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ETHYLENE AND CO_2 INHIBIT AFLATOXIN B_1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS* GROWN ON PEANUTS

presented by

AGNESIA GUNTERUS

has been accepted towards fulfillment
of the requirements for the

M.S. degree in FOOD SCIENCE

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Major Professor's Signature

4-29-05
Date

ETHYLENE AND CO_2 INHIBIT AFLATOXIN B_1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS* GROWN ON PEANUTS

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Department of Food Science and Human Nutrition

2005

ABSTRACT

ETHYLENE AND CO_2 INHIBIT AFLATOXIN B_1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS* GROWN ON PEANUTS

By

Agnesia Gunterus

Production of Aflatoxin B_1 by *Aspergillus parasiticus* is inhibited when the fungus is grown on raw peanuts under exposure to different concentrations of ethylene and/or CO_2. Ethylene, a natural plant growth hormone produced during fruit ripening, is food-safe and relatively inexpensive to generate in sufficient quantities for treatment of stored plant materials. Our objective was to determine if ethylene demonstrated potential for use in a modified storage atmosphere for reduction of aflatoxin production by *A. parasiticus* on stored crops.

Peanuts were used as a model crop. Conidiospores of *Aspergillus parasiticus* D8D3 were inoculated on peanuts contained in a Petri dish. Petri dishes were placed in a growth chamber and continually flushed with air containing variable concentrations of CO_2 and/or ethylene. The growth chamber was incubated at 30°C in the dark for 5 days unless otherwise noted. Aflatoxin in peanuts was quantified by ELISA (Enzyme Linked Immunosorbent Assay) and Thin Layer Chromatography (TLC).

Ethylene inhibited aflatoxin production and the levels of inhibition depended on the concentration used. The greatest effect of ethylene and CO_2 in reducing aflatoxin accumulation was about 80% and 85% reduction, respectively.

To my parents, Frans and Lenny Gunterus, my sister, Alicia Gunterus, and my brother, Aurel Gunterus for their unconditional love and support

ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. John Linz for all of his help during the two years of my graduate study, for his patience and his understanding during the challenging times. I would also like to thank my committee, Dr. James Pestka, Dr. Randy Beaudry, and Dr. Gale Strasburg who also have helped me in many different aspects during my study.

My great appreciation to Dr. Ludmila Roze who has helped me tremendously with her advice and physical strength during preparation of gas tanks for my experiments. I would also like to thank my fellow lab members, Sung Yong Hong, Joo-Won Lee, Suil Kang, and Anindya Chanda for their support. My special thanks to Anna Arthur and Eric Harper for their great assistance during my experiments.

I would also like to thank to Dr. Corley Holbrook who provided the peanuts for this study.

Lastly, I would like to acknowledge my family, my very good friends Maria Suparno and Rully Kusuma, for their endless support and for JC who made all of this possible for me.

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CHAPTER 1

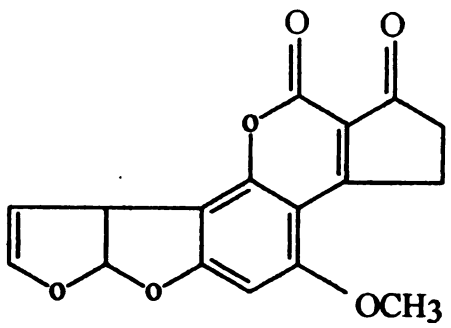
LITERATURE REVIEW

Aflatoxin

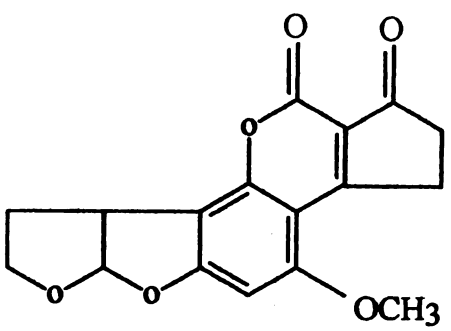
The producer

Aflatoxins, secondary metabolites produced by aflatoxigenic strains of *Aspergillus spp.,* are both mutagenic and carcinogenic to animals (reviewed in Cary et al., 2000). Fungi belonging to *Aspergillus spp.* are common contaminants of human food and animal feeds (reviewed in Gourama and Bullerman, 1995). The two fungi that most commonly produce aflatoxin are *Aspergillus parasiticus* and *A. flavus*; the first is known to be the more stable aflatoxin producer (reviewed in Gourama and Bullerman, 1995). Aflatoxins are major contaminants of corn, peanuts, cottonseed, and tree nuts (reviewed in Cary et al., 2000). Infection of the host plant can occur both before harvest and during storage (Wilson and Payne, 1994). *A. flavus* produces aflatoxin B_1 and B_2, while *A. parasiticus* produces aflatoxin G_1 and G_2 in addition to the two B toxins (reviewed in Dvorackova, 1990) (Figure 1.1).

Both of the primary aflatoxin producers occur mostly in the soil. However, conidiospores of *A. flavus* are found more often in air than in the soil and are found more in temperate regions while spores of *A. parasiticus* are found typically in soil and are more adaptable to warmer climates. These observations help explain why *A. parasiticus* is the main contaminant of peanuts and *A. flavus*



Aflatoxin B_1



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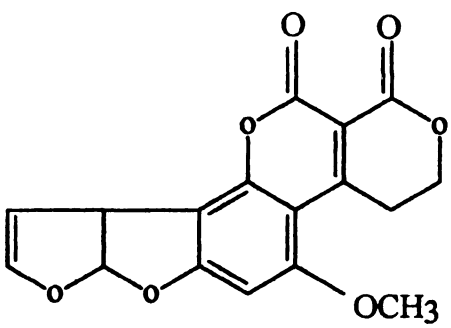
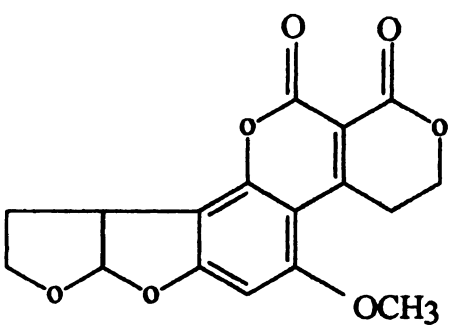


Aflatoxin G_1



Aflatoxin G_2

Figure 1.1. Chemical structures of Aflatoxin B_1, B_2, G_1, and G_2 (taken from dissertation of Michael Miller, 2003).

is more of a problem in corn (reviewed in Gourama and Bullerman, 1995).

A. parasiticus and *A. flavus* are members of the fungi imperfecti, the Deuteromycota, meaning that they do not have a known sexual stage in their life cycles (reviewed in Gourama and Bullerman, 1995). They reproduce by means of conidia, asexual reproductive structures. The conidia are produced on structures called phialides, in a chain-like manner, where the oldest conidia are at the apex of the chain (Figure 1.2). The phialide, that forms a bulbous structure, and the chain-like conidia are the main characteristics of fungi in the *Aspergillus* group.

History and Toxicology

Aflatoxins were first discovered in 1960 after an outbreak in England, known as "Turkey-X disease", where thousands of turkeys died as a result of consuming contaminated feed (Blount, 1961). The causal agent was later determined to be a secondary metabolite of *A. flavus*, and was named aflatoxin, *A. flavus* toxin (Asao et al., 1963).

Aflatoxin B_1 (AFB_1) is the most carcinogenic among the aflatoxin family (Bhatnagar et al., 1992). It is known to be the second most carcinogenic substance, the first being the synthetically derived polychlorinated biphenyls (PCBs) (reviewed in Cary et al., 2000). Aflatoxin B_1 can induce carcinomas in rats and trout when ingested at concentrations below 1µg/kg body weight (Robens and Richard, 1992).

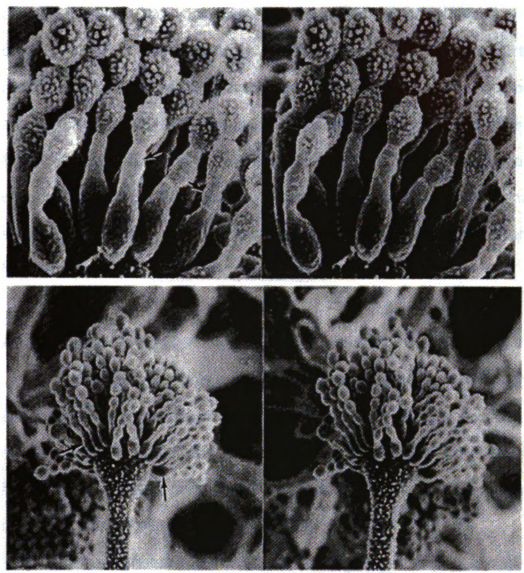


Figure 1.2. Phialides and conidia of *A. parasiticus* (top) *and A. flavus* (bottom). Arrow indicates a phialide.
(http://www.aflatoxin.info/aflatoxin.asp)

Toxicity of AFB_1 in animals depends on several factors including dose and susceptibility. Biological effects of the toxin may occur as an acute and clinically obvious disease, a chronic but less apparent impairment of health and productivity, or an impairment of resistance and immune responsiveness that is not clinically apparent as being associated with aflatoxin consumption (Roebuck and Maxuitenko, 1994). The organ that is mainly targeted for aflatoxicosis is the liver (Cullen and Newberne, 1994). Evidence of aflatoxicosis has also been observed in humans. Studies within a human population in mainland China showed a relationship between aflatoxin exposure and liver cancer (Cullen and Newberne, 1994).

Aflatoxin biosynthesis

Mycotoxins, secondary metabolites produced by several species of fungi, are not constitutively synthesized. Instead, they are produced in response to developmental, environmental, and nutritional conditions. Fungi control mycotoxin biosynthesis primarily by way of transcriptional regulation of the mycotoxin biosynthetic genes (Miller, 2003).

Aflatoxin synthesis does not appear to have any impact on physiological role in primary growth and metabolism of the fungi or survival (short term) of the producing fungi (reviewed in Cary et al., 2000). In other words, the producing fungi will still be able to survive even when aflatoxin production is inhibited. Aflatoxins, however, are toxic to other competitor organisms, thus they may have survival benefit for the fungi (Detroy et al., 1971).

At least 20 genes are involved in the aflatoxin biosynthetic pathway (Figure 1.3). Mutants of *A. parasiticus* that are unable to synthesize aflatoxin, as well as studies using radio labeled precursors and metabolic inhibitors, have been useful in identifying the genes and enzymes involved in the pathway (reviewed in Cary et al., 2000).

One of the genes involved in an early step in the aflatoxin biosynthetic pathway is the *nor-1* gene, which encodes norsolorinic acid reductase. Understanding how this gene is regulated may allow development of methods for reducing aflatoxin production by the fungi because this gene is involved early in the aflatoxin biosynthetic pathway. Creating a mutant that lacks this gene should result in a strain that is not able to synthesize aflatoxin. Our lab has done this work and the results indicated that aflatoxin production by this mutant was reduced but not entirely inhibited. Chiou et al., 2002 constructed a reporter gene with the *nor-1* promoter fused to β-glucuronidase gene (*uidA*) from *Escherichia coli*. Studies reported in this thesis were conducted using the D8D3 strain of *A. parasiticus* which carries one copy of the *nor-1* promoter fused with GUS (β-glucuronidase) gene integrated at the *nor-1* locus.

Due to its high toxicity, aflatoxin content in foods and feeds is highly regulated in the United States as well as many other countries (Cotty et al., 1994). In the United States, the U.S. Food and Drug Administration has issued a guideline of 20 ppb (parts per billion), which is the maximum allowable total aflatoxin content in foods and 20-300 ppb for feeds depending on the animal (Council for Agriculture Science and Technology, 2003). Most European

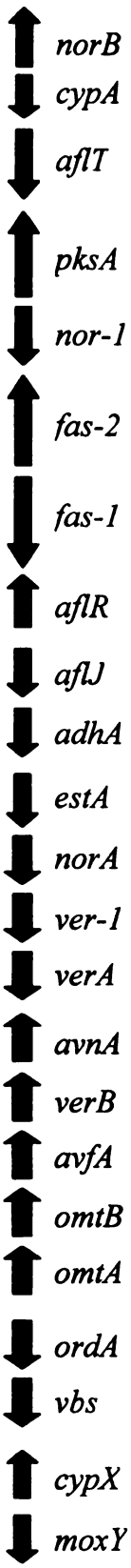


Figure 1.3. Genomic organization of the aflatoxin biosynthetic gene cluster in *A. parasiticus*. Arrows indicate direction of transcription. Drawn approximately to scale (taken from dissertation of Michael Miller, 2003)

countries only allow 3-5 ppb aflatoxin B_1(Council for Agriculture Science and Technology, 2003).

Economic significance

Because aflatoxin is highly regulated in the US, farmers suffer a great loss when their crops are infected. It was estimated that each year, 25% of the world's crops are contaminated by mycotoxins (Mannon and Johnson, 1985). Also, since aflatoxin is more tightly regulated in most European countries, significant quantity of the crops that could normally be exported to those countries are excluded. The total crop lost leads to a higher price for the commodities and a decrease in the supply. On top of that, mycotoxins also result in increased costs for research, regulatory enforcement, mitigation, lawsuits, testing, and quality control (Council for Agriculture Science and Technology, 2003).

It is not possible to calculate precisely the total economic loss due to mycotoxins because there are a lot of uncertainties surrounding the factors that play a role in determining the cost. These factors include the extent and level of contamination, the variability of contamination, the variability of price and quantity of the affected commodities, the costs of efforts to mitigate the contamination, and the loss in livestock value from contaminated feed (Council for Agriculture Science and Technology, 2003). These uncertainties were built into a cost model that utilized Monte Carlo computer simulations to estimate the distribution, prices, and contamination levels of commodity outputs (Council for Agriculture Science and Technology, 2003).

The potential value of crops lost due to aflatoxin contamination generated by the Monte Carlo computer simulations was $47 million per year in food crops (peanuts and corn) and $225 million per year in feed crops. The estimated livestock cost was about $4 million per year (Council for Agriculture Science and Technology, 2003). Although these numbers are estimates, it is very apparent that aflatoxin has a significant economic impact in the United States.

Although aflatoxin is often found in all susceptible crops at low concentration, it is most common at detectable levels in corn and peanuts. The reason is because these commodities are grown under climatic conditions which are favorable for *A. parasiticus* and *A. flavus* (Phillips et al. 1994). These conditions include a relative humidity of 88 to 95% surrounding the substrate and a storage temperature of 25°C to 30°C (Ellis et al., 1991).

Aflatoxin contamination varies by year and region, and is positively impacted by improvements in practices that are carried out by farmers and distributors (Council for Agriculture Science and Technology, 2003). Long periods of drought will induce aflatoxin contamination, which results in an increase in crop loss. Good storage practices will reduce the effect of initial contamination. However, poor storage practices will increase contamination even during non-drought periods (Council for Agriculture Science and Technology, 2003).

Current detoxification methods

Strategies to control aflatoxin contamination have primarily focused at the pre-harvest level since that is when the fungi first colonize host tissues (Cleveland and Bhatnagar, 1992; Cleveland et al., 1997). Several strategies have been utilized to reduce pre-harvest aflatoxin contamination in susceptible crops. These include the use of pesticides, altered cultural practices, and the use of resistant varieties (reviewed in Cary et al., 2000). However, these methods have demonstrated only a limited potential for reducing aflatoxin levels, especially when the environmental conditions favor the contamination process (reviewed in Cary et al., 2000). There are currently three ways to remove aflatoxin from infected crops: physical, biological, and chemical approaches.

a. Physical approaches

Physical approaches include thermal inactivation, irradiation, and solvent extraction (Council for Agriculture Science and Technology, 2003). Aflatoxins are heat stable and cannot be completely destroyed by heat treatment (Christensen et al. 1977). Incomplete and non-uniform reduction of aflatoxin may be obtained by oil roasting, dry roasting peanut and oilseed meals (Marth and Doyle, 1979) or roasting corn (Conway et al., 1978). This incomplete destruction of aflatoxin is affected by the commodity's temperature, heating interval, and moisture content (Mann et al., 1967). In peanuts, roasting conditions and initial aflatoxin concentration determine the degree of reduction (Lee, 1989). During processing of contaminated product, the process itself sometimes separated aflatoxin from

final product, for example in processing of peanut oil. Aflatoxin levels in dough are not significantly reduced by baking temperatures (Reiss, 1978).

