15

Phytoinhibition of Growth and Aflatoxin Biosynthesis in Toxigenic Fungi

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1. Introduction

Aflatoxins are primarily produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminate a wide variety of food and feed commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk and dried fruits [Strosnider et al., 2006].
Presence of aflatoxins in food chain is associated with decrease in quality and quantity of food and feed materials. In addition, consumption of aflatoxin-contaminated products can pose a risk of development of various diseases in human and animals. Aflatoxins are produced in toxigenic fungi after undergoing biosynthesis pathway involving several enzymes and reactions. Upon consumption of aflatoxin contaminated products by human and animals, the toxin undergoes metabolism via cytochrome P450 enzymes in the liver. Aflatoxin metabolism in mammalian organs is a committed process and different metabolites are produced which can exert adverse effects of toxic metabolites. Aflatoxin epoxide (8,9-epoxide) is the major toxic metabolite which can bind to DNA and induce hepatocellular carcinoms. The extent of aflatoxin toxicity and carcinogenicity in human and animals depends on several factors including the metabolic capacity of the organism. Aflatoxin contamination of food products is associated with health and socioeconomic costs which is difficult to valuate in the developing countries. Moreover, the current regulations do little to help reduce aflatoxin and related health effects. Therefore the focus should be on promoting the adaptation of strategies that can control aflatoxin and its associated health risks. According to Wu and Khlangwiset (2010), interventions to reduce aflatoxin-induced illness can be grouped into three categories; agricultural, dietary and clinical. Agricultural interventions are methods that can be applied either in the field (preharvest) or in drying, storage and transportation (postharvest) to reduce aflatoxin levels in food. The dietary and clinical interventioans are considered as secondary interventions by which the aflatoxin-related illness can be reduced. These two types interventions are associated with advantages and disadvantages.
Due to concern for the potential effects of aflatoxins on human health, most countries have legislation that restricts marketing of aflatoxin-contaminated grains [Van Egmond, 1989]. The United States Food and Drug Administration has set an aflatoxin limit of 20 μg/kg for

foods and for most feeds and feed ingredients. The European Union has enacted a very stringent aflatoxin tolerance threshold of 2 μg/kg aflatoxin B1 and 4 μg/kg total aflatoxins for nuts and cereals for human consumption [Bankole and Adebanjo, 2003].

The objective of the present article is to review different approaches by which aflatoxins can be reduced or eliminated in the food chain. The feasibility and the safety of aflatoxin detoxification process in food materials depend on different factors. The safety issue of food products that undergo detoxification treatment could be improved by using phytochemical agents with potential antimicrobial activities.

One of the characteristics of aflatoxin inactivation processes is that it should destroy the mycelia and spores of the toxic fungi, which may proliferate under favorable condition. The pH and moisture content of the foodstuffs have been reported as the main abiotic factors affecting the fungal infestation. [Prakash et al., 2011]. The chemical profile of the substrate may also play a major role in the growth and proliferation of moulds on the foodstuffs as has been emphasized by Singh et al. (2008).

Because of the toxic and carcinogenic potential of aflatoxins, much emphasis has been focused on the control or elimination of these fungi and/or their toxic metabolites in food grains and livestock feeds. Cultural practices, such as adjustments of sowing and harvesting time can be effective to a certain extent in preventing pre-harvest aflatoxin contamination. However, in case of inappropriate storage conditions, the fungi can invade the grains causing serious damage and toxin accumulation in the grains. Though some of the fungicides are effective in preventing the growth of *Aspergillus flavus* in storage especially as a fumigant [Paster et al., 1995], consumer concerns about possible risks associated with the use of fungicides have resulted in an intensive search for safer and more effective control options that pose minimal risk to human health and the environment. [Velazhahan et al., 2010,].

Whichever decontamination strategy is used, it must meet some basic criteria [Park, 1993; Beaver, 1991; Pomeranz et al., 1990]:

- The mycotoxin must be inactivated (destroyed) by transformation to non-toxic compounds.
- Fungal spores and mycelia should be destroyed, so that new toxins are not formed.
- The food or feed material should retain its nutritive value and remain palatable.
- The physical properties of raw material should not change significantly.
- It must be economically feasible (the cost of decontamination should be less than the value of contaminated commodity).

Principally there are three possibilities to avoid harmful effects of contamination of food and feed caused by mycotoxins:

- Prevention of contamination,
- Decontamination of mycotoxin-containing food and feed,
- Inhibition of absorption of mycotoxin in consumed food in the digestive tract [Bata et al 1999].

2. Mycology

It is well established that not all molds are toxigenic and not all secondary metabolites from molds are toxic. About 300 different secondary metabolites are known [Bhatnagar et al., 2002], however only a few of them play a role as contaminants in food. These are especially

aflatoxins, trichothecenes, fumonisins, ochratoxin A and patulin [Bennett et al., 2003]. For all of them statutory limits have been set or are under discussion within the European Union. The most important fungal genera, which produce these mycotoxins, belong to the genera *Aspergillus, Penicillium* or *Fusarium*. Aflatoxin is produced mainly by toxigenic strains of *Aspergillus flavus, Aspergillus.parasiticus and Aspergillus nomius* [Beck et al., 1990; Karolewiez and Geisen, 2005; Kimura et al., 2003; O'Callaghan et al., 2003; Proctor et al., 2003; Penalva and Arst Jr. et al., 2002; Yu et al., 2004a]. Accumulation of aflatoxin B has been reported from members of three diffrent groups of *Aspergilli: Aspergillus* section Flavi: *Aspergillus flavus, Aspergillus flavus var. parvisclerotigenus, Aspergillus parasiticus, Aspergillus toxicarius, Aspergillus nomius, Aspergillus pseudotamarii, Aspergillus zhaoqingensis, Aspergillus bombycis. Aspergillus* section Nidulantes*: Emericella astellata and Emericella venezuelensis. Aspergillus* section Ochraceorosei*: Aspergillus ochraceoroseus* and *Aspergillus rambellii*. G type aflatoxins have only been found in some of the spices in *Aspergillus* section Flavi, while B type aflatoxins are common in all three groups. However it is a well known fact that the presence of a mycotoxigenic fungus in a food sample does not ultimately indicate the production of the respective mycotoxin. The biosynthesis of secondary metabolites, like the mycotoxins, is tightly regulated depending on environmental conditions like substrate, pH, water activity or temperature [Hope et al., 2005]. These facts may suggest, that for a complete assessment of the mycotoxicological status of a food, not merely the detection of a putative mycotoxin producing fungus is important, but the knowledge about the ability of the fungus to activate mycotoxin biosynthesis genes under the environmental conditions suitable for the food chain. [Schmidt-Heydt et al., 2007]. Factors contributing to the presence or production of mycotoxins in foods or feeds include storage, environmental, and ecological conditions.

