IUPAC Conference Proceeding, Guaruja, SP, Brazil.

190. Trucksess, M.W. 2003. General Referee Replicateorts: Committee on Natural Toxins and Food Allergens: Mycotoxins. JAOAC Intl. 86 (1):1-10.

191. Tuite, J., Shaner, G., Rambo, G., Foster, J. and Caldwell, R.W. 1974. The Giberella ear rot epidemics of corn in Indiana in 1965 and 1972. Cereal Sci. Today. 19: 238-240.

192. Turner, W.B. 1978. Fungal Metabolites. Academic Press, London, UK.

193. Turner, W.B. and Alderidge, D.C. 1983. Fungal Metabolites II. Academic Press, London, UK.

194. Ueno, Y., Aoyama, S., Sugiura, Y, Wang, D.S., Lee, U.S., Hirooka, E.Y., Hara, S., Karki, T., Chen, G., and Yu, S.Z. 1993. A limited survey of fumonisins in corn and corn-based products in Asian countries. Mycotoxin Research. 9:27-34.

195. Urbain, W.M. 1986. Food Irradiation. Academic Press. Orlando, FL.

196. Van Egmond, H.P. 1991. Regulatory aspects of mycotoxins in Asia and Africa. *In:* Fungi and Mycotoxins in Stored Products: Proceedings of an International Conference, eds. B.R. Champ, E. Highley, A.D. Hocking and J.L. Pitt, ACIAR Proceedings no. 36, 198-204. Australian Centre for International Agricultural Research (ACIAR), Publ., Canberra, Australia.

197. Vasisht, K. 2004. Regional Workshop on quality control of medicinal plant products in South East Asia. United Nations Industrial Development Organization (UNIDO) and International Center for Science and Technology (ICS). Trieste, Italy.

198. VICAM. 1999. FumoniTest Instruction Manual. VICAM LP. Watertown, MA.

199. Vigers, A.J, Roberts, W.K., and Selitrennikoff, C.P. 1991. A new family of plant antifungal proteins. Molecular Plant-Microbe Interactions 4(4):315-323.

200. Visconte, A., and Boenke, A. 1995. Improvement of the determination of fumonisins (FB_1 and FB_2) in maize and maize-based feeds. European Commission BCR Information—Chemical Analysis, Brussels, Belgium.

201. Visconti, A. 1996. Fumonisins in maize genotypes grown in various geographic areas. Adv. Exp. Med. Biol. 392:193-294.

202. Vrabcheva, T & Gareis, M. Draft. 2002. Natural occurrence of ochratoxins A & B in spices and herbs. Submitted for publication to Mycopathol. 22.

203. Wicklow, D.T., Horn, B.W., Shotwell, O.L., Hesseltine, C.W., and Caldwell, R.W. 1988. Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. Phytopathol. 78(1): 8-74.

204. Windham, G. L., Williams, W. P., and Davis, F. M. 1999. Effects of the southwestern corn borer on Aspergillus flavus kernel infection and aflatoxin accumulation in maize hybrids. Pl. Dis. 83:535-540.

205. Widstrom, N.W., Wiseman, B.R., McMillian, W.W., Kwolek, W.F., Lillehoj, E.B., Jellum, M.D., and Massey, J.H. 1978. Evaluation of commercial and experimental three-way corn hybrids for aflatoxin B_1 production potential. Agron. J. 70:986-989.

206. Widstrom, N.W., Wilson, D.M, and McMillian, W.W. 1984. Ear resistance of maize inbreds to field aflatoxin contamination. Crop Sci. 24(6):1155-1157.

207. Widstrom, N.W., McMillian, W.W., and Wilson, D.M. 1987. Segregation for resistance to aflatoxin contamination among seeds on an ear of hybrid maize. Crop Sci. 27:961-963.

208. Wilson, D.M. and Abramson, D. 1992. Mycotoxins. In: Storage of Cereal Grains and Their Products, 2nd ed, ed. D.B. Sauer, 341-391. American Association of Cereal Chemists, Publ., St. Paul, MN.

209. Wilson, D.M., Sydenham, E.W., Lombaert, G.A., Trucksess, M.W., Abramson, D. and Benntee, G.A. 1998. Mycotoxin Analytical Techniques *In:* Mycotoxins in Agriculture and Food Safety, eds. K.K. Sinha and D. Bhatnagar, 135-182. Marcel Dekker Inc. Publ., New York, NY.

210. Wilson, D.M. 2002. Biology and ecology of mycotoxigenic Aspergillus species as related to economic and health concerns. *In*: Mycotoxins and Food Safety, eds. J.W. DeVries, M.W. Trucksess and L.S. Jackson, 1-17, Kluwer Academic/Plenum Publ., New York, NY.

211. Wilson, T.M., Nelson, P.E. and Knepp, C.R. 1985. Hepatic neoplastic nodules adenofribrosis, and cholangiocarcinomas in male Fisher rats fed corn naturally contaminated with *Fusarium moniliforme*. Carcinogenesis 6:1155-1160.

212. Witkowski, J.L. 1997. Bt corn & European corn borer. U. of Minnesota, St. Paul, Cooperative Ext. Serv. NCR-602.

213. Wood, G.M., Cooper, S.J., and Chapman, W.B. 1982. Problems associated with laboratory simulation of effects of food processes on mycotoxins. Proc. of V. Int. Symp. Mycotoxins, Vienna, Austria, 142.

214. Woods, R.J. and Pikaev, A.K. 1994. Selected Topics in Radiation Chemistry. *In*: Applied Radiation Chemistry radiation Processing, eds. R.J. Woods and A.K. Pikaev, 165-183, John T. Wiley & Sons, Inc. Publ. New York, NY.

215. Yat, P.N., Arnason, J.T., Lu, Z.Z., Fitzloff, J.F., Fong, H.H.S., and Awang, D.V.C. 2002. HPLC methods for separation and quantitative determination of ginsenosides used in the American Botanical Council's ginseng evaluation program. Herbalgram.org, Accession On-line: Ginsenoside Methodology.

216. Zummo, N. and Scott, G.E., 1992. Interaction of Fusarium moniliforme and *Aspergillus flavus* on kernel infection and aflatoxin contamination in maize ears. Pl. Dis. 76(8):771-773.