Some studies have been done to analyze the effects of irradiation in destruction of aflatoxin in infected crops. Gamma irradiation at 2.5 rad does not degrade aflatoxin in contaminated peanut meal. Similarly, UV light produced no significant change in fluorescence and toxicity (Feuell, 1977). Also, exposure of aflatoxin to UV light has been reported to activate the toxin to a mutagenic form (Stark et al., 1990). Farag et al. (1996) reported that aflatoxins B_1, B_2, G_1, and G_2 respond to microwave treatment in both model and actual food systems. The rate of aflatoxin destruction was positively correlated with the power setting and exposure time (Farag et al., 1996).

Another physical approach to reduce aflatoxin concentration in contaminated crops is solvent extraction. Examples of solvents include 95% ethanol, 90% aqueous acetone, 80% isopropanol, hexane-ethanol, hexane-methanol, hexane-acetone-water, and hexane-ethanol-water combinations. Although effective, solvent extraction is considered impractical for most applications because most useful solvents are toxic for food applications. (Shantha,1987).

b. Biological approaches

The strategies described above are considered post-harvest approaches. Some pre-harvest strategies have also been studied to control aflatoxin before they are produced. One study by Cole and Cotty, 1990, was conducted using

non-toxigenic strains of *A. parasiticus* and *A. flavus*. These organisms were impaired in their ability to produce aflatoxin. The objective was to have these organisms compete with the toxin-producing ones to decrease aflatoxin contamination in peanuts and cottonseed. The results indicated that aflatoxin contamination in peanuts and cottonseed is significantly decreased (Cole and Cotty, 1990).

Another biological approach to control aflatoxin is to use another microorganism that has the ability to inactivate aflatoxin. One microorganism that is being studied is *Flavobacterium aurantiacum* that was shown to significantly remove aflatoxin without producing toxic byproducts or metabolites (Ciegler et al., 1966). Aflatoxins in contaminated grains are degraded by fermentation (Dam et al., 1977). Other microorganisms have also been shown to interfere with aflatoxin production. This is thought to occur due to competition for nutrients and space or through production of substances that interfere with the infection process or aflatoxin production (Bhatnagar et al., 1994).

c. Chemical approaches

Ammoniation is one chemical approach that has been tested for ability to degrade aflatoxin in contaminated crops. Ammoniation decreased aflatoxin levels by 99% when performed under appropriate conditions (Brekke et al., 1977, 1979; Dollear et al., 1968; Gardner et al., 1971; Masri et al., 1969; Park et al., 1984; Phillips et al., 1994). Ammoniation converts aflatoxin B_1 to a less toxic product (Council for Agriculture Science and Technology, 2003). There are two different

ammoniation procedures that are currently used, high-pressure and high temperature (HP/HT) and atmospheric pressure and ambient temperature (AP/AT). The HP/HT method is frequently used in cottonseed and cottonseed meal while the AP/AT method is used mainly for whole cottonseed.

In 1976, the United States Food and Drug Administration (FDA) approved the use of ammonia-treated non-aflatoxin-contaminated cottonseed meal for ruminants (21 CFR 573.140) (Park et al., 1988). In 1979, the National Cottonseed Growers Association (NCGA) sent a petition to FDA to allow the use of the ammoniation decontamination method for controlling aflatoxin levels in cottonseed and cottonseed meal (Park et al., 1988). Numerous short and long-term toxicological studies have been done to support this FAP (Food Additives Petition). FDA concluded that the data provided to support this FAP did not completely respond to the concern on potential presence of toxic residues in human foods derived from animals fed ammoniated feed (Park et al., 1988). Therefore, FDA claimed jurisdiction over materials that are transported between states (Park et al., 1988). Arizona and California have approved this method for decontamination of cottonseed products. Texas, North Carolina, Georgia, and Alabama permit ammoniation of aflatoxin-contaminated corn. This method has also been used internationally in Mexico, Sudan, South Africa, Senegal, and Brazil. This method was extensively reviewed (Anderson, 1983; Goldblatt and Dollear, 1979; Palmgren and Hayes, 1987; Park et al., 1988).

Another chemical approach is to use sodium bisulfite, an accepted food additive. This substance is known to react with Aflatoxin B_1, G_1, and M_1, to form

water soluble products (Doyle and Marth, 1978a, 1978b; Hagler et al., 1982; Moerck et al., 1980). Ozonization is another chemical method that has been shown to effectively degrade aflatoxins in corn and cottonseed meals (Dollear et al., 1968; Dwarakanath et al., 1968) and in aqueous solution (Maeba et al., 1988). Other chemical approaches utilized adsorbents to absorb mycotoxins (Council for Agriculture Science and Technology, 2003).

Although the methods mentioned above are effective, the safety and application of the methods needs to be studied further. The use of ammonia is only approved for aflatoxin contaminated feed and is not approved for interstate distribution. The use of UV, gamma, and other irradiation methods may not be very safe for the handler. Other chemical approaches may not be suitable for decontamination of aflatoxin-contaminated crops that are intended for human consumption because most of the chemicals used are not food safe.

Ethylene

Biology of ethylene: plant hormone

Ethylene is a gaseous plant hormone. It can be produced by all parts of many plants although the rate depends on tissue type and the developmental stage (Taiz and Zeiger, 2002). Meristematic and nodal regions are the areas where ethylene is synthesized at highest levels. Ethylene production increases during leaf abscission, flower senescence, and fruit ripening (Taiz and Zeiger, 2002). Wounding and environmental stresses such as flooding, chilling, disease,

and temperature or drought stress also induce ethylene biosynthesis (Taiz and Zeiger, 2002).

The structure of ethylene is very simple (Figure 1.4). It can be completely oxidized to CO_2 in most plant tissues and it is readily released from the plant tissues (Taiz and Zeiger, 2002). Because it is very easily released from the plant tissue and it can affect other tissues or organs, ethylene trapping agents are normally used during storage of fruits, vegetables, and flowers. One example of an ethylene trapping agent is potassium permanganate ($KMnO_4$) that is sometimes used for apple storage (Taiz and Zeiger, 2002).

Besides plants, ethylene has been found to be synthesized in fairly large quantities by certain strains of the common enteric bacterium *Escherichia coli* and yeast using methionine as a substrate (Taiz and Zeiger, 2002). Ethylene production in mammalian cells has not been reported. However, it was recently observed that marine sponges and cultured mammalian cells could respond to ethylene, raising the possibility that ethylene might act as a signaling molecule in these cells (Perovic et al., 2001).

History of ethylene use in agriculture

Ethylene was not the first plant hormone to be used agriculturally, but it is the most widely used one due to its diverse range of beneficial effects on regulating plant growth responses (reviewed in Kays and Beaudry, 1987). The earliest report on the effect of ethylene in plants was by Girardin in 1864. At that time, illuminating gas was still being used for lighting and leakage of this gas was

a major problem. Although ethylene was not known at the time to be the active compound in this gas, it was noticed that rows of trees that were exposed to illuminating gas defoliated and eventually died (reviewed in Kays and Beaudry, 1987).

In the early 1900s, kerosene stoves were used in railroad cars to prevent freezing during shipment of citrus fruits. It was found that the gas produced from these stoves caused degreening in these fruits (Crocker and Knight, 1908; Harvey, 1915). Sievers and True (1912) suggested that this degreening was due to gaseous products that were produced as a result of incomplete combustion of the kerosene used in the stoves. This active gas compound was later found to be ethylene and was patented by Denny in 1923.

Effects on plant physiology

Ethylene promotes ripening in some fruits. Fruits that ripen in response to ethylene all have one common characteristic. That is, their respiratory rate rises before the ripening phase. This characteristic is called climacteric (Taiz and Zeiger, 2002). Examples of climacteric fruits include apples, bananas, avocados, and tomatoes. When unripe climacteric fruits are exposed to ethylene, an accelerated climacteric rise is observed (Taiz and Zeiger, 2002). When unripe nonclimacteric fruits are treated in the same manner, respiration increases as a function of ethylene concentration. However, treatment does not induce endogenous ethylene production and therefore does not induce ripening (Taiz and Zeiger, 2002).

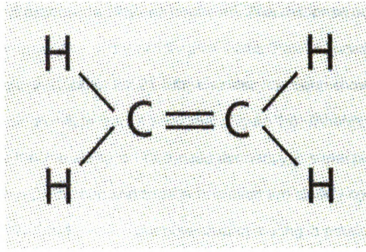


Figure 1.4. Structure of ethylene

In Arabidopsis, ethylene elicits what is called the triple response, characterized by inhibition and swelling of the hypocotyls, inhibition of root elongation, and exaggeration of the apical hook (Figure 1.5) (Taiz and Zeiger, 2002). A triple response was also observed in 6-day-old pea seedlings when treated with 10ppm ethylene (Figure 1.6) (Taiz and Zeiger, 2002). Epinasty, a condition where the leaves of the plants bend downward, was also observed in tomato plants in response to ethylene treatment. This response occurs because the cells on the upper side of the petiole grow faster than the ones on the bottom (Figure 1.7) (Taiz and Zeiger, 2002). Ethylene also has been shown to have an effect on root hair growth in lettuce seedlings (Figure 1.8) (Abeles et al., 1992).

Ethylene has the ability to break seed dormancy in some plant seeds. In peanuts, ethylene production and seed germination are closely correlated (Taiz and Zeiger, 2002). Ethylene also promotes bud sprouting in potato and other tubers and acts as a positive regulator in the differentiation of root hairs (Taiz and Zeiger, 2002). In ethylene-insensitive tobacco, the plant becomes susceptible to soil fungal pathogens that are not plant pathogens normally. This indicates that ethylene is involved in the resistance response to some pathogens, but not others (Taiz and Zeiger, 2002). Ethylene production generally increases in response to pathogen attack in both pathogenic and non-pathogenic interactions (Taiz and Zeiger, 2002).

Ethylene also plays a role in leaf abscission. In a study using birch (*Betaul pendula*), wild type and mutant trees carrying a mutated version of the *Arabidopsis* ethylene receptor gene (ETR1-1) were treated with 50ppm ethylene

for 3 days. The mutant plant did not drop their leaves while the wild type did (Taiz and Zeiger, 2002) (Figure 1.9).

Current application

Ethylene is widely used in agriculture because it has many physiological effects in plant development. However, ethylene is very difficult to apply in the field as a gas because of its high diffusion rate (Taiz and Zeiger, 2002). This problem has been solved with the use of ethylene generating compounds. The most widely used ethylene generating compound is ethephon, or 2-chloroethylphosphonic acid that was discovered in 1960 (Taiz and Zeiger, 2002). Ethephon is used in aqueous form by spraying it onto the plant. The plant will then absorb and transport the compound. Ethephon releases ethylene slowly by a chemical reaction so the hormone will exert its effects (Taiz and Zeiger, 2002).

Similar to ethylene, ethephon induces fruit ripening in apple and tomato (Taiz and Zeiger, 2002). It synchronizes flowering and fruit set in pineapple, and accelerates abscission of flowers and fruits (Taiz and Zeiger, 2002). It can be used to induce fruit thinning or fruit drop in cotton, cherry, and walnut (Taiz and Zeiger, 2002). It is also used to promote female sex expression, prevent self pollination and increase yield in cucumber (Taiz and Zeiger, 2002). Ethephon has numerous other applications in agriculture.

In storage facilities, ethylene inhibitors are often used to preserve fruits (Taiz and Zeiger, 2002). These storage facilities utilize a controlled atmosphere of low O_2 concentration and low temperature to inhibit ethylene biosynthesis (Taiz

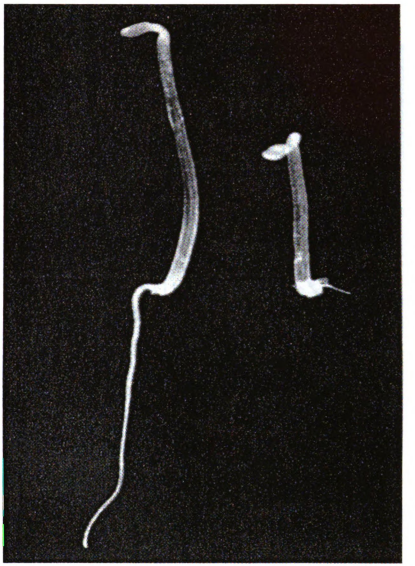


Figure 1.5. The triple response in *Arabidopsis*. Three-day-old etiolated seedlings grown in the presence (left) and absence (right) of 10ppm ethylene. Note that shortened hypocotyls, reduced root elongation, and exaggeration of the curvature of the apical hook all resulted from the presence of ethylene (Taiz and Zeiger, 2002 courtesy of S. Gepstein).

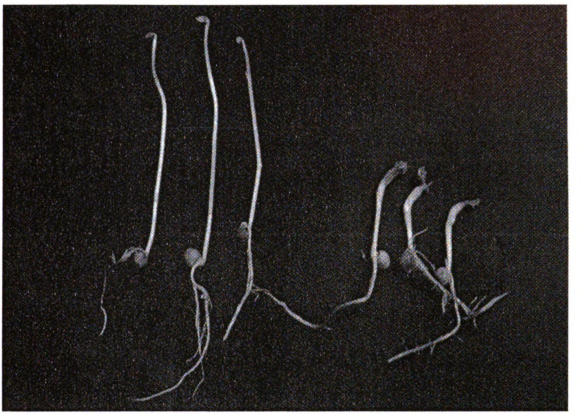


Figure 1.6. Triple response of etiolated pea seedlings. Six-day-old pea seedlings were treated with 10ppm ethylene (right) or left untreated (left) (Taiz and Zeiger, 2002 courtesy of S. Gepstein).

Figure 1.7. Symptom of epinasty, downward bending of the tomato leaves (right), caused by ethylene (Reid, 1995).

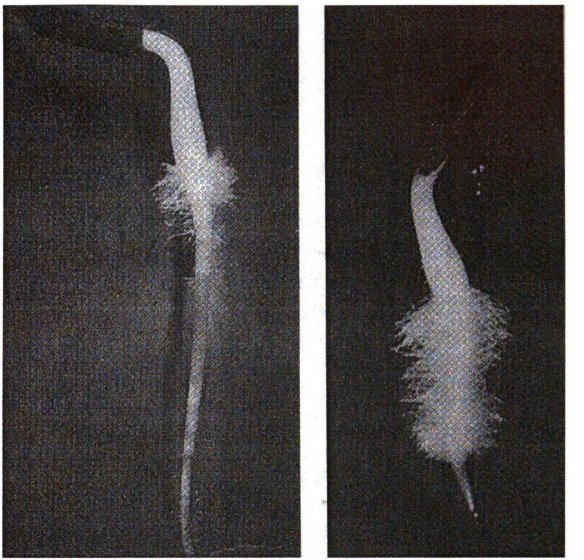


Figure 1.8. Excessive root hair formation caused by ethylene in lettuce seedlings. Two-day-old seedlings were treated with air (left) or 10ppm ethylene (right) for 24 hours (Abeles, 1992)

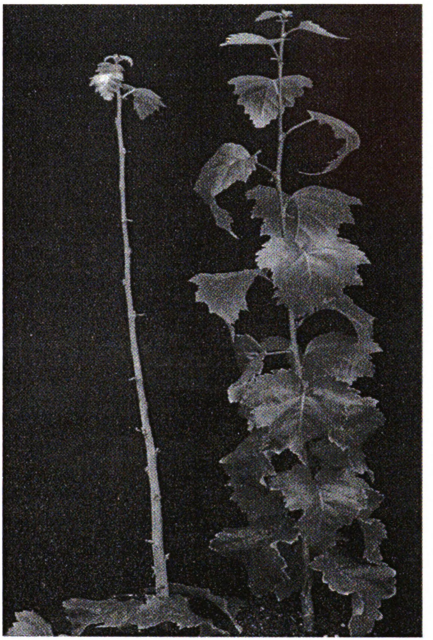


Figure 1.9. Abscission in birch (*Betaul pendula*) caused by ethylene. Plant on the left is wild type. Plant on the right was transformed with a mutated version of the *Arabidopsis* ethylene receptor, ETR1-1. The expression of this gene was under the transcriptional control of its own promoter. The mutant trees did not drop their leaves when fumigated with 50ppm ethylene for 3 days (Taiz and Zeiger, 2002).

and Zeiger, 2002). A CO_2 concentration of 3 to 5% will prevent ethylene effects as a ripening promoter (Taiz and Zeiger, 2002). One ethylene inhibitor that is commonly used currently in post harvest applications is 1-methylcyclopropene (MCP) (Taiz and Zeiger, 2002).