2.1 Biosynthesis

The structure of aflatoxins consists of a coumarin nucleus attached to a bifuran and either pentanone (aflatoxin B1 and aflatoxin B2) or a six-membered lactone (aflatoxin G1 and aflatoxin G2). Aflatoxin B1, B2, G1, and G2 are the four main naturally-occurring aflatoxins, among which aflatoxin B1 (C17H12O6) is known to be the most significant in terms of animal and human health risk [Pier, 1992; Coulombe 1993, Bluma et al., 2008].
Aflatoxins belong to the polyketide class of secondary metabolites produced by toxigenic strais of *Aspergillus flavus* and *Aspergillus parasiticus,* and are synthesized by enzymes encoded within a large gene cluster [Yabe and Nakajima 2004; Yu et al., 2004b]. As shown in figure 1, the initial step in the generation of the polyketide backbone of aflatoxins is proposed to involve polymerization of acetate and nine malonate units (with a loss of CO2) by a polyketide synthetase in a manner analogous to fatty acid biosynthesis [Dutton, 1988; Bhatnagar et al., 1992]. Aflatoxin synthesis is controlled by different enzymes which are expressed through gene expression processes. Genetic studies on aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus* led to the cloning of 25 clustered genes within a 70 kb DNA region responsible for the enzymatic conversions in the aflatoxin biosynthetic pathway. Regulatory elements such as aflR and aflS (aflJ), nutritional and environmental factors, fungal developmental and sporulation were also found to affect aflatoxin formation. In *Aspergillus flavus* there are eight chromosomes with an estimated genome size of about 33–36 Mbp that harbor an estimated 12,000 functional genes [Yu et al., 2004b].

Many inhibitors of aflatoxin biosynthesis may act at three levels: (1) Modulate environmental and physiological factors affecting aflatoxin biosynthesis, (2) inhibit signaling circuits upstream of the biosynthetic pathway, or (3) directly inhibit gene expression or enzyme activity in the pathway. The known inhibitory compounds either alter known environmental and physiological modulators of aflatoxin biosynthesis or they alter signal transduction pathways in the upstream regulatory network [Holmes et al., 2008]. Each step in gene expression, transcription, RNA transport and processing, translation, protein processing and localization can be inhibited by natural plant products or other agents [Trail et al., 1995].

2.2 Toxicity and detoxification of aflatoxin in mammalian organs

Aflatoxins, which are known to be potent mutagenic, carcinogenic, teratogenic, hepatotoxic, immunosuppressive, also inhibit several metabolic systems [International Agency for Research on Cancer, 1993] and causing damages such as toxic hepatitis, hemorrhage, and edema [Santos et al., 2001]. Aflatoxins have been detected in cereal grains, oil seeds, fermented beverages made from grains, milk, cheese, meat, nut products, fruit juice and numerous other agricultural commodities [Bullerman, 1986].

As shown in figure-2, aflatoxin B1 undergoes metabolism in mammalian liver leading to the formation of metabolites such as aflatoxin B1-epoxide and hydroxylated metabolites (aflatoxin M1, aflatoxin P1, aflatoxin Q1, and aflatoxicol). The metabolites produced in phase-I undergo phase II biotransformation involving the enzymes glutathione S-transferase (GST), β-glucuronidase, and/or sulfate transferase which produce conjugates of aflatoxin B1-glutathione, aflatoxin B1-glucuronide, and aflatoxin B1-sulfate, respectively.

Aflatoxin B1 caused damage by two different ways in the cells. Firstly, it is activated to aflatoxin B1-8, 9-epoxide and forms adduct primarily at N7 position of guanine and is responsible for its mutagenic and carcinogenic effects [Wang and Groopman, 1999; Denissenko et al., 1999]. Secondly, aflatoxins especially aflatoxin B1, produce reactive oxygen species such as superoxide radical anion, hydrogen peroxide and lipid hydroperoxides; though these do not appear to interact with DNA, but they are precursors to the hydroxyl radical. The hydrox radicals interact with DNA which may cause mutations [Halliwell and Gutteridge, 1999]. The major conjugate of aflatoxin B1-epoxide identified is the aflatoxin-B1-glutathione conjugate [Monroe & Eaton, 1987].

To control the level of reactive oxygen species and to protect cells under stress conditions, living tissues contain enzyme systems such as, superoxide dismutase, glutathione peroxidase, and catalase as well as antioxidant substances. The effect of reactive oxygen species is balanced by the antioxidant action of non-enzymatic antioxidants, as well as by antioxidant enzymes. Such antioxidant defenses are extremely important as they represent the direct removal of free radicals (prooxidants), thus, providing maximal protection for biological sites [Valko et al., 2006].

It is worth mentioning that glutathione conjugation system is present in aflatoxin-producing fungi which can facilitate detoxification of the toxic metabolite of aflatoxin from the mycelia. Aflatoxin B1-glutathione conjugation in the toxigenic fungi depends on the levels of fungal glutathione and glutathione S-transferase which are inducible in fungi cultured in presence of classic inducers. Likewise the fungi may express enzymes which are involved in inactivation of free radicals as a result of metabolic functions [Saxena et al. 1989; Ziglari et al. 2008].

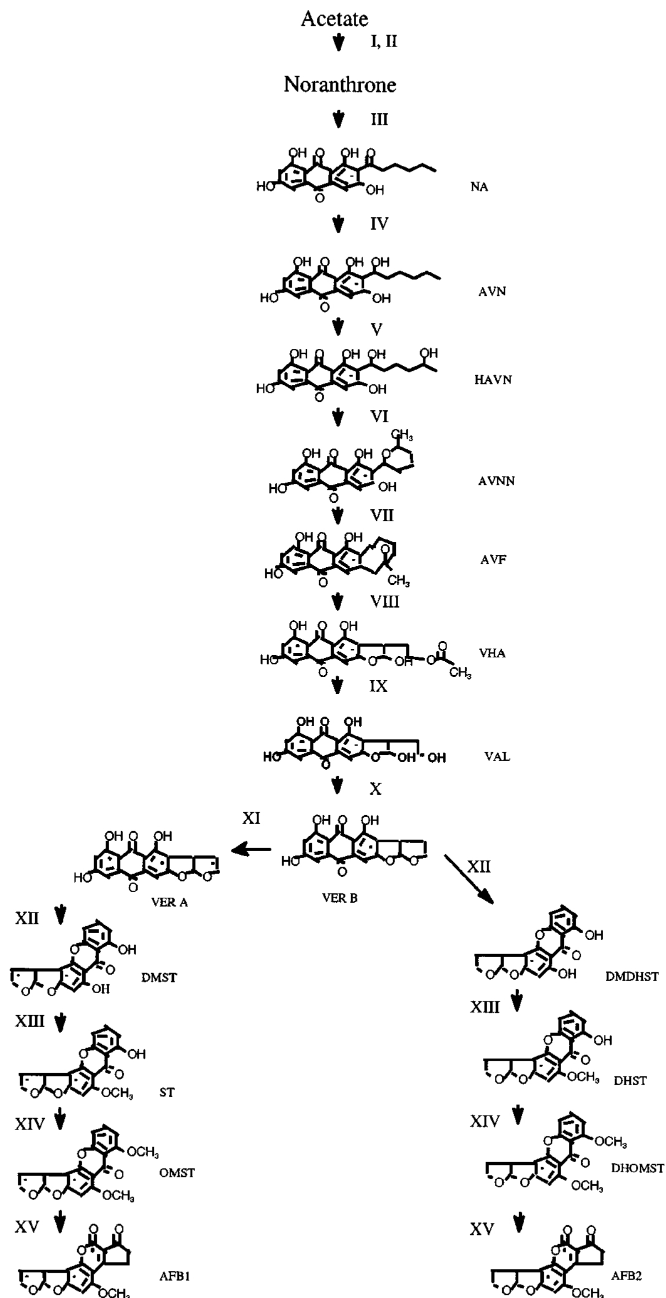

Fig. 1. Biosynthesis of aflatoxins in toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Adopted from Payne and Brown, 1998. The numbers show the metabolites formed during the biosynthesis.

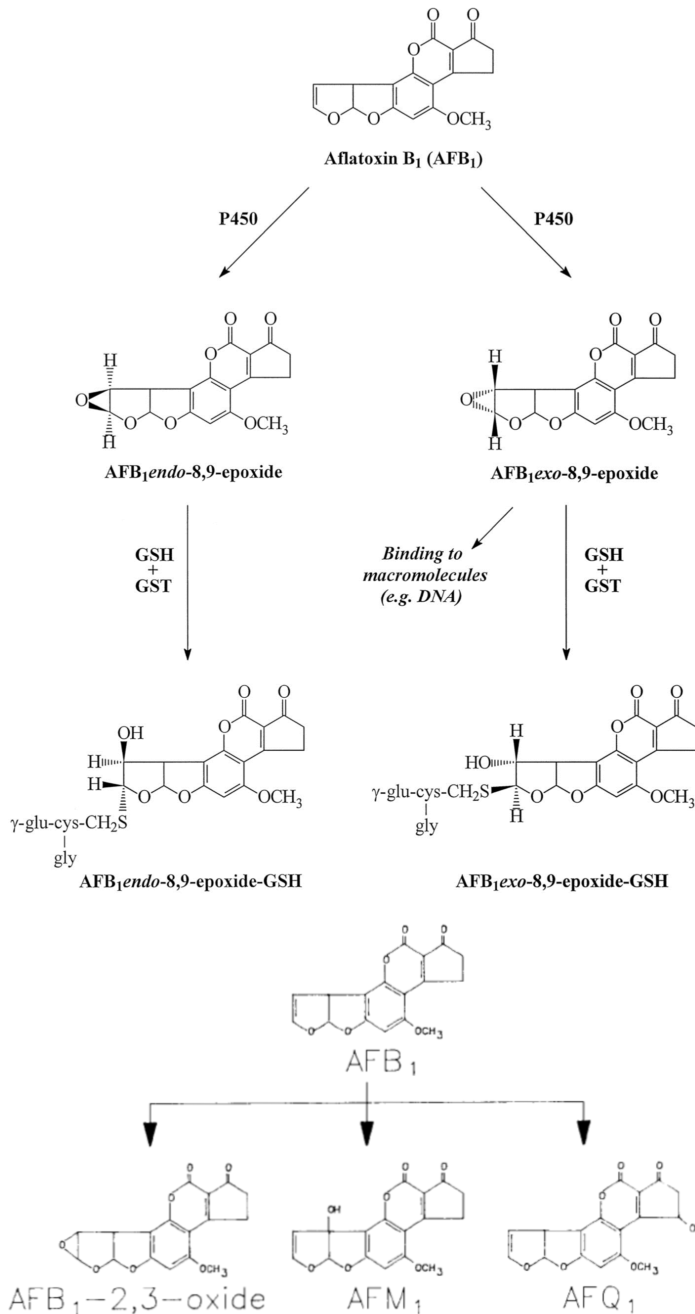

Fig. 2. Biotransformation aflatoxin B1 by phase I and phase II xenobiotic metabolizing enzymes in mammalian cells. [Daniels et al., 1990]

3. Aflatoxin elimination methods

Contamination of food commodities with aflatoxin resulting from fungal attack can occur before, after and during the harvest and storage operations. The enormous health and economic significance of food and feed contaminants have become steadily clearer since the 1960s, when mycotoxin was first discovered.
There are two strategies to reduce the levels of aflatoxins in food and feed materials. The first approach is to control or prevent food contamination in aflatoxin producing fungi. This strategy so called preharvest strategy is relatively easy and can be implicated during cultivation and harvesting. However, the postharvest strategy which deals with elimination of aflatoxins and aflatoxin-producing fungi in the agricultural products appears to be more complicated and not recommended for human consumption. The complexity of elimination methods varies depending on the quantity and the nature of the food material. The safety of the products undergoing elimination is currently suggested for animal feed and their use for human consumption is not recommended. Inactivation or removal of aflatoxins in food and feed commodities at a large scale can recycle a major part of the protection for animal consumption. Chemical and physical treatment are currently major procedures used at large scale. However, the safety of these methods can be increased by using phytochemical agents to reduce aflatoxin production in food and feed products. Phytochemical agents which may directly or indirectly enter to human food may not pose a toxicity threat to humans. Hence, replacement of phytochemicals is in the benefit of human safety.

3.1 Physical control

Various physical techniques have been devised to remove, destroy or suppress the toxicity of the mycotoxins. These techniques include physical removal of the contaminated portions of the foodstuffs, treatment with heat, and radiation in order to convert the toxins into relatively innocuous compounds or the addition of adjuvants to suppress or otherwise mask the ill effects of toxins [Park et al., 2007]. Many physical methods such as microwave heating and treatments with ozone (ozonation) have been recommended for detoxification of aflatoxin contaminated food [Farag et al., 1996; Xu, 1999; Prudente and King, 2002, Inan et al., 2007]. Washing the grain, heating and drying are other traditional methods used to reduce mycotoxins in food products. Physical method is believed to be the most effective method for the reduction of mycotoxins in contaminated commodities. However, a technique such as, gamma radiation is limited due to high cost of equipment [Jalili et al., 2010].