Ethylene and aflatoxin

In 1985, Sharma et al., found that CEPA (2-chloroethylphosphonic acid) at a concentration of 3200ppm inhibited aflatoxin biosynthesis in *A. parasiticus* to non-detectable levels in both stationary and shaken cultures. A study done by one of our lab members, Dr. Ludmila Roze, demonstrated that treatment with 100 ppm ethylene reduced aflatoxin production in *A. parasiticus* by 90%. These observations will be discussed in more detail in the introduction of Chapter 2.

Application of ethylene

Ethylene producing compounds can be grouped into three classes based on the site of origin of the ethylene molecule. The first class includes compounds that break down or are metabolized to release the ethylene (ethylene-releasing compounds). The second class induces ethylene formation by the target tissue (ethylene-inducing compounds) and the third class releases ethylene held by an absorbent (reviewed in Beaudry and Kays, 1988).

Some examples of ethylene releasing compounds evaluated for commercial use are (2-chloroethyl)phosphonic acid (Ethrel®, Amchem 66-329, CEPA, and ethephon), (2-chloroethyl)methylbis(phenylmethoxy)silane (Silaid®, or

CGA-15281), and (2-chloroethyl)tris(2-methoxyethoxy)silane (Alsol®, CGA-13285, and etacelasil) (reviewed in Beaudry and Kays, 1988). Ethylene-inducing compounds act through either direct or relatively direct effects on the synthesis pathway. Compounds that act directly affect the formation of ACC, which under normal conditions appears to be the rate-limiting step in ethylene biosynthesis (reviewed in Beaudry and Kays, 1988). These compounds may also affect production of auxin, another plant hormone that has similar responses in plant tissues as those of ethylene (Morgan, 1976). Compounds that induce ethylene indirectly do so by causing wounds or stress to the plant tissue which in turn will promote ethylene synthesis (Saltveit et al., 1978; Goeschl et al., 1966; Hoffman and Yang, 1982).

Carbon dioxide (CO_2)

Effects of CO_2 on fungi

CO_2 has been used in modified atmosphere packaging (MAP) due to its bacteriostatic and fungistatic properties to control food spoilage organisms (Daniels et al., 1985; Nguyen-the and Carlin, 1994; Ashie et al., 1996). There are several mechanisms proposed to explain the inhibitory effect of CO_2: it may affect the function of biological membranes, interfering with cell division, substrate uptake or transport; it may cause acidification of internal pH which can affect carboxylation/ decarboxylation reactions or have more specific effects on enzymes that do not involve CO_2; or it also affect the physicochemical properties

of enzymes altering for example, solubility or structure (Daniels et al., 1985; Dixon and Kell, 1989; McIntyre and McNeil, 1997).

Growth inhibition or stimulation depends on the concentration of CO_2 and the effect varies across microorganisms and may be dependent on other environmental parameters, especially temperature (Becard and Piche, 1989; Lannelongue and Finne, 1986; Eyles et al., 1993). In fungi, changing CO_2 concentration has been recognized as a regulatory factor in the control of fungal morphology and differentiation (Bartnicki-Garcia and Nickerson, 1962; Zonneveld, 1988). In many *Alternaria spp*, sporulation is induced when CO_2 is removed from filamentous cells growing on sealed plates using a KOH/CO_2 sink (Cotty, 1987). In *Aspergillus nidulans*, sexual differentiation was inhibited in plate cultures when the CO_2 level during growth was lowered using 5% KOH. The maximum dry weight obtained was 20% lower than in the control (Zonneveld, 1988). This inhibition of sexual differentiation was thought to be due to inhibition of α-1,3 glucan synthesis (Zonneveld, 1988). No CO_2 responsive genes that regulate fungal growth and development have yet been identified (reviewed in Stretton and Goodman, 1997). Clevstrom et al. (1983) incubated *A. flavus* on a synthetic low salt medium with traces of air for 3 days followed by a flow of nitrogen that contained <100ppm oxygen. These conditions resulted in formation of only small amounts of both Aflatoxin B_1 and B_2 (<100µg/liter after 2 weeks) compared to higher quantities of these toxins (>1000µg/liter) when the fungus was only treated with traces of air continuously for 2 weeks. This means that the fungi require oxygen during to synthesize these toxins.

Under practical storage conditions, an atmosphere of nitrogen or carbon dioxide with low oxygen content cannot prevent aflatoxin formation but can reduce aflatoxin contamination, even if the product is highly contaminated with a potent aflatoxin producer (Davis and Diener, 1970; Fabbri et al., 1980).

In 1993, Ellis et al., did a study on controlling growth and aflatoxin production by *A. flavus* using modified atmosphere packaging (MAP) conditions. They analyzed the effect of water activity (a_w), pH, storage temperature, headspace oxygen and carbon dioxide concentration on the growth and aflatoxin production by *A. flavus* on synthetic media. All conditions were found to be highly significant factors ($p<0.01$) in controlling growth of *A. flavus* in synthetic media. At 20°C, and 5% and 15% O_2 (balance CO_2:N_2 = 60%:40%), no growth was observed even when a_w and pH were varied. No growth was observed under 10% O_2, at 15°C and 25°C with an a_w at 0.96 or below at all pH treatments. The optimum conditions for growth were a_w 0.964, pH 6.86, temperature of 31.7°C, and 12.9% O_2.

Other factors influencing aflatoxin production

Other factors that influence aflatoxin production include nitrate, benzoic acid, sorbic acid, BHA, trace metals, caffeine, and light. Kachholz and Demain (1983) reported that biosynthesis of averufin, an early intermediate in aflatoxin biosynthesis, was regulated by the nitrogen source. Nitrate had a negative effect on averufin production and this effect was due to repression of enzyme(s) involved in averufin formation.

Benzoic acid and sodium benzoate reduced aflatoxin production in *A. flavus* in synthetic media accompanied by accumulation of a yellow pigmented compound (Uraih et al., 1977). Although, this compound has not been identified, it is thought to be an intermediate in aflatoxin biosynthesis. Benzoic acid blocks an enzymatic step in the biosynthetic pathway resulting in accumulation of this intermediate (reviewed in Buchanan and Zaika, 1987).

Butylated hydroxyanisole (BHA), an antioxidant, inhibited growth and aflatoxin production by *A. parasiticus* and *A. flavus* (Cheung and Sim, 1964; Foudin et al., 1978; Fung et al., 1977; Lin et al., 1983). A larger reduction was observed in aflatoxin G production than in aflatoxin B production (Fung et al., 1977; Lin et al., 1983). A possible explanation for this observation was that BHA inhibits an oxidative process proposed to be responsible for converting B aflatoxins to G aflatoxins and therefore more aflatoxin B accumulated (Yousef and Marth, 1983).

Zinc is an essential element for cellular growth and metabolism (Failla, 1977). It is also essential for aflatoxin biosynthesis (Gupta et al., 1977; Lee et al., 1966; Marsh et al., 1975; Mateles and Adye, 1965). The required zinc concentration for optimum aflatoxin production varied between studies. Mateles and Adye suggested that the minimum zinc concentration is 0.4mg/L while Lee et al., 1966 suggested that the level is 0.8mg/L. A minimum concentration as high as 50mg/L has also been reported (Lin et al., 1983). Bennett et al., 1979 showed that zinc was essential for versicolorin production, another intermediate in aflatoxin biosynthesis. They used a mutant of *A. parasiticus* that accumulates

versicolorin A and C. Stimulation of versicolorin A production was observed only when zinc was present at the early period of vegetative growth, between 20-30 hours post inoculation, similar to results seen by Failla and Niehaus (1986). Versicolorin synthesis, which began at 50 hours post inoculation, was directly proportional to the zinc content at 30 hours. This suggested that zinc may be acting at the pre-transcriptional or transcriptional level. Several enzymes, including the glycolytic enzymes (Gupta et al., 1976) and enzymes that are involved in the mannitol cycle and pentose phosphate pathway (Niehaus and Dilts, 1982, 1984), in *A. parasiticus* have also been reported to be affected by zinc. These authors suggested that the effect of zinc on polyketide synthesis was mediated by inhibition of these enzymes that caused lowering of the cellular NADPH/NADP ratio and therefore prevent conversion of malonyl coA to fatty acids.

Other trace metals such as magnesium, manganese, molybdenum, iron, and zinc are important and are generally included in defined medium for Aspergilli (Adye and Mateles, 1964).

Caffeine-containing commodities generally do not have high incidences of aflatoxin contamination (Lenovich, 1981; Levi, 1980) although *A. flavus* is one species associated with the mycoflora isolated from these agricultural materials (Mislivec, 1983; Hansen and Welty, 1980). Several studies also showed that coffee and cocoa beans are poor substrates for aflatoxin production (Lenovich, 1981; Lenovich and Hurst, 1979; Llewellyn et al., 1978; Nartowicz et al., 1979; Wildman et al., 1967). Buchanan and Fletcher (1978) evaluated the effects of

caffeine on *A. parasiticus* cultured in synthetic media. They found that both growth and toxin production were inhibited by caffeine; toxin production was affected to a greater extent. Other studies also showed that removal of caffeine from green and roasted coffee beans greatly increased the potential for aflatoxin production (Nartowicz et al., 1979).

Another environmental factor that could also influence aflatoxin production is light. Light is an essential factor in many molds for its involvement in induction and completion of sporulation. It also affects vegetative growth and aflatoxin production of toxin-producing strains in both liquid and solid media (reviewed in Ellis et al., 1991). The role of light may be both inhibitory and stimulatory in some species due to the photochemical effects on the medium (Carlile, 1970). Bennett et al. (1978) reported that conidiospores of *Aspergillus* were more abundant when the molds were exposed to light. They also reported that the blue wavelengths contained in white light were most effective in eliciting photoresponses in fungi. They indicated that aflatoxin production was inhibited by light at either high or low temperatures but not at intermediate temperatures, 20-25°C. The type of substrate also determined the effect of light on aflatoxin production. Aflatoxin biosynthesis was completely inhibited when the fungus was grown on Czapek's medium under light (Joffe and Lisker, 1969). Reiss (1975), on the other hand, found that light had no effect on aflatoxin production in bread. Nkama et al. (1987) reported that the rate of reduction of aflatoxin B_1 increased with increasing light intensity.

CHAPTER 2

Effect of CO_2, ethylene, and combination of the two on Aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*

INTRODUCTION

In 1985, Sharma et al. conducted a study on the possible association between ethylene and aflatoxin biogenesis in toxigenic *Aspergillus parasiticus* and non toxigenic *A. flavus*. In this study, the authors measured the concentration of ethylene that is generated by these fungi. They also studied the effect of CEPA (2-chloroethyl phosphonic acid), an ethylene generating compound, on aflatoxin biosynthesis in *A. parasiticus* in stationary and shake cultures.

They observed that ethylene was generated during the first 24 hours of growth in both fungi. The toxigenic *A. parasiticus* strain stopped producing ethylene at this time while the non toxigenic *A. flavus* strain continued to produce ethylene throughout growth although in a smaller amount (~4ppm). Lower concentrations of CEPA (0-1200ppm) seemed to stimulate growth of the organism, but aflatoxin biosynthesis was drastically reduced. However, higher concentrations of CEPA (1400-1600) inhibited both growth and aflatoxin biosynthesis. At a CEPA concentration of 3200ppm, no toxin could be detected in either stationary or shake cultures. The authors also tried adding CEPA at

different times during growth. When CEPA was added before 48 hours of growth, aflatoxin production was reduced. However, when it was added after that period, aflatoxin production was not reduced. This suggests that ethylene production by the toxigenic *A. parasiticus* started and ended prior to synthesis of the toxin.

A member of our laboratory, Dr. Ludmila Roze, conducted a study on ethylene and CO_2 effects on aflatoxin biosynthesis by *A. parasiticus* in defined solid growth media. She initially analyzed the effects of ethylene doses on aflatoxin biosynthesis. She found that aflatoxin level was reduced in a dose-dependent manner, with the greatest reduction (90% reduction) obtained when the fungus was treated using 146ppm ethylene. In a study on the effect of CO_2 on aflatoxin production, CO_2 was most effective at 0.1% and adding more CO_2 reduced the inhibitory effect. Dr. Roze also found that when CO_2 and ethylene were used together, there was an additive inhibitory effect.

Ethylene was also observed to decrease the level of transcription of the *nor-1* gene in *A. parasiticus* (Roze et al., 2004). Studies were done by measuring GUS activity, which indirectly measures *nor-1* promoter function. In colonies that were treated with 0.6, 5.6, and 12.6ppm ethylene, GUS activity was undetectable. Since *nor-1* is one of the early genes in the aflatoxin biosynthetic pathway, reduction in transcription of this gene, indicated that ethylene may negatively affect the function of this gene and result in lower aflatoxin production.

The objective of this study was to observe the effects of ethylene, CO_2, and a combination of the two on Aflatoxin B_1 biosynthesis by *Aspergillus parasiticus* grown on peanuts. The earlier studies described above had indicated

that ethylene reduced aflatoxin production by *A. parasiticus*. However, both of the studies were conducted using synthetic media. The hypothesis of the current study was that ethylene reduces aflatoxin synthesis when the fungus is grown on a typical plant substrate.

We observed that ethylene reduced aflatoxin in a dose-dependent manner and the highest reduction was obtained when *A. parasiticus* was treated with 100ppm ethylene (approximately 85% reduction). CO_2 (0.1%) also inhibited aflatoxin production by *A. parasiticus*. An additive effect of the two was shown in one experiment.

MATERIALS AND METHODS

Fungal strain, spore numbers, inoculation method

The *A. parasiticus* strain (D8D3) used in this study was isogenic and derived from the parent strain SU-1 (ATCC 56775), a wild type aflatoxin producer (Chiou et al., 2002). *A. parasiticus* D8D3 contains the GUS (*uidA*; encodes β-D-glucuronidase) reporter fused to the *nor-1* promoter. Either approximately 10^3 conidiospores/g peanuts or approximately 10^6 conidiospores/g peanuts were inoculated depending on the experiment. Conidiospores of D8D3 were stored frozen (-80°C) in 20% glycerol-water solution until use. Spores for inoculation were prepared by diluting the spore stock with sterilized deionized distilled water (ddH_2O) to the desired spore concentration (10^4 and 10^7). Spores were quantified using hemacytometer. Frozen spores were thawed prior to diluting. Dilutions

were done using 1.5ml micro-centrifuge tubes. The spores were diluted 10 times (100µl spore stock in 900µl dH_2O) until the desired concentration was obtained. 100µl of the diluted spores were added to 4 grams of peanuts in a 50ml conical centrifuge tube. The spores and peanuts were mixed by light agitation. The inoculated peanuts were then placed onto a sterile Petri plate lined with wet filter paper. 2 ml of sterilize ddH_2O were added to wet the filter paper prior to adding the inoculated peanuts.