3.1.1 Drying and roasting of food products

Traditionally food and feed materials are dried using sunlight. The level of aflatoxins was reduced to over 40% by roasting and heating peanuts [Rustom, 1997]. Buser and Abbas reported that an extrusion process is able to decrease the level of aflatoxins to 33%. [Hwang et al., 2006]. The influence of temperature and pressure was also examined in an extruder which is a bioreactor that transforms cereals, under high temperature and pressure. As a hydrated powder, the crude material feeds the extruder, undergoing chemical and physical transformations because of the thermal effect and severe shear stress [Chiruvella et al., 1996]. Thus, extrusion-cooking is an attractive process for continuous food/feed processing,

and has been developed extensively in recent years as an efficient manufacturing process. High temperature/short time extrusion-cooking is commonly used in the industry to directly produce expanded products such as snack foods, breakfast cereals and pet foods [Miller, 1990; Moore, 1994; Rokey, 1994; Rahman, 1995]. For temperatures between 140 and 200˚C and moisture content ranging from 170 to 270 g/kg, reductions in aflatoxin levels between 50 and 75% were obtained. Cazzaniga et al. (2001) reported that extrusion of maize flour with low levels of aflatoxin B1 (50 ng/g) was partially successful (10–25%) for the decontamination of aflatoxins with metabisulphite addition (1%) at temperatures of 150 and 180 ◦C, respectively. [Mendez-Albores et al., 2009]. The level of aflatoxin B1 in dried wheat was decreased to 50% and 90% by heating at 150 and 200˚C, respectively. However, the reduction of aflatoxin B1 in wet wheat in which water (10%) was intentionally added was higher by heating than in dried wheat. The reduction of aflatoxin B1 was increased by 8% and 23% in wet United States wheat (soft red white wheat) and Korean wheat (Anbaekmil) compared to dry United States and Korean wheat, respectively, through heat treatment. Traditional processing used in Korean foods such as Sujebi (a soup with wheat flakes) and steamed bread caused 71% and 43% decrease in aflatoxin B1 content. Reduction of aflatoxin B1 toxicity was directly proportional to washing time in both Korean and United States wheat. [Hwang, et al., 2006].

3.1.2 Irradiation

It has been shown that gamma ray treatment of food products is effective in reducing mycotoxin concentration in different foods. It was found that by increasing the gamma doses from 10 to 60 kGy, mycotoxin reduction significantly increased, however, there was no reduction in the mycotoxin content at doses less than 10 kGy. In a related study, doses of 15, 20, 25 and 30 kGy were used to destroy aflatoxin B1 in peanut sample by 55–74% [Prado et al., 2003]. Aflatoxin B2 and aflatoxin G2 in all of the treatments showed lower reduction comparing with other mycotoxins. According to Aziz and Youssef (2002) a dose of 20 kGy was sufficient for complete destruction of aflatoxin B1 in peanut, yellow corn, wheat and cotton seed meal. Ghanem et al. (2008) demonstrated that aflatoxin B1 degradation in food samples was inversely related to the oil content in irradiated samples. It has been suggested that water content is an important factor in the destruction of mycotoxin by gamma rays, since the radiolysis of water leads to the formation of highly reactive free radicals that can readily attack aflatoxin B1 at the terminal furan ring and produce molecules with lower biological activity [Jalili et al., 2010]. EL-Bazza et al. (1996) reported that gamma irradiation dose level of 3.0 kGy proved to be effective in decontamination of Carum carvi and Matricaria chamomilla samples from fungi, while, Ammi visnaga and Artimisia judica samples could only be decontaminated at a higher dose level of 4.0 kGy. A gamma radiation dose of 6.0 kGy was found to be sufficient to free the tested seed samples from fungi [El-Bazza et al., 2001]. The adverse effects of gamma radiation on food quality have been demonstrated by treating Ashanti pepper with the optimal gamma radiation dose. Both ground and whole forms of Ashanti pepper were subjected to 0, 2.5, 5.0, 7.5 and 10 kGy doses of gamma rays from a 60Co source, showed that, the 2.5-kGy doses reduced the fungal and bacterial load by 2 log cycles and 7.5 kGy eliminated the fungal population. A dose of 10 kGy was required to decontaminate the samples irrespective of sample form, although grinding and not irradiation affected the essential oil composition of the spice [Onyenekwe et al., 1997]. Different combinations of temperature and pressure on the

influence of gamma radiation have been also studied. Combination treatment of heat and irradiation (3.5 kGy) reduced the A*spergillus flavus* spore inoculum size by about 4 log cycles and yielded the highest amount (41.1 µg/ml) of aflatoxin B1 in Maize Meal broth supplemented with 2% glucose and 2% peptone (AMMB). However, moist heat treatment of spores receiving the same dose (3.5 kGy) reduced toxin formation by 25%. Aflatoxin B1 formation by *Aspergillus flavus* spores incubated in AMMB was completely prevented by a combination treatment of moist heat and 4.0 kGy of gamma irradiation. A similar treatment attenuated aflatoxins B2, G1 and G2 production which were formed with B1 by *Aspergillus flavus* NRRL 5906 [Odamtten et al., 1986].
The self-designed microwave-induced argon plasma system [Park et al., 2007] is a technique required much less exposure time for mycotoxin degradation than other methods, such as visible or UV light and gamma ray. The UV irradiation and etching by plasma may be responsible for degrading and removing the mycotoxins. This plasma system has many advantages, such as increased ionization by reactive species and relatively high intensity of UV light (75–102 mW/cm2), low average temperature (75–130°C) and easy operation. In summary, the mycotoxins, aflatoxin B1, deoxynivalenol, and nivalenol were completely removed after 5 seconds of plasma treatment. Moreover, the cytotoxicity of mycotoxins was significantly reduced with progress in the treatment time [Park et al., 2007].

3.1.3 Bioabsorption of aflatoxins in animal feed

One of the most important approaches aimed at reducing the risk of aflatoxicosis or in limiting decrease in animal performance and toxic metabolite carry-over in milk, meat and eggs, is the use of clays in contaminated feeds to reduce aflatoxin absorption in the intestine. Some in vitro tests [Philips et al., 1988] showed that various absorbing materials such as alumina, silica and aluminosilicate are capable of binding aflatoxin in solution. Extraction using various solvents at different temperatures and pH showed a release which varied in intensity in function of the type of material used. It has been demonstrated that the hydrated sodium calcium aluminosilicates (HSCAS) were particularly efficacious in binding aflatoxin. Analogous detoxification trials have been performed using zeolites, bentonites and modified phylloaluminosilicates. A micronized zeolite [Stankov et al., 1992] was tested as an aflatoxin sorbent in feeds for weaning piglets and it induced a marked reduction in mortality rate and increase of feed consumption and body weight. In contrast, a study on dairy cows [Pietri et al., 1993] did not detect any zeolite induced reducing action on carry-over, while a test on broilers of domestic fowl [Sova et al., 1991] showed the total absence of beneficial effects determined by addition of zeolite. In fact, in synthetic zeolites, as opposed to natural ones, the pore size distribution varies very little and is generally concentrated within a narrow diameter range. If the size of the pores is compatible with those of the aflatoxin molecules, conspicuous adsorption occurs. In contrast, adsorption can be easily nil because no intermediate sized pores are present. A test on bentonite as an aflatoxin sorbent conducted on dairy cows [Veldman, 1992] revealed a 33% carryover reduction; while *in vitro* trials on trout feed [Winfree & Allred 1992] achieved adsorption of 70% the aflatoxin B, present in the feed. An *in vitro* test [Sison., 1992] demonstrated the efficacy of a commercial product (Mycobond) made of chemically modified phylloaluminesilicate combined with multilayered montmorillonite and detected the formation of an inert, and stable complex capable of preventing absorption of mycotoxins in the intestine [Piva et al., 1995].

3.1.4 Other methods

Practical methods to degrade mycotoxins using ozone gas (O3) have been limited due to low O3 production capabilities of conventional systems and their associated costs. Recent advances in electrochemistry (i.e. proton-exchange membrane and electrolysis technologies) have made available a novel and continuous source of O3 gas up to 20% by weight. It is possible that the rapid delivery of high concentrations of O3 will result in mycotoxin degradation in contaminated grains-with minimal destruction of nutrients. Results indicated that aflatoxin B1 and aflatoxin G1 were rapidly degraded using 2% O3, while aflatoxin B2 and aflatoxin G2 were more resistant to oxidation and required higher levels of O3 (20%) for rapid degradation [McKenzie et al., 1997]. Ozonation, an oxidation method, has been developed for the detoxification of aflatoxins in foods [Samarajeewa et al., 1990]. Ozone, or triatomic oxygen, is a powerful disinfectant and oxidising agent [McKenzie et al., 1997]. It reacts across the 8, 9-double bond of the furan ring of aflatoxin through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids [Proctor et al., 2004]. As a disinfectant, ozone is 1.5 times stronger than chlorine and is effective over a much wider spectrum of micro-organisms [Xu, 1999, Maeba et al., 1988] have confirmed the destruction and detoxification of aflatoxins B1 and G1 with ozone. Aflatoxin B1 and G1 were sensitive to ozone and degraded with 1.1 mg/l of ozone in 5 min in model experiments. The reductions of content of aflatoxin B1 levels in flaked and chopped red peppers around 80% and 93% after exposures to 33 mg/l ozone and 66 mg/l ozone for 60 min, respectively was shown. [Inan et al., 2007].

3.2 Chemical control

A number of chemicals have been investigated for their ability to destroy, transform, or inactivate aflatoxin [Dollear, 1969; Mann et al., 1970]. Developing measures to control mycotoxin contamination is a high priority for the food and animal feed industries. The most reliable method to prevent mycotoxicosis is to avoid the use of contaminated materials to disinfect fungi and to inactivate mycotoxin. Most of the chemical treatments proposed are not necessarily practical, however, because they not only decompose aflatoxin but also deplete the quality of the food and feed materials themselves. The chemical used for elimination of aflatoxins are mainly antifungal agents, but they can also be exclusively used for inhibition of aflatoxin biosynthesis and destruction of the toxins.

3.2.1 Antifungal agents

So far a large number of compounds have been found to inhibit aflatoxin production. Most of them appear to do so by inhibiting fungal growth. For example, some surfactants have suppressed the growth of *Aspergillus flavus* and aflatoxin synthesis. [Bata et al., 1999]. Among the chemical compounds screened, propionic acid (0.1–0.5%), ammonia (0.5%), copper sulphate (0.5–1%) and benzoic acid (0.1–0.5%) completely inhibited *A. parasiticus* growth. It has been shown that sodium benzoate has antimicrobial effect on the growth, survival and aflatoxin production of *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* in packaged garri (2 kg/pack) during storage at ambient temperature (30±2 ˚C) [Ogiehor et al., 2004]. Sodium hypochlorite (0.1–0.5%) exhibited high anti-fungal property (68–84%). Urea (0.1–0.5%), citric acid (0.2–0.5%) and sodium propionate (0.1–0.5%) were moderate in inhibiting fungal growth. Citric acid below 0.2% had poor anti-fungal effect [Gowda et al., 2004]. Ammonia at 0.2% level and copper sulphate below 0.08% level had moderate anti-fungal activity (60 and 36%, respectively).

3.2.2 Inhibition of aflatoxin biosynthesis and degredation of the toxin

Two extensively studied inhibitors of aflatoxin synthesis are dichlorvos (an organophosphate insecticide) and caffeine [Hsieh, 1973]. A large number of chemicals can react with aflatoxins and convert them to less toxic and mutagenic compounds. These chemicals include acids, bases, oxidizing agents, bisulphites and gases [Dollear et al., 1968; Mann et al., 1970; Mendez-Albores, 2007]. The components of the neem tree (Azadirachta indica) is well known for its interference in aflatoxin biosynthesis with very little action on the fungal mycelia [Bhatnagar et al., 1988., Allameh et al., 2001] . There are evidences which show that neem leave extracts exclusively inhibit aflatoxin biosynthesis in toxigenic fungi without a major change in the mycelia. Information about the effectiveness and mode of action of the neem components will be discussed under section "3-3-3 Interference of natural products in aflatoxin biosynthesis" of this chapter.

In addition, most of the antifungal agents can also inhibit aflatoxin biosynthesis and cause destruction of aflatoxin structures. For example, ammoniation process is believed to detoxify aflatoxins in various raw materials with high efficiency [Buser and Abbas, 2002]. Alkaline compouds, such as ammonia, sodium- and calcium hydroxide etc, were used particularly for destruction of aflatoxin (for a review, see Samaraeva et al., (1990). Elimination of aflatoxins in feed by ammonia treatment is one of the approaches to reduce aflatoxicosis. After replacing the aflatoxin-containing maize with ammoniated grains (1%v/w) in diet of one-day-old broiler chicks, the mortality rate significantly decreased [Allameh et al., 2005]. Treatment with ammonia in the gaseous phase, in solution, or with substances capable of releasing it, achieved optimum results in detoxifying peanut, cotton and corn meals. It was observed that the aflatoxin B1 molecular structure is irreversibly altered when exposure to ammonia lasts long enough. In contrast, if exposure is not sufficiently protracted, the molecule can revert to its original state [Piva et al., 1995]. Our experience showed that ammonia vapors reduce aflatoxins after destruction of fungal mycelia and spores of the toxic fungi [Namazi et al., 2002]. Efficient detoxification of aflatoxin-contaminated groundnut meal with ammonia during pelleting using 5% NH3 and 200 g water/kg during a 10-day period further confirm the effectiveness of ammonia [Thiesen, 1977].