Peanut cultivars

Peanuts used in initial studies were bought locally. However, since the genotype of these peanuts was unknown, the remainder of the studies were conducted using the near isogenic, Georgia Green peanut cultivar. This was kindly provided by Dr. Corley Holbrook, USDA-ARS, Tifton, GA.

The peanuts already had the shells removed. Prior to use, the testa were removed by soaking the peanuts in water for approximately 5 seconds, 3 times to soften the testa. The testa were removed by hand and the peanuts were air-dried under the hood for at least 2 hours.

Growth conditions, flow through system

Fungal growth experiments were conducted in desiccator jars (Figure 2.1D). Each desiccator was flushed with air mixed with different amounts of CO_2 and/or ethylene depending on the treatment. The gas flow in the desiccators was

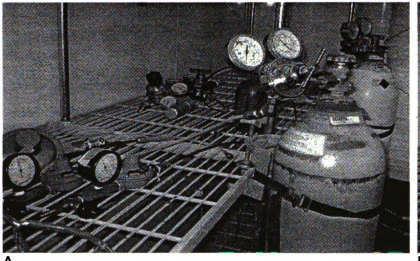
maintained at 100ml/min. Three Petri plates with inoculated peanuts were placed in each chamber resulting in triplicate samples for each treatment.

In each chamber where no CO_2 and/or ethylene was necessary, a CO_2 and ethylene scrubber was placed in the chamber.

Gas flowed from tanks through 2 regulators and through a reservoir of water (Figure 2.1B). This water served as a source of moisture in the desiccators. From here, the gas flowed through different size capillaries that determined the final concentration of each gas. The gas then flowed to small mixing chambers where the different gases were mixed according to the desired concentration (Figure 2.1C). From each mixing chamber, the gas flowed to desiccators carrying the inoculated and control treatments (Figure 2.1D). Desiccators carrying inoculated peanuts were incubated for 5 days (unless otherwise noted) in the dark. The room was maintained at 30°C at 100% relative humidity.

Aflatoxin extraction

Extraction of aflatoxins from peanuts was carried out using AOAC official method 990.34 with slight modifications. After incubation, inoculated peanuts were removed using heat-sterilized forceps and ground using sterilized mortar and pestles. The ground peanuts were then placed onto 50ml conical tubes (Corning Sterile Disposable Polypropylene Centrifuge Tubes, Corning Inc., Corning, NY 14831). 8ml of 80% methanol-water was added into each tube. The tubes were then mixed by vortexing for 3 minutes. The samples were allowed



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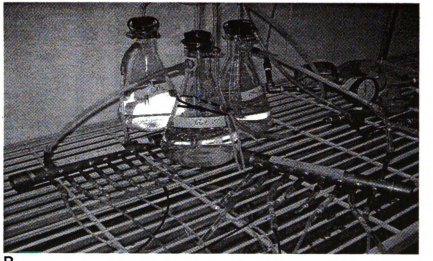

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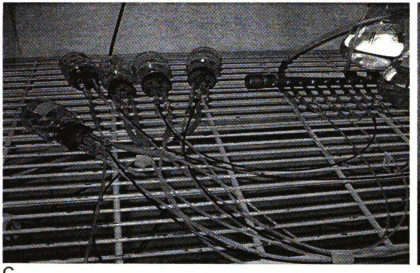

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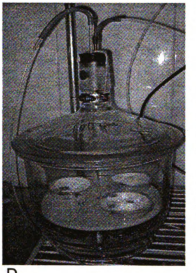


D

Figure 2.1. Flow Through System
A. Gases flow from tanks through 2 regulators. B. From regulators, gases flow through flask of water to C. mixing chambers, where the gases are mixed according to the concentration desired for each treatment. From each mixing chamber, the gas flows into D. desiccator (in-port). The out-port in the desiccator allows the gas from the desiccator to flow out.

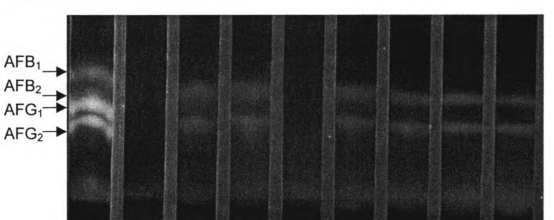
to incubate for 15 minutes and the liquid phase was then removed into another tube. These tubes were centrifuged in an IEC PR-600 Centrifuge (Thermo Electron Corp., Marietta, OH) at 2500rpm, 10°C for 10 minutes to remove spores, mycelia, peanut materials, and other solids from the supernatant. After centrifugation, the supernatant, that was now virtually free from foreign materials, was collected into a scintillation vial (wide mouth 20ml low background glass vial, Research Products International Corp., Mt. Prospect, IL). These supernatants were then evaporated using the Meyer N-Evap Analytical Evaporator (Organomation Associates, Inc., South Berlin, MA) under a stream of 99.8% oxygen free nitrogen in a hot water bath (approximately 50°C). After all liquids were completely evaporated, the toxin was resuspended in 1ml 70% methanol-water. Samples prepared using this protocol were left for at least 1 day prior to analysis. Just before analysis, mixtures were placed into 1.5ml screw-cap micro centrifuge tubes and were centrifuged for 5 minutes (13,000rpm, room temperature) to remove unwanted materials that might remain.

Aflatoxin analysis

Aflatoxin was quantified using ELISA (Enzyme Linked Immuno-Sorbent Assay). TLC (Thin Layer Chromatography) was used to confirm the trends observed in the ELISA data. ELISA was conducted using the method of Pestka et al. (1980). Antibody against Aflatoxin B_1 was purchased from Sigma (Anti-aflatoxin B_1-peroxidase conjugate antibody produced in rabbit, Sigma-Aldrich, St. Louis, MO) and HRP (Horse Radish Peroxidase) conjugate was prepared in the

lab by Skory (Skory, 1992). Aflatoxin concentrations reported are representative of three plates. Error bars represent standard error of the means. This ELISA method has been used extensively in our laboratory (Chiou et al., 2002; Roze et al., 2004; Miller, 2003)

TLC plates used were Partisil® LHPKD silica gel 60Å, 10 X 10cm, 200µm thick, plates (Whatman Inc., Clifton, NJ). Samples (5µl for each lane) were spotted in the loading zone while maintaining the smallest spot size possible. Plates were placed in a chamber containing 95% chloroform-acetone until the solvent front traveled appropriate distance. Plates were analyzed under UV light to observe bands. Each of the bands was then analyzed using a densitometer to quantify band intensity. A true reading of the band intensity was obtained by subtracting reading from the intensity of the background since a significant portion of the signal came from the background. The images were taken using Kodak DC 290 Zoom Digital Camera and band intensity was analyzed using Kodak 1D 3.6 Image Analysis Software.



Above is an example of TLC analysis. Although the samples and standards did not comigrate at the same rate in certain experiments, we were able to identify

the top band as aflatoxin B_1 and the lower band as aflatoxin G_1 based on the color of the bands under UV light. The difference in comigration between the samples and the standards in certain experiments could not be corrected even when we treated the samples with a clean up step prior to analysis.

Statistical analysis

When the data followed a normal distribution and displayed equal variances, one-way ANOVA followed by Boneferonni's multiple comparison tests were used to analyze significant differences between treatments. When data did not follow normal distribution or did not display equal variances, ANOVA for heterogenous variances was used. Below is a table of the test used for analyzing ELISA results from each experiment. Since all TLC data followed normal distribution and displayed equal variances ANOVA with Boneferonni's multiple comparison tests were used to analyze densitometry results.

CO_2-store bought peanuts	ANOVA with Boneferonni's
CO_2-Georgia Green (1)	ANOVA for heterogeneous variances
CO_2-Georgia Green (2)	ANOVA with Boneferonni's
Ethylene dose response (1)	ANOVA for heterogeneous variances
Ethylene dose response (2)	ANOVA with Boneferonni's
Spore load study (1)	ANOVA for heterogeneous variances
Spore load study (2)	ANOVA for heterogeneous variances

Table 2.1. Statistical analysis of ELISA results

Most data that did not follow normal distribution or have equal variances were data generated in early experiments. This indicated that the experimental methods were improved throughout the study and yielded more reproducible results. The variation in the results of early studies may also be due to our inability to effectively control CO_2 levels during the experiment that resulted in

variation in aflatoxin produced. Also, since ELISA is more sensitive, it is more susceptible to variation within the experiment and therefore the variation in the ELISA results was greater than variation in the TLC densitometry results. However results of both types of analysis produced similar trends.

Gas and gas analysis

All gases (medical air, CO_2, and ethylene/air mixture) were purchased from Linde gas (Cleveland, OH) unless otherwise specified. In some experiments, the ethylene/air gas mixtures were made in the lab by mixing medical air and a certain amount of ethylene, according to the desired final concentration of ethylene in the tank. These gas mixtures, either made in the lab or purchased, yielded no differential effects in the experiments.

O_2, CO_2, and ethylene concentrations were monitored daily (with minor exceptions as indicated) using GC (Gas Chromatography). There were 2 different GCs, one for ethylene only (Carle AGC series 400, HACH Carle Chromatography, Loveland, CO) and the other for CO_2 and O_2 (ADC.225.MK3, The Analytical Dev. Co. Ltd., Hoddesdon, England). Samples were injected into the sample ports. Standards were analyzed prior to running samples. Sample concentrations were then calculated using the standards. Samples were taken from both the in-port and out-port of each desiccator.

RESULTS

1. Ethylene, CO_2, and ethylene combined with CO_2 inhibited aflatoxin production in *A. parasiticus*.

a. Store-bought peanuts

In our first experiment, we inoculated store-bought peanuts with 10^6 conidiospores/g peanuts. The treatments used were 0.05% CO_2, 0.1% CO_2. 0.05% CO_2 + 2 ppm ethylene, 0.1% CO_2 + 2 ppm ethylene, and 2 ppm ethylene. The treatments were chosen based on a study conducted previously by Dr. Ludmila Roze using solid defined growth media (GMS). The Petri plates were incubated for 6 days in the dark, and other conditions were carried out as stated in the materials and methods section.

Gas concentrations for this particular experiment were measured at day 3, at the in-port and on day 5 of the experiment, at the in-port and out-port (Table 2.2). The ethylene concentration on the last day (1.07 ppm) was below the expected value, 2 ppm. The reason was unknown but predicted to result from a failure of the gas regulators. The time when the ethylene concentration started decreasing was also not known because gas concentrations were not analyzed on days 4 and 5 due to instrument malfunction.

CO_2 (0.05%) decreased aflatoxin production by approximately 75%, while 0.1% CO_2 decreased it by approximately 60%. Adding 2 ppm ethylene together with CO_2 decreased aflatoxin further at 0.1% CO_2 but not at 0.05% CO_2. 2ppm ethylene alone decreased aflatoxin production by 80%. The highest reduction

was obtained by using 2 ppm ethylene and 2 ppm ethylene + 0.1% CO_2 (see Figure 2.2). These trends observed by ELISA were confirmed by TLC (Figure 2.3). Although all treatments did not yield significant statistical differences, the trend shows differences between them.

Because aflatoxin produced in this study was low compared to studies conducted using GMS defined medium (Roze et al., 2004), we thought that this may be due to the inoculum level. Sharma et al. (1980) and Clevstrom and Ljunggren (1983) found that when the inoculum was reduced by half, aflatoxin increased 8 to 10 fold over 5 days. A study to confirm the effect of inoculum size was conducted (described later in this chapter) and the results suggested that the highest amount of aflatoxin produced with an even growth of mold on the peanuts, was at 10^3 conidiospores/g peanuts. This was consistent with a study conducted by Karunaratne and Bullerman (1990) who suggested that maximum aflatoxin production occurred at spore loads of 10^3spores/ml. For the remainder of the studies, an inoculum of 10^3 conidiospores/g peanuts was used instead of 10^6 conidiospores/g peanuts to better demonstrate the effects of CO_2 and ethylene.

The peanuts used in our initial studies were bought locally. The cultivar was unknown and the composition could be different throughout the package. Therefore, the peanut cultivar Georgia Green was obtained from USDA- ARS in Tifton, GA and was used in latter studies. This cultivar is a commonly used, commercially sold, peanut cultivar.

Treatments	CO_2 concentration (%)		
	Day		
	3 (in-port)	6 (mixing chambers)	6 (out-port)
Air	0.03	0.04	0.05
0.05% CO_2	0.43	0.04	1.01
0.1% CO_2	0.14	0.05	0.24
0.05% CO_2 + 2 ppm ethylene	0.05	0.05	0.25
0.1% CO_2 + 2 ppm ethylene	0.15	0.07	0.27
2 ppm ethylene	0.03	0.05	0.27

A.

Treatments	Ethylene concentration (ppm)		
	Day		
	3 (in-port)	6 (mixing chambers)	6 (out-port)
Air	~0.0063	ND	ND
0.05% CO_2	~0.0063	ND	ND
0.1% CO_2	~0.0063	ND	ND
0.05% CO_2 + 2 ppm ethylene	2.09	0.826	0.82
0.1% CO_2 + 2 ppm ethylene	1.87	0.759	0.79
2 ppm ethylene	2.25	0.888	1.07

B.

Table 2.2. Gas concentrations during the CO_2, ethylene, and CO_2 + ethylene experiment using store-bought peanuts. A. CO_2 concentrations and B. Ethylene concentrations. ND denotes there are no detectable peaks.

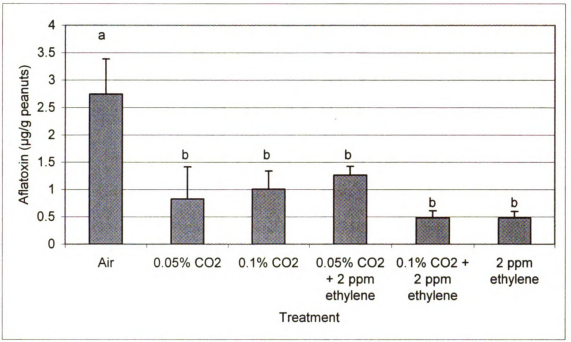


Figure 2.2. Effect of CO_2, ethylene, and mixture of ethylene and CO_2 on aflatoxin accumulation by *A. parasiticus* grown on store-bought peanuts analyzed by ELISA. *A. parasiticus* D8D3 (10^6/g peanuts) was grown on raw peanuts in the flow through system for 6 days at 30°C. Aflatoxins from three replicate samples were extracted and analyzed by ELISA. Bars represent standard error for each treatment. a is statistically different than b ($P<0.05$).

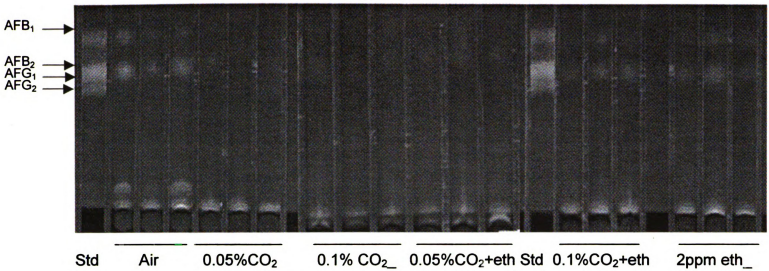


Figure 2.3. Effect of CO_2, ethylene, and mixture of ethylene and CO_2 on aflatoxin accumulation by *A. parasiticus* grown on store-bought peanuts (TLC). *A. parasiticus* D8D3 (10^6/g peanuts) was grown on raw peanuts in the flow through system for 6 days at 30°C. Ethylene concentration used in 0.05%CO_2+eth and 0.1% CO_2+eth was 2 ppm. Aflatoxins from three replicate samples were extracted and analyzed by TLC.

b. Georgia Green peanuts with lower inoculum size

Our next experiment was designed to confirm effects of CO_2, ethylene, and combination of the two using Georgia Green and the new inoculum size. 0.05% CO_2 treatment was excluded in the remainder of the study because the aflatoxin reduction resulted by this treatment was not sufficient to be implemented in commercial use.