Sodium bisulfite treatment is a common aflatoxin B1 detoxification method [Moerck et al., 1980; Sommartya et al., 1988; Hagler et al., 1982]. Although it is less efficacious than ammonia detoxification it overcomes some of the typical disadvantages of ammonia methods and also has much lower costs. The main reaction product has been isolated and identified as a sulfonate, called 15α-sodium sulfonate or aflatoxin B1S (aflatoxin B1S) [Hagler et al., 1983; Yagen et al., 1989], which forms by insertion of NaHSO, at the double bond of fitrofuranic ring, depriving the aflatoxin B1 molecule of main DNA molecule reaction site, thus reducing its mutagenic potential. The efficacy of nixtamalization, a traditional practice widely used in South America to prepare typical corn tortillas consisting of cooking the corn in boiling water supplemented with calcium hydroxide has been reviewed [Piva et al., 1995].

Formaldehyde is a compound which is moderately efficacious in attacking and neutralizing the aflatoxin B1 molecule, even if no data on its reaction mechanism are available. Studies showed its enhanced efficacy in association with ammonia [Frayssinet et al., 1972] and calcium hydroxide [Codifer et al. 1976]. In contaminated milk samples addition of 0.5% formaldehyde could reduce 1.1 µg aflatoxin M, to 0.05 µg [Heimbecher et al., 1988]. Bleaching of flour with chlorine in a commercial mill resulted in a 10% reduction of

deoxynivalenol content. Aqueous sodium bisulfate caused the greatest reduction in mycotoxin levels [Bata and Laszitity, 1999].

Aqueous citric acid exhibits detoxifying activity in aflatoxin B1-contaminated feeds and protects animals from chronic aflatoxin toxicity [Mendez-Albores, 2007]. The aqueous citric acid had detoxification activity in treating aflatoxin contaminated maize [Méndez-Albores et al., 2005]. The detoxification of aflatoxin B1 initially involves the formation of the β-keto acid structure, catalyzed by the acidic medium, followed by hydrolysis of the lactone ring yielding aflatoxin D1 (a nonfluorescent compound, which exhibits phenolic properties and lacks the lactone group derived from the decarboxylation of the lactone ring-opened form of aflatoxin B1); and to a lesser extent, a second compound (a nonfluorescent phenol, commonly known as aflatoxin D2), which retains the difurane moiety but lacks both the lactone carbonyl and the cyclopentenone ring, characteristic of the aflatoxin B1 molecule The addition of different amounts of citric acid in the milled sorghum resulted in a moderated improvement in the extent of detoxification when using concentrations of 0.5–2 N [Mendez-Albores et al., 2009].

Anti-aflatoxigenic activity of certain chemicals such as eugenol [Jayashree and Subramanyam, 1999] and hydrolysable tannins [Mahoney and Molyneux, 2004] as well as some plant components [Joseph et al., 2005] is due to their antioxidant capacities. Epoxides, which can lead to lipid peroxidation of fungal cells, stimulated aflatoxin biosynthesis [Fanelli et al., 1983]. Inhibition of aflatoxin B1 production by 2-chloroethyl phosphoric acid revelaed that this compound can greatly reduce the expression of two aflatoxin biosynthetic genes, aflR and AflD, indicating that ethylene-related inhibition in aflatoxin biosynthesis is partly due to transcriptional inhibition of aflatoxin biosynthetic genes [Huang et al., 2009].

3.3 Biological control

Biological factors possess antimicrobial properties can be classified based on their source and the mechanism of action. Certain bacteria, fungi and yeast have been identified for their potential action of aflatoxin producing fungi. The mechanisms of action of biological agents to control aflatoxins is mainly through, biodegradation of the secondary metabolites and antifungal activity. Great successes in reducing aflatoxin contamination have been achieved by application of nontoxigenic strains of Aspergillus *flavus* and Aspergillus *parasiticus* in fields of cotton, peanut, maize and pistachio. According to Yin and co-workers (2008), the nontoxigenic strains applied to soil can occupy the same niches as the natural occurring toxigenic strains. Therefore, they may be capable of competing and displacing toxigenic strains.

3.3.1 Biodegradation of aflatoxins

Inactivation of aflatoxin by physical and chemical methods has not yet proved to be effective and economically feasible [Mishra and Das, 2003]. Microorganisms, especially bacteria, have been studied for their potential to either degrade mycotoxins or reduce their bioavailability. In recent years scientists focused on identification and application of natural products for inactivation of aflatoxins. It has been suggested that the biological detoxification offers an attractive alternative for eliminating toxins and safe-guarding the quality of food and feed. Ciegler et al. (1979) screened over 1000 microorganisms for the ability to degrade aflatoxins. Only one bacterium, *Flavobacterium aurantiacum* B-184, was able to irreversibly remove aflatoxin from solutions. The early investigations showed that pH

and temperature influenced the uptake of the toxin by the cells. The first important question which must be answered is whether *Flavobacterium aurantiacum* actually degrades the aflatoxin or whether the disapperance of the toxin resulted from adsorption to the cells.

Detoxification of aflatoxin B1 by *Enterococcus faecium* is probably due to the binding of the mycotoxin to the bacterial cell wall components, a mechanism which has also been postulated by other studies [Haskard et al., 2001]. Bacterial cell wall peptidoglycans and polysaccharides have been suggested to be responsible components for the mycotoxin binding by bacteria [Hosono et al., 1988].

It has been demonstrated that *Bacillus subtilis* could reduce the aflatoxin quantity in co-culture with *Aspergillus* producing aflatoxin. Perhaps, likely, the *Bacillus subtilis* metabolites inhibit both spore germination and hyphal elongation, which induces the decrease of fungal development and consequent reduction of the aflatoxin production.

According to Teniola and co-workers (2005), *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* are able to degrade aflatoxin B1 more effectively and within a shorter time than the two *Nocardia corynebacterioides* strains. It was particularly interesting to notice up to 70% aflatoxin B1 elimination within 1 h of applying cell free extracts from the two strains, and >90% degradation was observed within 4 h. There was no detectable aflatoxin B1 from any strain after 24 h, with the exception of *Nocardia corynebacterioides* DSM 12676 (formerly *Flavobacterium aurantiacum*). These results are similar to the observations of Smiley and Draughon (2000), who showed that about 74.5% aflatoxin B1 degradation by the bacterial cell free extract obtained by lysozyme treatment after 24 h of incubation. It has been observed a diminishing aflatoxin B1 degradation which was attributed to the effects of heat treatment and incorporation of proteinase K into their extract. Liquid cultures of *Rhodococcus erythropolis* were also able to degrade aflatoxin B1 very effectively. Optimal degradation by the four isolates occurred at 30 ˚C which makes them applicable in food in the tropical environment like West Africa [Teniola et al., 2005].

It has been demonstrated that the *Bacillus subtilis* could reduce the aflatoxin levels directly without affecting the fungal development. The probiotic activity of bacteria depends on the bacterial strain and the density of bacteria used.

Some of the species of bacteria and fungi have been shown to enzymatically degrade mycotoxins (Bata and Lasztity, 1999]. However, question remains on the toxicity of products of enzymatic degradation and undesired effects of fermentation with non-native microorganisms on quality of food.

Isolates of yeasts belonging to different species including *Saccharomyces cerevisiae* and *Candida krusei* were tested for aflatoxin binding, some of the isolates from West African maize were found to bind more than 60%(w/w) of the added toxins. Most of the yeast strains bound more than 15% (w/w) of aflatoxin B1 and the toxin binding was highly strain specific. There are many reports on use of physically separated yeast cell walls obtained from brewery as feed additive in poultry diet resulting in amelioration of toxic effects of aflatoxins (Santin et al., 2003). When dried yeast and yeast cell walls were added to rat diet along with aflatoxin B1, a significant reduction in the toxicity was observed (Baptista et al., 2004). In an *in vitro* study with the cell wall material, there was a dose-dependent binding of as much as 77% (w/w) and modified mannan-oligosaccharides derived from the *Saccharomyces cerevisiae* cell resulted in as much as 95% (w/w) binding (Devegowda et al., 1996). Available experimental supports suggest the role of both peptidoglycon and polysaccharides in toxin binding (Zhang & Ohta, 1991). Based on some of the studies reported, it is confirmed that removal of mycotoxins is by adhesion to cell wall components

rather than by covalent binding or by metabolism, as the dead cells do not lose binding ability (Baptista et al., 2004; Santin et al., 2003). Reported literature indicates that mannan components of cell wall play a major role in aflatoxin binding by *Saccharamyces cerevisiae* (Devegowda et al., 1996). Animal feeding experiments with whole yeast and yeast cell wall [Santin et al., 2003) show that addition of *Saccharomyces cerevisiae* in the diet resulted in reduced mycotoxin toxicities, indicating possible stability of the yeast-mycotoxin complex through the gastrointestinal tract. Similarly, Gratz et al., 2004 showed that pre-exposure of cells of *Lactobacillus rhamnosus* strain GG to aflatoxin B1 reduces its binding with intestinal mucus, resulting in faster removal. (Shetty et al., 2006).

In recent years it became clear that fungi play a major role in the degradation of aflatoxin B1. Fungi have been implicated in aflatoxin B1 degradation include zygomycetous fungi (*Rhizopus sp*. and *Mucor sp*.), ascomycetous fungi (*Aspergillus niger* and *Trichoderma sp*.), plant pathogens (*Phoma sp*. and *Alternaria sp*.), as well as basidiomycetous fungi (*Armillariella tabescens* and other white rot fungi) (Leonowicz et al., 1999). When the degradation of polyphenolic xenobiotics are considered, fungi is considered as one of the major groups responsible for their degradation, presumably due to the large repertoire of extracellular enzymes produced by these fungi (Arora and Sharma, 2009). Treatment of aflatoxin B1 with laccase enzyme produced by white rot fungi in unconcentrated culture filtrates, pure fungal laccase as well as with recombinant laccase enzymes decreased the fluorescence properties of the aflatoxin B1 molecule as determined with HPLC. It has been shown that treatment of aflatoxin B1 with fungal laccase enzymes targets and changes the double bond of the furofuran ring of the aflatoxin B1 molecule causing changes in aflatoxin fluorescence and mutagenicity properties (Alberts et al., 2009).

The use a yeast strain, *Pichia anomala*, to reduce spore production of *Aspergillus flavus* on pistachio nut fruits, leaves, and flowers has also been reported (Hua, 2004). Another approach involves competitive exclusion of toxigenic strains with a nonaflatoxigenic isolate. Using nonaflatoxigenic *Aspergillus flavus* isolates to competitively exclude toxigenic *Aspergillus flavus* isolates in agricultural fields has become an adopted approach to reduce aflatoxin contamination. From screening subgroups of nonaflatoxigenic *Aspergillus flavus*, an *Aspergillus flavus* isolate, (TX9-8), has been identified, which competed well with three *Aspergillus flavus* isolates producing low, intermediate, and high levels of aflatoxins, respectively. TX9-8 has a defective polyketide synthase gene (pksA), which is necessary for aflatoxin biosynthesis. Co-inoculating TX9-8 at the same time with large sclerotial (L strain) *Aspergillus flavus* isolates at a ratio of 1:1 or 1:10 (TX9-8: toxigenic) prevented aflatoxin accumulation. The intervention of TX9-8 on small sclerotial (S strain) *Aspergillus flavus* isolates varied and depended on isolate and ratio of co-inoculation. At a ratio of 1:1 TX9-8 prevented aflatoxin accumulation by *Aspergillus flavus* CA28 and caused a 10-fold decrease in aflatoxin accumulation by *Aspergillus flavus* CA43. No decrease in aflatoxin accumulation was apparent when TX9-8 was inoculated 24 h after toxigenic L- or S strain *Aspergillus flavus* isolates started growing. The competitive effect is likely due to TX9-8 outgrowing toxigenic *Aspergillus flavus* isolates. [Chang, Hua 2007].

According to the literature, *Armillariella tabescen* (Scop. ex Fr.) *Sing.,* is a non-toxic, edible fungus possesses detoxification activity towards aflatoxin B1 contaminated media. The detoxification activity of the extracts obtained from *Armillariella tabescen* mycelium pellets is assigned to the enzymes in the active extracts [Liu et al., 1998]. There are also other microorganisms such as soil or water bacteria, fungi, and protozoa and specific enzymes isolated from microbial systems can degrade aflatoxin group members with varied efficiency to less- or nontoxic products [Wu et al., 2009].