Gas concentrations were analyzed at day 1, 3, 4, and 5. The CO_2 concentration in the 0.1% CO_2 + 2ppm treatment chamber was not stably maintained. The CO_2 concentration was highest at day 3 (0.9%). The CO_2 concentration was adjusted and was measured at 0.5% throughout the remainder of the experiment. Also, the CO_2 concentration in the 0.1% CO_2 treatment chamber was maintained at around 0.3% throughout the experiment. At that time we could not obtain a concentration of 0.1% CO_2 because the capillaries were not appropriate to produce the desired concentration. In the ethylene only treatment, the CO_2 scrubber was also not used to represent the same condition as in experiment in above experiment (a.). However, in the next experiments, a CO_2 scrubber was used in the ethylene only treatment to differentiate CO_2 effect from ethylene effect (refer to Table 2.3, for gas concentration throughout the experiment).

Treatment with 0.1% CO_2 + 2ppm ethylene reduced aflatoxin production by more than 90%. Ethylene alone and 0.1% CO_2 alone also reduced aflatoxin by 80% and 85% respectively (Figure 2.4). These ELISA results were confirmed

by TLC (Figure 2.5). TLC bands were analyzed using a densitometer and the results agreed with visual analysis (Figure 2.6).

Treatments	CO_2 concentration (%)							
	Day							
	1	2	3		4		5	
	Mixing chambers	In-port	In-port	Out-port	In-port	Out-port	In-port	Out-port
Air	0.036	0.038	0.041	0.011	0.042	0.004	0.043	ND
0.1% CO_2	0.35	0.32	0.286	0.755	0.273	0.004	0.273	0.621
0.1% CO_2 + 2 ppm ethylene	0.104	0.038	0.896	0.264*	0.485	0.507	0.492	0.528
2 ppm ethylene	0.039	0.033	0.043	0.686	0.035	0.71	0.034	0.702

A.

Treatments	Ethylene concentration (ppm)							
	Day							
	1	2	3		4		5	
	Mixing chambers	In-port	In-port	Out-port	In-port	Out-port	In-port	Out-port
Air	ND	0.03	0.04	ND	ND	ND	ND	ND
0.1% CO_2	ND	0.07	ND	ND	ND	ND	ND	ND
0.1% CO_2 + 2 ppm ethylene	1.947	2.47	1.99	2.22	2.44	2.34	2.131	2.131
2 ppm ethylene	1.956	2.44	2.22	2.35	2.31	2.24	2.1	2.124

B.

Table 2.3. Gas concentrations during the CO_2, 2ppm ethylene, and CO_2 + 2ppm ethylene experiment using Georgia Green peanuts. A. CO_2 concentrations and B. Ethylene concentrations. ND denotes there are no detectable peaks.

*This gas concentration measured at the in-port was adjusted to 0.096.

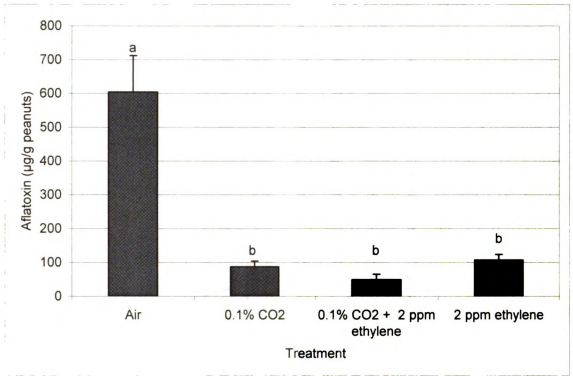


Figure 2.4. Effect of CO_2, ethylene, and mixture of 2 ppm ethylene and CO_2 on aflatoxin accumulation by *A. parasiticus* grown on Georgia Green peanuts with lower inoculum size (ELISA). *A. parasiticus* D8D3 (10^3/g peanuts) was grown on raw peanuts in the flow through system for 5 days at 30°C in the dark. Aflatoxins from three replicate samples were extracted and analyzed by ELISA. Bars represent standard error of the means for each treatment. a is statistically different than b ($P<0.05$)

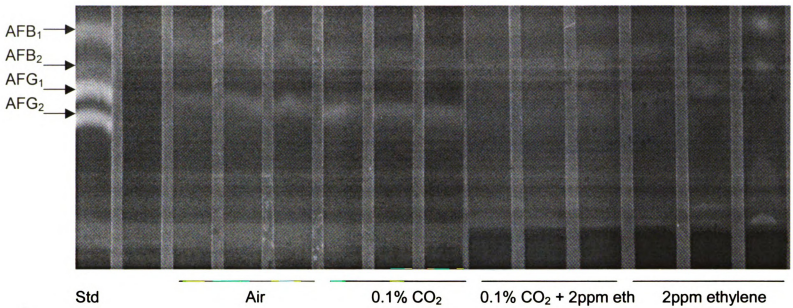


Figure 2.5. Effect of CO_2, ethylene, and mixture of ethylene and CO_2 on aflatoxin accumulation by *A. parasiticus* grown on Georgia Green peanuts with lower inoculum size (TLC). *A. parasiticus* D8D3 (10^3/g peanuts) was grown on raw peanuts in the flow through system for 5 days in the dark at 30°C. Aflatoxins from three replicate samples were extracted and analyzed by TLC.

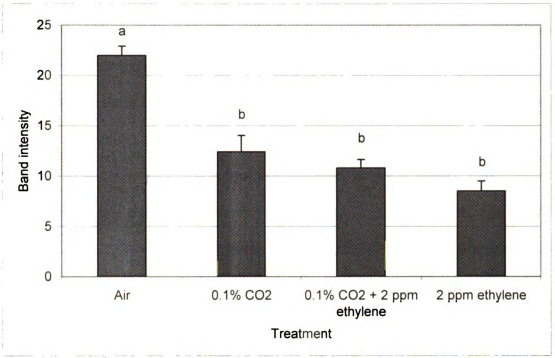


Figure 2.6. Densitometry analysis of TLC bands from Figure 2.5. a is statistically different than b ($p<0.05$). Bars represent standard error of the means.

c. New CO_2 scrubber, new CO_2 scrubbing method, two different doses of ethylene

The next experiment was conducted to correct problems with experimental protocol from the experiments described above. Two different ethylene concentrations were used in this experiment to observe effects of 0.1% CO_2 + 100 ppm as the 100 ppm treatment has repeatedly generated the highest reduction in aflatoxin production (data shown later in this chapter). The treatments for this experiment were: air, 0.1% CO_2, 2 ppm ethylene, 100 ppm ethylene, 0.1% CO_2 + 2 ppm ethylene, and 0.1% CO_2 + 100 ppm ethylene.

Gas concentrations for this experiment were measured every day with the exception of day 4. The ethylene gas tank was found empty on day 5. However, when measured at the out-port of all chambers receiving ethylene, measurable ethylene remained suggesting that the tank had only been empty for a short period of time (Table 2.4). The ethylene concentration was not measured at the out-port on day 1 because gas concentrations measured at the in-port were as expected. At day 5, ethylene was not measured at the in-port because the tank was empty, which means there was no ethylene going into the desiccator jars.

The biggest effect on reduction of aflatoxin was in the 100 ppm ethylene treatment. Adding CO_2 in addition to the ethylene did not seem to enhance the effect as in the previous experiment (Figure 2.7). TLC results (Figure 2.8), when analyzed using a densitometer confirmed the ELISA results (Figure 2.9).

Treatments	CO_2 concentration (%)								
	Day								
	0	1		2		3		5	
	Mixing chambers	In-port	Out-port	In-port	Out-port	In-port	Out-port	In-port	Out-port
Air	0.01	0.009	0.009	0.01	0.02	0.01	0.02	0.01	0.03
2 ppm ethylene	0.01	0.009	0.009	0.01	0.02	0.01	0.02	0.01	0.03
100 ppm ethylene	0.01	0.009	0.009	0.01	0.02	0.02	0.02	0.01	0.03
0.1% CO_2	0.11	0.08	0.16	0.06*/0.09	0.29	0.08	0.37	0.1	0.35
0.1% CO_2 + 2ppm	0.13	0.08	0.19	0.06*/0.09	0.28	0.08	0.38	0.1	0.38
0.1% CO_2 + 100ppm	0.12	0.08	0.13	0.05*/0.07	0.18	0.07	0.22	0.15	0.43

A

Treatments	Ethylene concentration (ppm)						
	Day						
	0	1	2		3		5
	Mixing chambers	In-port	In-port	Out-port	In-port	Out-port	Out-port
Air	ND	ND	ND	ND	ND	ND	ND
2 ppm ethylene	1.67	1.13	2.23	2.17	2.18	2.2	0.09
100 ppm ethylene	116.1	83.85	121.45	126.97	122.37	121.33	6.23
0.1% CO_2	ND	ND	ND	ND	ND	ND	ND
0.1% CO_2 + 2ppm	1.53	1.02	2.08	2.09	1.97	2.13	0.09
0.1% CO_2 + 100ppm	106.05	90.35	120.41	135.94	126.16	125.47	5.12

B

Table 2.4. Gas concentrations during CO_2, ethylene, and CO_2 + ethylene experiment with new CO_2 scrubber. A. CO_2 concentrations and B. Ethylene concentrations. (*CO2 concentrations in these chambers were adjusted accordingly). ND denotes there are no detectable peaks.

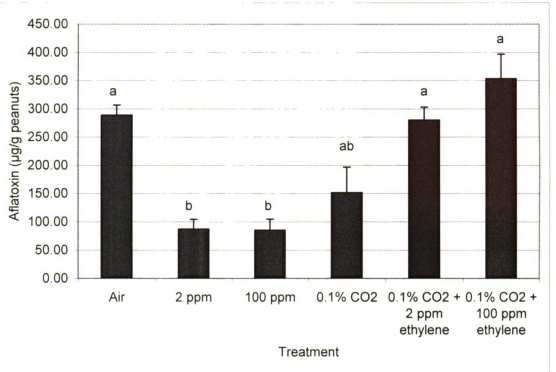


Figure 2.7. Effect of CO_2, ethylene, and mixture of ethylene and CO_2 on aflatoxin accumulation by *A. parasiticus* grown on peanuts using new CO_2 scrubber (ELISA). *A. parasiticus* D8D3 (10^3/g peanuts) was grown on Georgia Green peanuts in the flow through system for 5 days at 30°C in the dark. Aflatoxins from three replicate samples were extracted and analyzed by ELISA. Bars represent standard error of the means for each treatment. a is statistically different than b ($P<0.05$). For the 0.1% CO_2 and 2ppm treatments, there were only 2 samples for each treatment because one was contaminated by another fungus, resulting in very low aflatoxin (outliers). These samples therefore were excluded from analysis.

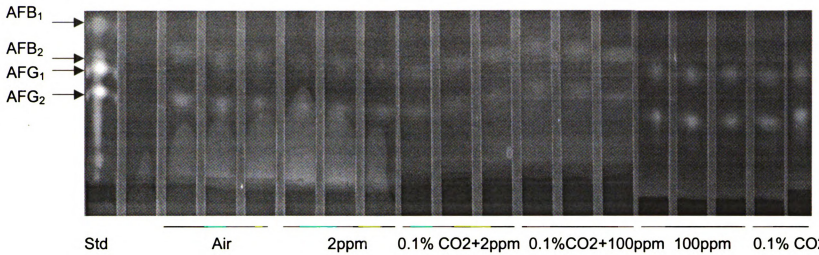


Figure 2.8. Effect of CO_2, ethylene, and mixture of ethylene and CO_2 on aflatoxin accumulation by *A. parasiticus* grown on Georgia Green peanuts with new CO_2 scrubber (TLC). *A. parasiticus* D8D3 (10^3/g peanuts) was grown on raw peanuts in the flow through system for 5 days in the dark at 30°C. Aflatoxins from three replicate samples were extracted and analyzed by TLC. One of the samples from 0.1% CO_2 was contaminated by another fungus, aflatoxin was very low in this sample and therefore this sample was excluded from analysis. The first lane in the 2ppm treatment was also contaminated and was also excluded from analysis.

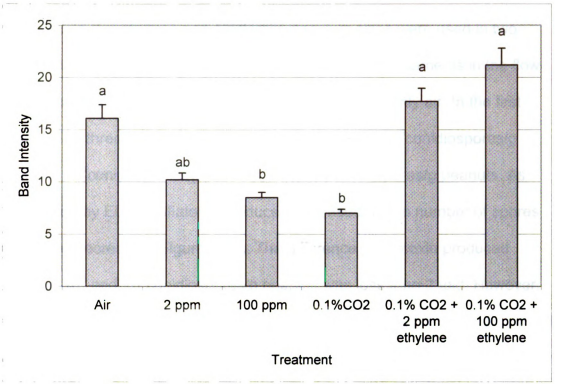


Figure 2.9. Densitometry analysis of TLC bands from Figure 2.8. a is statistically different than b ($p<0.05$). Bars represent standard error of the means.

2. Effect of inoculum size on aflatoxin production

A study was conducted to verify the effect of inoculum size on aflatoxin produced. In this study, 3 and 4 different inoculum sizes were used in two independent experiments. All growth conditions were the same as in the flow through system, but no CO_2 or ethylene were included, only air. In the first experiment, three different inoculum sizes were used: 10^5conidiospores/g peanuts, 10^4conidiospores/g peanuts, and 10^3conidiospores/g peanuts. As determined by ELISA, aflatoxin produced increased as the number of spores inoculated decreased (Figure 2.10). The difference in aflatoxin produced between 10^5 and 10^4conidiospores/g peanuts was less than 2 fold. However, aflatoxin increased almost 10 fold with 10^3conidiospores/g peanuts compared to 10^4conidiospores/g peanuts. This ELISA result was confirmed using TLC (Figure 2.11). In a repeat of this experiment, 4 different inoculum sizes were used; 10^5conidiospores/ g peanuts, 10^4conidiospores /g peanuts, 10^3conidiospores /g peanuts, and 10^2conidiospores/g peanuts. The 10^2conidiospores/g peanuts inoculum size was included to see if this treatment would extend the trend observed above. This experiment verified the trend obtained from the previous experiment (Figure 2.12); that is lower inoculum size resulted in higher levels of aflatoxin produced. The 10^2conidiospores/g peanuts inoculum produced 20 fold more aflatoxin than the 10^3 inoculum size. However, growth at the 10^2 inoculum size did not evenly coat the peanuts. Therefore, the inoculum size chosen to carry out subsequent experiments was the 10^3conidiospores/g peanuts.

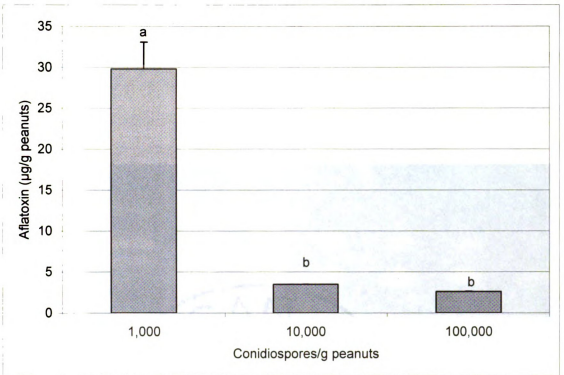


Figure 2.10. Effect of inoculum size on aflatoxin production by *A. parasiticus* – first study (ELISA). Each inoculum was grown on 4 grams of raw peanuts in the dark for 5 days at 30°C. There were 2 plates for each treatment. Bars represent standard error of the means for each treatment. a is statistically different than b ($p<0.05$).

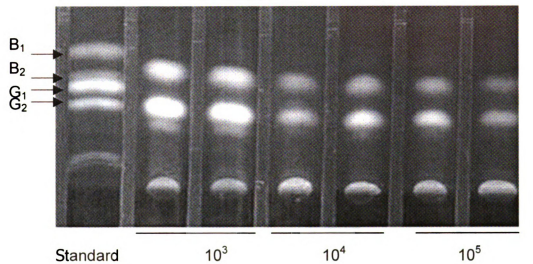


Figure 2.11. TLC analysis of effect of inoculum size on aflatoxin production. The top band represents aflatoxin B_1 and the second band represents aflatoxin G_1. Both bands were more intense when inoculum size decreased.

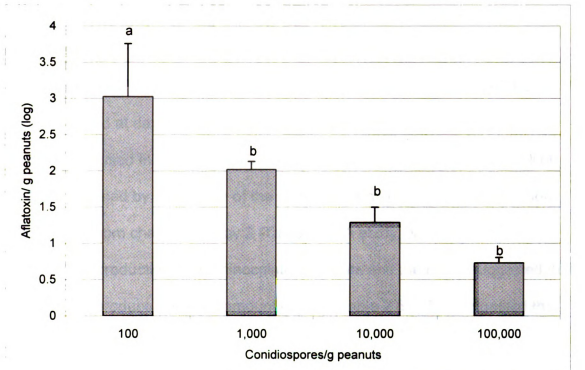


Figure 2.12. Effect of inoculum size on aflatoxin production by *A. parasiticus* – repeat of study in Figure 2.10 (ELISA). Each inoculum was grown on 4 grams of raw peanuts in the dark for 5 days at 30°C. There were 3 plates for each treatment. Bars represent standard error of the means for each treatment. a is statistically different than b ($p<0.05$).

3. Effect of ethylene concentration on aflatoxin produced by *A. parasiticus*

Five different ethylene concentrations were used: 2 ppm, 10 ppm, 25 ppm, 70 ppm, and 100 ppm. A CO_2 scrubber (soda lime) was used in all treatments to exclude the effects of CO_2. These ethylene concentrations were chosen to represent different ranges of ethylene between 2 to 100 ppm based on results from the study conducted by Dr. Ludmila Roze.

Analysis of gas concentration was conducted at the beginning of the experiment and at day 1, 3, and 5 (refer to table 2.5). Even though a CO_2 scrubber was used in each chamber, the scrubber was unable to remove all of the CO_2 produced by respiration of the fungus. The highest CO_2 concentration was in the 10ppm chamber at day 3 (Table 2.5). A study was conducted to observe CO_2 production from uninoculated peanuts and the result suggested that peanuts only produce a minute amount of CO_2 (Table 2.6). This suggested that the CO_2 produced during the incubation period was predominantly produced by the fungus.

The greatest aflatoxin reduction was obtained under 100 ppm ethylene, although significant differences were not observed between treatments with 70 ppm, 100 ppm, and 25 ppm (Figure 2.13). 100 ppm, 70 ppm, and 25 ppm ethylene reduced aflatoxin production by approximately 20% while 2 ppm and 10 ppm reduced aflatoxin production by 50% and 70% fold respectively (Figure 2.13). ELISA results were confirmed using TLC (Figure 2.14).

Treatments	CO_2 concentration (%)			
	Day			
	0 (Mixing chambers)	1 (Out-port)	3 (Out-port)	5 (Out-port)
Air	0.03	0.01	0.06	0.05
2 ppm	0.03	0.01	0.05	0.05
10 ppm	0.03	0.01	0.08	0.06
25 ppm	0.02	0.004	ND	ND
70 ppm	0.02	0.003	ND	ND
100 ppm	0.01	0.004	0.05	0.04

A.

Treatments	Ethylene concentration (ppm)			
	Day			
	0 (Mixing chambers)	1 (Out-port)	3 (Out-port)	5 (Out-port)
Air	ND	ND	ND	ND
2 ppm	2.28	1.53	1.57	1.45
10 ppm	8.65	6.31	6.32	6.91
25 ppm	31.51	29.8	27.43	29.75
70 ppm	74.45	74.98	66.57	70.4
100 ppm	111.02	106.04	99.43	107.12

B.

Table 2.5. Gas concentrations for ethylene dose response experiment. A. CO_2 concentrations and B. Ethylene concentrations. ND denotes there are no detectable peaks.

Chamber	CO_2-day 1 (%)	CO_2-day 3 (%)	CO_2-day 5 (%)
With scrubber – In port	0.03	0.03	0.05
With scrubber – Out port	0.009	0.009	0.007
Without scrubber – In port	0.03	0.04	0.04
Without scrubber – Out port	0.07	0.06	0.05

A.

Chamber	CO_2-day 1 (%)	CO_2-day 3 (%)	CO_2-day 5 (%)
With scrubber – In port	0.03	0.04	0.04
With scrubber – Out port	0.009	0.009	0.02
Without scrubber – In port	0.04	0.04	0.03
Without scrubber – Out port	0.06	0.08	0.10

B.

Table 2.6. CO_2 concentrations during the study to observe CO_2 accumulation by peanuts. Data in B represent a repeat of experiment. Experiments were conducted in the flow through system with air only; all other conditions remained as in other experiments conducted in the flow through system. Three plates of uninoculated peanuts were placed in each chamber. There were two chambers; one with CO_2 scrubber and the other without.

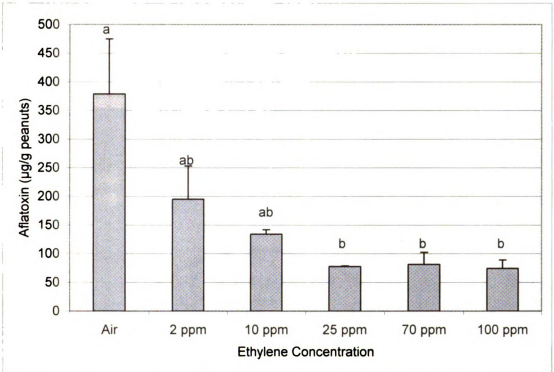


Figure 2.13. Effect of ethylene concentration on aflatoxin accumulation by *A. parasiticus* grown on peanuts – first study (ELISA). *A. parasiticus* D8D3 (10^3/g peanuts) was grown on Georgia Green peanuts in the flow through system for 5 days at 30°C in the dark. Aflatoxins from three replicate samples were extracted and analyzed by ELISA. Bars represent standard error of the means for each treatment. a is statistically different than b ($P<0.05$).

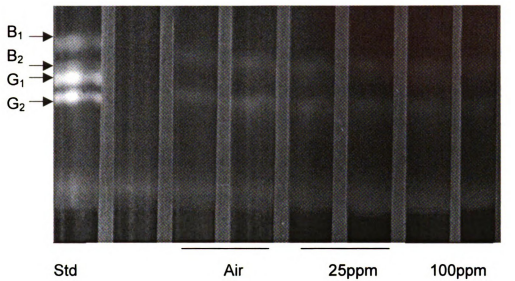


Figure 2.14. Effect of of ethylene concentrations on aflatoxin accumulation by *A. parasiticus* grown on peanuts (TLC). *A. parasiticus* D8D3 (10^3conidiospores/g peanuts) was grown on raw peanuts in the flow through system for 5 days in the dark at 30°C. Aflatoxin extracts from two replicate samples were analyzed by TLC.

We repeated the experiment to verify the effect of ethylene concentrations on aflatoxin biosynthesis by *A. parasiticus*. Since the CO_2 scrubber used in the previous experiment above did not efficiently scrub all of the CO_2 in the desiccator jars, a new CO_2 scrubber was used for the repeat. The new CO_2 scrubber material was hydrated lime (CaOH). Also, instead of placing a plate of the CO_2 scrubber only on the bottom of each desiccator, three small pouches filled with this scrubber material were placed between the three plates. Finally, the air used for the repeat of this experiment was obtained from atmospheric air and not from medical air tanks as in previous experiments. A tube of CO_2 scrubber was placed in the incoming air line to eliminate any CO_2 coming into the system. The same thing was done for the ethylene tank.

Gas concentrations were measured on day 1, day 2, and day 5 (Table 2.7). In this experiment, the CO_2 concentration was very low across all treatments, indicating that the new CO_2 scrubber and the minor modifications of the system worked very well in eliminating the CO_2 coming into the system and accumulation of CO_2 in the system due to fungal respiration.

The results from this experiment were similar to the previous experiment. Lowest aflatoxin levels were obtained after treatment with 25 and 100ppm, although treatment with 70ppm yielded no significant difference with these two. However, the aflatoxin concentration in the 70ppm treatment was significantly higher than in the 25ppm treatment (Figure 2.15). TLC (Figure 2.16) results appeared to agree with ELISA results, when analyzed using a densitometer, and confirmed the trend observed in the ELISA results (Figure 2.17)

Treatments	CO2 concentration (%)						
	Day						
	0	1		2		5	
	In-port	In-port	Out-port	In-port	Out-port	In-port	Out-port
Air	ND	ND	ND	ND	0.02	0.007	0.02
2 ppm	ND	ND	ND	ND	0.02	0.005	0.02
10 ppm	ND	ND	ND	ND	0.01	0.005	0.02
25 ppm	ND	ND	ND	ND	0.02	0.005	0.03
70 ppm	ND	ND	ND	ND	0.02	0.007	0.02
100 ppm	ND	ND	ND	ND	0.02	0.003	0.02

A.

Treatments	CO2 concentration (%)					
	Day					
	0	1		2		5
	In-port	In-port	Out-port	In-port	Out-port	Out-port
Air	ND	ND	ND	ND	ND	ND
2 ppm	1.5	2.08	2.27	1.92	1.95	1.83
10 ppm	5.8	9.17	9.17	8.4	8.35	8.04
25 ppm	22.15	31.8	31.21	30.1	26.03	27.69
70 ppm	63.7	83.92	75.38	74.03	71.61	69.94
100 ppm	88.3	111.9	112.49	107.7	108.52	103.39

B.

Table 2.7. Gas concentrations during the repeat of the dose response experiment. Ethylene at the in-port for day 5 was not measured because the ethylene concentrations at the out-port were as expected. A. CO_2 concentrations and B. Ethylene concentrations. ND denotes there are no detectable peaks.

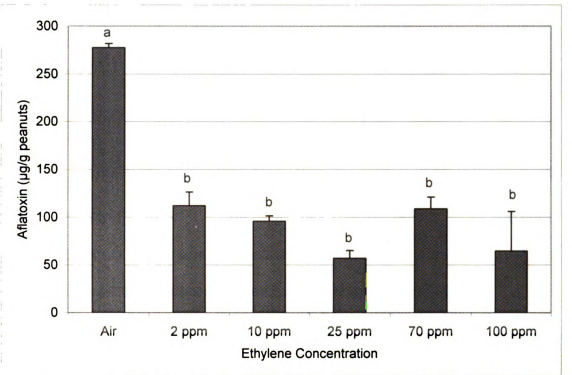


Figure 2.15. Effect of ethylene concentrations on aflatoxin accumulation by *A. parasiticus* grown on peanuts – repeat of study in Figure 2.13 (ELISA). *A. parasiticus* D8D3 (10^3conidiospores/g peanuts) was grown on Georgia Green peanuts in the flow through system for 5 days at 30°C in the dark. Aflatoxins from three replicate samples were extracted and analyzed by ELISA. Bars represent standard error of the means for each treatment. a is statistically different than b ($P<0.05$).

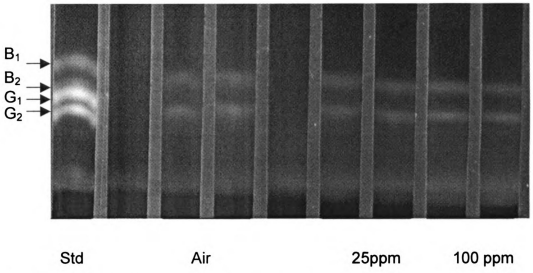


Figure 2.16. Effect of ethylene concentration on aflatoxin accumulation by *A. parasiticus* grown on peanuts analyzed by TLC. *A. parasiticus* D8D3 (10^3conidiospores/g peanuts) was grown on Georgia Green peanuts in the flow through system for 5 days in the dark at 30°C. Aflatoxin extracts from two replicate samples were analyzed by TLC.

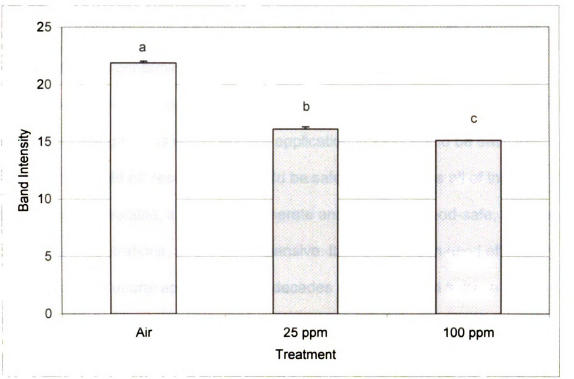


Figure 2.17. Densitometry analysis of TLC bands from Figure 2.16. a is statistically different than b and c, b is statistically different than c ($p<0.05$). Bars represent standard error of the means.

DISCUSSION

The ultimate goal of this study was to find a new, more efficient way to reduce aflatoxin contamination in susceptible food and feed crops. Aflatoxin contamination poses a significant threat to farmers and consumers around the world. An ideal gaseous compound for application in food would be easy to apply, would yield no residue, and would be safe. Ethylene has all of those properties. It is volatile, it is easy to generate and apply, it is food-safe, it works at very low concentrations, and is.not expensive. It also has been used effectively for several agricultural applications for decades and was found to have no negative impact on the nutritive value of some fruits (reviewed in Kays and Beaudry, 1987).

Our data strongly indicated that ethylene significantly reduced aflatoxin production in *A. parasiticus* grown on peanuts similar to the results when the fungus was grown in media. Growth of fungi is associated with production of CO_2 and other volatiles, and uptake of oxygen. To minimize these effects, our experiments were performed in a flow through system which allowed us to study the effects of ethylene only on the fungus. In our experiments, effects of ethylene were observed at very low concentration, 2 ppm.The dose response experiments demonstrated that the ethylene effect is likely saturated at 25ppm because further reduction of aflatoxin at ethylene concentrations higher than 25ppm was not observed.

In our experiments, we saw clear aflatoxin reduction with ethylene treatment only when the air flowing through the desiccators containing inoculated peanuts contained very low level of CO_2 (less than 0.06%). In certain experiments where the level of CO_2 in the gas mixture was higher than 0.09%, we saw reduced level of aflatoxin in the control (approximately 60%) and no further reduction by ethylene (data not shown).

Our data also provide strong evidence that 0.1% CO_2 significantly reduced aflatoxin biosynthesis by *A. parasiticus* grown on peanuts. We also studied the effect of the addition of the two gases, CO_2 and ethylene. However, data from the two experiments performed with both gases did not allow us to draw a clear conclusion because the results were not consistent. In previous experiments conducted in our lab with the fungus grown on chemically defined medium (GMS), however, the addition of CO_2 together with ethylene caused an additive effect on aflatoxin reduction by *A. parasiticus*. The effects of a combination of gases deserves further study.

The aflatoxin concentrations in this study were analyzed using ELISA as well as TLC. ELISA allowed us to detect aflatoxin B_1 primarily while TLC detected all four aflatoxins that were present in the samples. The results from TLC were analyzed using a densitometer. Although the magnitude of the differences in aflatoxin concentrations between control and treated samples shown by TLC were not as great as the ELISA results indicated, the trends that were obtained from the ELISA results were confirmed by the trends from densitometry analysis of TLC bands for the corresponding samples. When different concentrations of

aflatoxin B_1 standards were analyzed using TLC, a 2 fold increase in aflatoxin concentration was reflected by a 1.5 fold increase in band intensity (data not shown). When plotted, the relationship between aflatoxin concentration (ng/ml) and band intensity was linear with R^2= 0.9786.

How does *A. parasiticus* sense ethylene? What is the mechanism? Dr. Ludmila Roze conducted a GUS reporter analysis study in *A. parasiticus* D8D3, where the GUS reporter gene is fused with the *nor-1* promoter (Roze et al., 2004). GUS activity, in this study, indicates level of *nor-1* gene expression. *nor-1* is one of the genes responsible for an early step in the aflatoxin biosynthesis pathway. Results indicated that *nor-1* gene expression decreased to non detectable levels when the fungus was treated with 0.6, 5.6, or 12.6ppm ethylene. A decrease in the expression of the *nor-1* gene suggests that ethylene affects aflatoxin biosynthesis at the level of transcription of the gene. Ludmila also did a study using 1-MCP (methyl-cyclopropene), which is a compound that has similar structure to ethylene and was predicted to act as inhibitor of the ethylene effect because it could compete for the ethylene binding site in the fungus. Addition of this compound to ethylene treated samples showed a decrease in the inhibitory effect of ethylene (Roze et al., 2004). Therefore, ethylene may be sensed by the fungus via an ethylene receptor.

A study that was conducted by Flaishman and Kolattukudy (1994) indicated that some strains of *Colletotrichum* that normally infect climacteric fruits could respond to ethylene produced by this fruit or ethylene produced by the ethylene generating compound, ethephon. Climacteric fruits are fruits that are

characterized by an increase in respiration rate prior to ripening. Flaishman and Kolattukudy suggested that ethylene induces production of appresoria in the fungus, a structure that plays a role during the infection process to enable the fungus to obtain nutrients from the host. Amagai (1987) indicated that ethylene induced macrocyst formation in the slime mold *Dictyostelium mucoroides*.

The mechanism by which CO_2 inhibits aflatoxin biosynthesis is still unknown. Clevstrom et al. (1983) observed that *A. flavus* produced less aflatoxin in an environment with limited oxygen supply, although they did not explain the reason for such results. In bacteria, CO_2 has been found to have negative effects on various enzymatic and biochemical pathways and this results in the anti-microbial property of CO_2 (Daniels et al., 1984). This anti-microbial property observed in bacteria may not explain the inhibitory effect on aflatoxin production by *A. parasiticus* observed in our study because no apparent reduction in growth was observed. However, mycelial dry weight was not analyzed in our experiments. Zonneveld (1988) suggested that when CO_2 is removed during the growth of *A. nidulans*, sexual differentiation was inhibited. This also may not have any impact in the reduced aflatoxin production by *A. parasiticus* since *A. parasiticus* is not known to have a sexual stage.

Studies conducted by Flaishman and Kollatukudy (1994), as well as the work that was done by Amagai (1987), demonstrate that fungi possess an ethylene signal transduction pathway including an ethylene sensor that is likely located on the outer surface of the membrane or inside the cell. We thought that the ethylene receptor in fungi might be similar to receptors in plants. However,

work performed in our lab allowed us to conclude that *A. parasiticus* and *A. nidulans* do not posses a homolog of plant ethylene receptors (Kang, unpublished data). We believe that Aspergilli have ethylene sensors of another nature; they could be protein kinases or more specifically, histidine kinases. Dr. Suil Kang, a member our lab, found novel genes with histidine kinase domains in *A. parasiticus*. One or more of these genes may encode an ethylene receptor but the function of these genes has not yet been identified. Further study is required.

CHAPTER 3

Future Implications

Our data strongly suggest that ethylene and CO_2 inhibit aflatoxin biosynthesis in *A. parasiticus*. However, for these gases to be used effectively in the field or during storage, additional information is required. More experiments will need to be conducted to confirm the additive effect of the two gases, CO_2 and ethylene because the results from our study did not provide strong evidence of this additive effect. It is important to understand the additive effect between the two gases because it is necessary to find the best method in reducing aflatoxin contamination.

More studies will need to be done to observe effects of ethylene on nutritive values and seed composition of crops that are susceptible to aflatoxin contamination. The effects of CO_2 or ethylene on other peanut cultivars, corn, and cotton seed also need to be studied further because these represent crops that are affected by aflatoxin to the greatest extent.

Based on our results, the best inhibitory effect, while maintaining lowest concentration of ethylene, was obtained at 25ppm. Although, ethylene gas is not expensive, it may be important to keep the exposure level as low as possible because excessively high levels of ethylene released or prolonged exposure to lower levels may damage the crops as observed in fruits (reviewed in Beaudry and Kays, 1988).

Applying ethylene in a storage facility may be accomplished by several methods. Sources of ethylene for this application include pressurized cylinders of diluted concentrations of ethylene with non-flammable gas, ethylene generating compounds, and the use of ethylene that is produced from a plant source.

Application of ethylene using pressurized cylinders can be done using two different methods: the shot method where the gas is rapidly released into a sealed room or the flow through method where the gas is slowly metered into a vented room where fresh air is continuously supplied (Gull, 1981). The first of the two could induce CO_2 build up in the system that might interfere with ethylene function. CO_2 concentrations of 2% were observed to inhibit action of ethylene (reviewed in Kays and Beaudry, 1987). The amount of gas that needs to be introduced into the room depends on the volume of the room, concentration of ethylene in the source tank, and final concentration desired in the room. The flow through method is more suitable for our purposes. This method will enable us to introduce ethylene and fresh air over the product while maintaining a low CO_2 level in the room.

Another method is to place an ethylene generating compound inside the storage facility where the susceptible crops are stored. The most common ethylene releasing compounds are 2-chlorophosphonic acid, which has the commercial names of Ethrel® and etephon. Also, since these compounds are now available in aqueous forms, it may also be applied by spraying the crops just after the harvest, prior to storage so that these compounds could start releasing

ethylene early during storage of the crops and the ethylene will inhibit aflatoxin production if any infection occurred in the field.

It may also be beneficial to store crops that are susceptible to aflatoxin contamination together with fruits that produce ethylene. The ethylene produced by these fruits will inhibit aflatoxin production since some fruits have been found to produce high levels of ethylene. Avocadoes, for example, have been shown to have an internal ethylene concentration between 300-700ppm at their climacteric peak (Burg and Burg, 1962).

Because CO_2 may modulate the effect of ethylene, CO_2 concentrations in the storage system will need to be monitored to maintain CO_2 levels lower than 0.05%. Any concentration higher than 0.07% CO_2 appears to act as an antagonist to the ethylene effect. Control of CO_2 levels may be accomplished by placing an appropriate quantity of CO_2 scrubber in the storage facility.

Application of ethylene in the field to inhibit aflatoxin production will need to be studied further. Ethylene clearly affects plant development. Therefore, the timing and dose of ethylene application in the field will be crucial factors to analyze.

The use of ethylene to reduce aflatoxin production in infected crops during storage is very promising. Ethylene is food-safe, very easy to apply, works at very low levels, inexpensive, and has no known negative effects on nutritive value of fruits. More importantly, methods suitable for application of ethylene in a storage facility are available and are currently being used.

LIST OF REFERENCES

Abeles, F.B.; Morgan, P.W.; Saltveit, M.E., Jr. *Ethylene in Plant Biology*, 2nd ed; Academic Press: San Diego, CA; 1992.

Adye, J.; Mateles, R.I. Incorporation of labeled compounds into aflatoxins. *Biochem. Biophys. Acta*, 1964, 86, 418-420.

Amagai, A. Regulation of the developmental modes of Dictyostelium mucoroides by cAMP and ethylene. *Differentiation*, 1987, 36, 111-115.

Anderson, R.A. Detoxification of aflatoxin-contaminated corn; In *Aflatoxin and Aspergillus flavus in Corn*; Diener, U.; Asquith, R.; Dickens, J. Eds.; Southern Cooperative Series Bulletin 279: Auburn University, Auburn, AL; 1983; 87-90.

AOAC Official Methods 990.34 Aflatoxins B_1, B_2, and G_1 in Corn, Cottonseed, Peanuts, and Peanut Butter; In *Official Methods of Analysis of AOAC International*; Horwitz, W. Ed.; AOAC International: Gaithersburg, MA; vol. II; ch. 49; pp. 7-8.

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

Ashie, I.N.A.; Smith, J.P.; Simpson, B.K. Spoilage and shelf-life extension of fresh fish and shellfish. *Crit. Rev. Food Sci. Nut.* 1996, 36, 87-121.

Bartnicki-Garcia, S.; Nickerson, W.J. Induction of yeastlike development in *Mucor* by carbon dioxide. *J.Bacteriol.* 1962, 84, 829-940.

Beaudry, R.M.; Kays, S.J. Application of ethylene-releasing compounds in agriculture. In *Plant Growth and Leaf-Applied Chemicals*; Neumann, P.M. Ed.; CRC Press: Boca Raton, FL; Ch. 8; 127.

Becard, G.; Piche, Y. Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. *Appl. Environ. Microbiol.* 1989, 55, 2320-2325.

Bennett, J.W.; Fernholz, F.A.; Lee, L.S. Effect of light on aflatoxins, Anthraquinones and sclerotia in Aspergillus flavus and *Aspergillus parasiticus*. *Mycologia*, 1978, 70, 104.

Bennett, J.W.; Rubin, P.L.; Lee, L.S.; Chen, P.N. Influence of trace elements and nitrogen sources on versicolorin production by a mutant strain of *Aspergillus parasiticus*. *Mycopathologia*, 1979, 69, 161-166.

Bhatnagar, D.; Cleveland, T.E.; Cotty, P.J. Mycological aspects of aflatoxin formation in foods and feeds. In *The Toxicology of Aflatoxins, Human Health, Veterinary Agricultural Significance*; Eaton, L.D.; Groopman, J.D. Eds.; Academic Press: New York, NY; 1994; 327-346.

Bhatnagar, D.; Ehrlich, K.C.; Cleveland, T.E. Oxidation-reduction reactions in biosynthesis of secondary metabolites; In *Mycotoxins in Ecological Systems*; Bhatnagar, D; Lillehoj, E.B.; Arora, D.K. Eds.; Marcel Dekker, Inc.: New York, NY; 1992; 255-286.

Blount, W.P. Turkey "X" disease. *J. Brit. Turkey Fed.* 1961. 9 (2). 52-54.

Brekke, O.L.; Peplinski, A.J.; Nofsinger, G.W.; Conway, H.F.; Stringfellow, A.C.; Montgomery, R.R.; Silman, R.W.; Sohns, V.E.; Bagley, E.B. Aflatoxin inactivation in corn by ammonia gas: A field trial. *Trans Am Soc Ag Engr*, 1979, 22, 425-432.

Brekke, O.L.; Sinnhber, R.O.; Peplinski, A.J.; Wales, J.H.; Putnam, G.B.; Lee, D.J.; Ciegler, A. Aflatoxin in Corn: Ammonia inactivation and bioassay with rainbow trout. *Appl Environ Microbiol*, 1977, 34, 34-37.

Buchanan, L.L; Zaika, R.L. Review of Compounds affecting the biosynthesis or bioregulation of aflatoxins. *Journal of Food Protection*, 1987, 50 (8), 691-708.

Buchanan, R.L.; Fletcher, A.M. Methylxanthine inhibition of aflatoxin production. *J. Food Sci.*, 1978, 43, 654-655.

Burg, S.P.; Burg, E.A. Post-harvest ripening of avocados. *Nature*, 1962, 148, 1190-1196.

Carlile, M.J. The photoresponses of fungi; In *Photobiology of microorganisms*; Halldal, P. Ed.; John Wiley & Sons: New York, NY; 1970, 310.

Cary J.W.; Linz, J.E.; Bhatnagar, D. Aflatoxins: Biological significance and Regulation of Biosynthesis. In *Microbial foodbourne diseases:mechanisms of pathogenesis and toxin synthesis;* Technomic Publishing Co: Lancaster, PA; 2002; Ch. 10; pp. 317-361.

Cheung, K.K.; Sim, G.A. Aflatoxin G1: Direct determination of the structure by the method of isomorphous replacement. *Nature* (London), 1964, 201, 1185-1188.

Chiou, C.H.; Miller, M.; Wilson, D.L.; Trail, F.; Linz, J.E. Chromosal Location Plays a Role in Regulation of Aflatoxin Gene Expression in Aspergillus parasiticus. *Appl. Environ. Microbiol.* 2002, 68, 306-315.

Christensen, C.M.; Mirocha, C.J.; Meronuck, R.A. *Molds, Mycotoxins, and Mycotoxicoses*; Agricultural Experiment Station miscellaneous Report 142. University of Minnesota, St. Paul, MN; 1977.

Ciegler, A.; Lillehoj, E.B.; Peterson, R.E.; Hall, H.H. Microbial Detoxification of Aflatoxin. *Appl Microbiol*, 1966, 14, 934-939.

Clevstrom, G.; Ljunggren, H.; Tegelstrom, S.; Tideman, K. Production of Aflatoxin by an *Aspergillus flavus* Isolate cultured under a limited oxygen supply. *Applied and Environmental Microbiology*. 1983, 400-405.

Cole, R.J,; Cotty, P.J. Biocontrol of aflatoxin production by using biocompetitive agents; In *Perspectives on Aflatoxin in Field Crops and Animal Food Products in the United States* (ARS-83). National Technical Information Services: Springfield, VA; 1990; 62-66.

Conway, H.F.; Anderson, R.A.; Bagley, E.B. Detoxification of aflatoxin-contaminated corn by roasting. *Cereal Chemistry*, 1978, 55, 115-117.

Cotty, P.J. Modulation of Alternaria tagetica by carbon dioxide. *Mycologia*. 1987, 79, 508-513.

Cotty, P.J.; Bayman, P.; Egel, D.S.; Elias, K.S. Agriculture, Aflatoxins, and Aspergillus; In *The Genus Aspergillus*; Powell, K, Ed; Plenum Press: New York, NY; 1994.

Council for Agricultural Science and Technology. Mycotoxins: Risks in Plant, Animal, and Human Systems. CAST: Ames, IA; 2003.

Crocker, W.; Knight, L.I. Effect of illuminating gas and ethylene upon flowering carnations. *Bot. Gaz.*, 1908, 46, 259-276.

Cullen, J.M; Newberne, P.M.; Acute hepatotoxicity of aflatoxins; In *The Toxicology of Aflatoxins*; Eaton, D.L; Goopman, J.D. Eds; Academic Press: New York, NY; 1994; 3-26.

Dam, R.; Tam, S.W.; Satterlee, L.D. Destruction of aflatoxins during fermentation and by-product isolation from artificially contaminated grain. *Cereal Chem*, 1977, 54, 705-714.

Daniels, J.A.; Krishnamurthi, R.; Rizvi, S.S.H. A review of effects of carbon dioxide on microbial growth and food quality. *J Food Prot*, 1985, 48, 532-537.

Davis, N.D.; Diener, U.L. Environmental factors affecting the production of aflatoxin; In *Proceedings of the First U.S.-Japan Conference on Toxic Microorganisms, Honolulu, 7-10 October 1968*; Herzberg, M. Ed; U.S. Government Printing Office: Washington, D.C.; 1970; 43-47.

Denny, F.E. Coloring citurs fruit. U.S. Patent 1, 475,938.

Detroy, R.W.; Lillehoj, E.B.; Ciegler, A. Aflatoxins and related compounds; In *Microbial Toxins*; Ciegler A.; Kadis, S.; Ajl, S.J. Eds; Academic Press: New York, NY; 1971; 3-178.

Dixon, N.M.; Kell, D.B. The inhibition by CO_2 of the growth and metabolism of micro-organisms. *J. Appl. Bacteriol.* 1994, 67, 109-136.

Dollear, F.G.; Mann, G.E.; Codifer Jr., L.P.; Gardner Jr., J.K.; Koltun, S.P.; Vix, H.L.E. Elimination of aflatoxins from peanut meal. *J Am Oil Chem Soc*, 1968, 45, 862-865.

Doyle, M.P.; Marth, E.H. Bisulfite degrades aflatoxin: Effects of temperature and concentration of bisulfite. *J Food Prot*, 1978a, 41, 774-480.

Doyle, M.P.; Marth, E.H. Bisulfite degrades aflatoxin: Effects of citric acid and methanol and possible mechanisms of degradation. *J Food Prot*, 1978b, 41, 891-896.

Dvorackova, I. Aflatoxin and Human Health; CRC Press: Boca Raton, 1990.

Dwarakanath, C.T.; Rayner, E.T.; Mann, G.E.; Dollear, F.G. Reduction of aflatoxin levels in cottonseed and peanut meals by ozoniation. *J Am Oil Chem Soc*, 1968, 45, 93-95.

Eaton, D.L.; Ramsdell, H.S.; Neal, G.E. Biotransformation of aflatoxins; In *The Toxicology of Aflatoxins*; Eaton, D.L; Goopman, J.D. Eds; Academic Press: New York, NY; 1994; 45-72.

Ellis, W.O.; Smith, J.P.; Simpson, B.K. Aflatoxins in Food: Occurrence, Biosynthesis, Effects on Organisms, Detection, and Methods of Control. *Critical Reviews in Food Science and Nutrition*, 1991, 30 (3), 403-439.

Ellis, W.O; Smith, J.P.; Simpson, B.K.; Khanizadeh, S.; Oldham, J.H. Control of growth and aflatoxin production of Aspergillus flavus under modified atmosphere packaging (MAP) conditions. *Food Microbiology*, 1993, 10, 9-21.

Eyles, M.J.; Moir, C.J.; Davey, J.A. The effects of modified atmospheres on the growth of psychotrophic pseudomonads on a surface in a model system. *Int J. Food Microbiol.* 1993, 20, 97-107.

Fabbri, A.A.; Fanelli, C.; Serafini, M.; Di Maggio, D.; Pirazzi, R. Aflatoxin production on wheat seed stored in air and nitrogen. *Trans. Br. Mycol. Soc.*, 1980, 74, 197-199.

Failla, L.J.; Niehaus Jr. W.G. Regulation of Zn2+ uptake and versicolorin A synthesis in a mutant strain of *Aspergillus parasiticus. Exp. Mycol.* 1986, 10, 35-41.

Failla, M.L. Zinc: Functions and transport in microorganisms. In *Microorganisms and minerals*; Weinberg, E.D. Ed.; Marcel Dekker, Inc: New York, NY; 1977; 151-214.

Farag, R.S.; Rashed, M.M.; Abo-Hagger, A.A. Aflatoxin destruction by microwave heating. *Intl J Food Sci Nutri*, 1996, 47, 197-208.

Feuell, A.J. Aflatoxin in groundnuts. IV. Problems of detoxification. *Trop Sci*, 1966, 8, 61-70.

Flaishman, M.A.; Kollatukudy, P.E. Timing of fungal invasion using host's ripening hormone as a signal. *Proc. Nat. Acad. Sci.*, 1994, 91, 6579-6583.

Foudin, A.S.; Leaich, L.L.; Ayres, J.C. Effects of ethoxyquin on aflatoxin-producing strains of Aspergillus parasiticus and Aspergillus flavus. *J. Food Sci.*, 1978, 43, 731-734.

Fung, D.Y.C.; Taylor, S.; Kahan, J. Effects of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on growth and aflatoxin production of Aspergillus flavus. *Journal of Food Safety*, 1977, 1, 39-51.

Gardner Jr., H.K.; Koltun, S.P.; Dollear, F.G.; Rayner, E.T. Inactivation of aflatoxins in peanut and cotton seed meals by ammoniation. *J Am Oil Chem Soc*, 1971, 48, 70-73.

Girardin, J.P.L. Einfluss des Leuchtgases auf die Promenaden und Strassen-baume. *Jahresker Agrikultur. Chem. Versuchssta,* Berlin, 1864, 7, 199-200.

Goeschl, J.D.I Rappaport, L.; Pratt, H.A. Ethylene as a factor regulating the growth of pea epicotyls subjected to physical stress. *Plant Physiol.*, 1966, 41, 877.

Goldblatt, L.A.; Dollear, F.G. Modifying mycotoxin contamination in feeds: Use of mold inhibitors, ammoniation, roasting; In *Interactions of Mycotoxins in Animal Production*. National Academy of Sciences: Washington, D.C.; 1979; 167-184.

Gourama, H.; Bullerman, L.B. Aspergillus flavus and Aspergillus parasiticus: Aflatoxigenic fungi of concern in foods and feeds: A review. *Journal of Food Protection*. 1995, 58 (12), 1395-1404.

Gull, D.D. Ripening tomatoes with ethylene. Univ. Florida Vegetable Crops Fact Sheet VC-29; p. 4

Gupta, S.K.; Maggon, K.K.; Venkitasubramanian, T.A. Effect of zinc on tricarboxylic acid cycle intermediates and enzymes in relation to aflatoxin biosynthesis. *J. Gen. Microbiol.*, 1977, 99, 43-48.

Gupta, S.K.; Maggon, K.K.; Venkitasubramanian, T.A. Zinc dependence of glycolytic enzymes of an aflatoxigenic strain of *Aspergillus parasiticus. Microbios Lett.*, 1976, 3, 89-92.

Hagler Jr., W.M.; Hutchins, J.E.; Hamilton, P.B. Destruction of aflatoxin in corn with sodium bisulfite. *J Food Prot*, 1982, 45, 1287-1291.

Hansen, A.P.; Welty, R. Microflora of raw cocoa beans. *Mycopathol. Mycol.* Appl., 1970, 44, 309-316.

Harvey, E.M. Some effects of ethylene on the metabolism of plants. *Bot. Gaz.*, 1915, 60, 193-214.

Hoffman, N.E.; Yang, S.F. Enhancement of wound-induced ethylene synthesis by ethylene in preclimacteric cantaloupe. *Plant Physiol.*, 1982, 69, 317

.

Joffe, A.Z.; Lisker, N. Effect of light, temperature, and pH value on aflatoxin production *in vitro. Appl. Microbiol.*, 1969, 18, 517.

Kachholz, T.; Demain, A.L. Nitrate repression of averufin and aflatoxin biosynthesis. *J. Nat. Products* (Lloydia), 1983, 46, 499-506.

Karunaratne, A.; Bullerman, L.B. Interactive effects of spore load and temperature on aflatoxin production. *J. Food Prot.*, 1990, 53, 227.

Kays, S.J.; Beaudry, R.M. Techniques for inducing ethylene effects. In *Symposium on Manipulation of Ethylene Responses in Horticulture*; Reid, M.S. Ed.; XXII International Horticultural Congress: Davis, CA; Sec. 3; p.77.

Lannelongue, M.; Finne, G. Effect of carbon dioxide on growth rates of selected microorganisms isolated from black drum pogonias-cromis. *J. Food. Protec.* 1986, 49, 806-810.

Lee, E.G.H.; Townsley, P.M.; Walden, C.C. Effect of bivalent metals on the production of aflatoxin in submerged cultures. *J. Food Sci.*, 1981, 31, 432-436.

Lee, L.S. Aflatoxin. *J Am Oil Chem Soc*, 1989, 66, 1398-1413.

Lenovich, L.M. Effect of caffeine on aflatoxin production on cocoa beans. J. *Food Sci.*, 1981, 46, 655 and 657.

Lenovich, L.M.; Hurst, W.J. Production of aflatoxin on cocoa beans. *J. Assoc. Off. Anal. Chem.*, 1979, 62, 1076-1079.

Levi, C. Mycotoxins in coffee. *J. Assoc. Offi. Anal. Chem.*, 1980, 63, 1282-1285.

Llewellyn, G.C.; Benevides, J.; Eadie, T. Differential production of aflatoxin on natural and heat-treated cocoa beans. *J. Food Prot.*, 1978, 41, 785-787.

Maeba, H.; Takamoto, Y.; Kamimura, M.; Miura, T. Destruction and detoxification of aflatoxins with ozone. *J Food Sci*, 1988, 53, 667-668.

Mann, G.E.; Codifer Jr., L.P.; Dollear, F.G. Effet of heat on aflatoxins in oilseed meals. *Journal of Agriculture and Food Chemistry*, 1967, 15, 1090.

Mannon, J.; Johnson, E. Fungi down on the farm. *New Sci.* 1985, 105, 12-16.

Marsh, P.B.; Simpson, M.E.; Trucksess, M.W. Effects of trace metals on the production of aflatoxins by *Aspergillus parasiticus. Appl. Microbiol.*, 1975, 30, 52-57.

Marth, E.H.; Doyle, M.P. Update on molds: Degradation aflatoxin. *Food Tenchnology*, 1979, 33, 81-87.

Masri, M.S.; Vix, H.L.E.; Goldblatt, L.A. Process for detoxifying substances contamintated with aflatoxin. United States Patent 3, 429, 709. 1969.

Mateles, R.I.; Adye, J.C. Production of aflatoxins in submerged culture. *Appl. Microbiol.*, 1965, 13, 208-211.

McIntyre, M.; McNeil, B. Dissolved carbon dioxide effects on morphology, growth, and citrate production in *Aspergillus niger* A60. *Enzyme Microb. Technol.* 1997, 20, 135-142.

Miller, M. Transcriptional regulation of the Aspergillus parasiticus aflatoxin biosynthetic pathway gene *nor-1. Ph.D. Thesis*. Michigan State University, East Lansing, MI.

Mislivec, P.B.; Bruce V.R.; Gibson, R. Incidence of toxigenic and other molds in green coffee beans. *J. Food Prot.*, 1983, 46, 969-973.

Moerck, K.P.; McElfresh, P.; Wohlman, A.; Hinton, B.W. Aflatoxin destruction in corn using sodium bisulfite, sodium hydroxide and aqueous ammonia. *J Food Prot*, 1980, 43, 571-574.

Morgan, P.W. Effects on ethylene physiology. In *Herbicides: Physiology, Biochemistry, Ecology*; v. 1 2nd ed.; Audus, L.J. Ed; Academic Press: New York, NY; 1976; 255.

Nartowicz, V.B.; Buchanan, R.L.; Segall, S. Aflatoxin production in regular and decaffeinated coffee beans. *J. Food Sci.*, 1979, 44, 446-448.

Nguyen-the, C.; Carlin, F. The microbiology of minimally processed fresh fruits and vegetables. *Crit. Rev. Food Sci. Nut.* 1994, 34, 371-401.

Niehaus Jr., W.G.; Dilts Jr., R.P. Purification and characterization of glucose-6-phosphate dehydrogenase from *Aspergillus parasiticus. Arch. Biochem. Biophys.*, 1984, 228, 113-119.

Niehaus Jr., W.G.; Dilts Jr., R.P.; Purification and characterization of mannitol dehydrogenase from *Aspergillus parasiticus. J. Bacteriol.*, 1982, 151, 243-250.

Nkama, I.; Nobbs, J.H.; Muller, H.G. Destruction of aflatoxin B_1 in rice exposed to light. *J. Cereal Sci.*, 1987, 5, 167.

Palmgren, H.S.; Hayes, A.W. Aflatoxin in food. In *Mycotoxins in Food*. Krogh, P. Ed; Academic Press: New York, NY; 1987; 65-95.

Park, D.L; Lee. L.S.; Price, R.L.; Pohland, A.E. Review of the Decontamination of Aflatoxins by Ammoniation: Current Status and Regulation. *J. Assoc. Off. Anal. Chem.* 1988, 71 (4), 685-703.

Perovic, S.; Seack, J.; Gamulin, V.; Muller, W.E.G.; Schroder, H.C. Modulation of intracellular calcium and proliferative activity of invertebrate and vertebrate cells by ethylene. *BMC Cell Biol*, 2001, 2, 7.

Pestka, J.J.; Gaur, P.K.; Chu, F.S. Quantitation of aflatoxin B_1 and aflatoxin B_1 antibody by an enzyme-linked immunosorbent microassay. *Appl. Environ. Microbiol.*, 1980, 40, 1027-1031.

Phillips, T.D.; Clement, B.A.; Park, D.L. Approaches to reduction of aflatoxins in food and feeds; In *The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance*; Eaton, L.D.; Groopman, J.D. Eds; Academic PressL New York, NY; 1994; 383-406

Reid, M.S. Ethylene in plant growth, development, and senescence. In *Plant Hormones: Physiology, Biochemistry and Molecular Biology*, 2nd ed; Davies, P.J. Ed.; Kluwer: Dordrecht, Netherlands; 1995; 486-508.

Reiss, J. Mycotoxins in foodstuffs. Eur. *J. Appl. Microbiol.*, 1975, 2, 183.

Reiss, J. Mycotoxins in foodstuffs. XI. Fate of aflatoxin B_1 during preparation and baking of whole wheat bread. *Cereal Chemistry*, 1978, 55, 421-423.

Robens, J.F.; Richards, J.L. Aflatoxins in animal and human health. *Rev. Environ. Contam. Tox.* 1992, 127, 175-181.

Roebuck, B.D.; Maxuitenko, Y.Y. Biochemical mechanisms and biological implications of the toxicity of aflatoxins as related to aflatoxin carcinogenesis; In *The Toxicology of Aflatoxins*; Eaton, D.L.; Groopman, J.D. Eds; Academic Press: New York, NY; 1994; 27-43.

Roze, L.V.; Calvo, A.M.; Gunterus, A.; Beaudry, R.; Kall, M.; Linz, J.E. Ethylene modulates development and Toxin biosynthesis in *Aspergillus* possibly via an ethylene sensor-mediated signaling pathway. *Journal of Food Protection*. 2004, 67, 438-447.

Saltveit Jr., M.E.; Dilly, D.R. Rapidly induced wound ethylene from excised segments of etiolated Pisum sativum L. cv. Alaska I. characterization of the response. *Plant Physiol.*, 1978, 61, 447.

Shantha, T. Detoxification of groundnut seed and products in India; In *Summary and Recommendations of the International Workshop on Aflatoxin Contamination of Groundnut*; ICRISAT Center, India; 1987; p.16.

Shantha, T.; Sreenivasa, M. Photo-destruction of aflatoxin in groundnut oil. *Indian J Tehncol*, 1977, 15, 453.

Sharma, A.; Padwal-Desai, S.R.; Nadkarni, G.B. Possible implications of reciprocity between ethylene and aflatoxin biogenesis in Aspergillus flavus and *Aspergillus parasiticus. Applied and Environmental Microbiology*, 1985, 49, 79-82.

Sievers, A.F.; True, R.H. A preliminary study of the forced curing of lemons as practiced in California. USDA Bur. Plant Ind. Bull., 1912, 232, 38.

Stark, A.A.; Gal, Y.; Shaulsky, G. Involvement of singlet oxygen in photoactivation of aflatoxins B_1 and B_2 to DNA-binding forms in vivo. *Carcinogenesis*, 1990, 11, 529-534.

Stretton, S.; Goodman, A.E. Carbon dioxide as a regulator of gene expression in microorganisms. *Antonie van Leeuwenhook*, 1998, 73, 79-85.

Taiz, L.; Zeiger, E. Ethylene: The Gaseous Hormone; In *Plant Physiology*, 3rd ed; Sinauer Asoociates, Inc., Publishers: Sunderland, MA; 2002; Ch. 22; 519-538.

Uraih, N.; Cassity, T.R.; Chipley, J.R. Partial characterization of the mode of action of benzoic acid on aflatoxin biosynthesis. *Can. J. Microbiol.*, 1977, 23, 1580-1584.

Wildman, J.D.; Stoloff, L.; Jacobs, R. Aflatoxin production by a potent *Aspergillus flavus* Link isolate. *Biotechnol. Bioeng.*, 1967, 9, 429-437.

Yousef, A.E.; Marth, E.H. Incorporation of [^{14}C]acetate into aflatoxin by resting cultreus of *Aspergillus parasiticus* in the presence of antifungal agents. Eur. J. *Appl. Microbiol. Biotechnol.*, 1983, 18, 103-108.

Zonneveld, B.J.M Effect of carbon dioxide on fruiting in Aspergillus nidulans. *Trans. Br. Mycol. Soc.*, 1988, 91, 625-629.