3.3.2 Antifungal agents

Antifungal agents with natural sources, which prevent the contamination of food by controlling the growth of *Aspergillus flavus* and *Aspergillus parasiticus*, is probably the most rational to prevent the growth of toxic fungi during storage.Inactivation of aflatoxin by physical and chemical methods has not yet proved to be effective and economically feasible (Mishra and Das, 2003). In recent years scientists focused on identification and application of natural products for inactivation of aflatoxins. It has been suggested that the biological detoxification offers an attractive alternative for eliminating toxins and safe-guarding the quality of food and feed.

Essential oils with antimicrobial properties are probably promising for growth inhibition of potentially toxigenic fungi. However, limited studies carried out on the mechanism of action of essential oils on fungal mycelia growth show that probably the cell wall and cell membrane are the main targets of the oil compartments. The plasma membrane of *Aspergillus parasiticus*, in the presence of thyme essential oils at 250 ppm, was seen to be irregular, dissociated from the cell wall, invaginated and associated with the formation of lomasomes. These lomasomes are usually found in fungi treated with imidazole components. The marked action of oil components might have conferred lipophilic properties and the ability to penetrate the plasma membrane. It has been shown that essential oil derived from Hyssopus officinalis affected the wall synthesis of *Aspergillus fumigatus.* The presence of the oil in the culture medium induced marked changes in the content of galactose and galactosamine. The alterations were related to changes in the structure of the cells. Such modifications induced by essential oils may be related to the interference of essential oil components with enzymatic reactions of wall synthesis, which affects fungal morphogenesis and growth [Ghfir et al., 1997].

Kurita et al. (1981) suggested that the antifungal activity of essential oil components, particularly aliphatic aldehydes, might be due to their ability to form charge transfer complexes with electron donors in the fungus cell [Rasooli et al., 2005]. The action of the oils on the integrity of nuclear membrane has not been ruled out. Changes in ultrastructure of the aflatoxin-roducing fungi treated with neem leaf extracts showed that the mycelia membrane is very susceptible to this treatment [Allameh et al. 2002].

3.3.3 Interference of natural products in aflatoxin biosynthesis

Numerous studies have been conducted to determine the effects of various food additives, preservatives, chemical, and environmental condition to effectively inhibit fungal growth and aflatoxin production. Despite the efficiency of chemicals in removal of aflatoxin-producing fungi and aflatoxins, the residues of chemicals can pose serious hazards to human and animal health. Meanwhile considerable pressure from consumers to reduce or eliminate chemically synthesized additives in their foods has led to a renewal of scientific interest in natural substances [Nychas, 1995; Bluma et al., 2008]. Some studies have concluded that whole essential oils have a greater antibacterial activity than the major components mixed, which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence. Among the thousands of naturally occurring constituents so far identified in plants and exhibiting a long history of safe use, there are none that pose, or reasonably might be expected to pose a significant risk to human health at current low levels of intake when used as flavoring substances [Rasooli

et al., 2007]. Numerous diverse compounds and extracts containing effects inhibitory to aflatoxin biosynthesis have been reported. The most of these inhibitors are plant-derived such as phenylpropanoids, terpenoids and alkaloids [Holmes et al., 2008]. Most plants produce antimicrobial secondary metabolites, either as part of their normal program of growth and development or in response to pathogen attack or stress. A novel way to reduce the proliferation of microorganism and/or their toxins production is the use of essential oils, which are mixtures of different lipophilic and volatile substances, such as monoterpenes, sesquiterpenes, and/or phenylpropanoids, and have a pleasant odor. Furthermore, they are considered to be part of the preformed defense system of higher plants [Reichling et al., 2009]. They are usually obtained by steaming or hydro-distillation which was first developed in the middle Ages by the Arabs. Essential oils can contain about 20-60 components in quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20-70%) compared to others present in trace amounts [Alpsoy, 2010]. A range of synthetic preservatives are being used to prevent the growth of food spoiling microbes causing different food borne diseases. However, most of them have been reported to cause different side effects after application. They are also responsible for the enhancement of reactive oxygen species molecules causing oxidative diseases by damaging the proteins, lipids, nucleic acids and more importantly stimulation of aflatoxin biosynthesis [Prakash, et al., 2011]. Until now, many studies have revealed that Aspergillus growth was completely inhibited by many plants essential oils. The effects of essential oils of 58 plant species were examined on the development of Aspergillus flavus and/or Aspergillus parasiticus by Alpsoy et al (2010). Different concentrations of the essential oils was found to inhibit the development of Aspergillus species. It is possible to use a combination of essential oils to increase their effects on fungal growth and aflatoxin production. The antifungal efficacy of plant essential oils varies depending on the concentration and composition of the oils. The inhibitory effects of the components of essential oil on growth rate of Aspergillus flavus and Aspergillus parasiticus has also been reported. Some essential oils and other extracts (vitamins, riboflavin, carotenoids, beta-carotene, alfa-carotene, lycopene, ascorbic acid, curcumin, several flavonoids, phenolic compounds and synthetic phenolic compounds) of plants could potentially provide protection against aflatoxins especially aflatoxin B1 [Rasooli et al. 2004; Rasooli and Owlia, 2005; Rasooli et al., 2008; Bluma and Etcheverry, 2008]. Phenolic compounds such as acetocyringone, syringaldehyde and sinapinic acid not only inhibited aflatoxin B1 biosynthesis, but also reduced production of intermediate metabolites namely, norsolinic acid. It was observed that the oils of cassia, clove, star-anise, geranium and basil inhibited the mycelial growth of established seed-borne infections of Aspergillus flavus, Curvularia pallescens and Chaetomium indicum as well as preventing infection following inoculation with Aspergillus flavus, Aspergillus glaucus, Aspergillus niger and Aspergillus sydowi. These oils also preserved the grain from natural Aspergillus flavus infection during the experimental period. Many natural compounds found in dietary plants, such as extracts of herbs and fruit extracts, possess antimicrobial activities against Aspergillus parasiticus [Soliman and Badeaa, 2002; Rasooli & Owlia, 2005]. Also spices and herbs, such as cloves, anise and star anise seeds, basil, cinnamon, marigold and spearmint [Soliman and Badeaa, 2002], garlic and onion, thyme [Rasooli & Owlia, 2005; Rasooli et al., 2006a], cassia and sweet basil [Atanda et al., 2007] have been reported to inhibit toxigenic and foodborne moulds[Rasooli et al., 2007]. Alderman and Marth (1976), who tested the antimicrobial

activity of citrus oils and D-limonene- the major constituent of citrus oils against an aflatoxin-producing strain of Aspergillus parasiticus, found that citrus oils at a concentration of 3000-3500 ppm, suppressed mold growth and aflatoxin production. Likewise, growth and aflatoxin formation in the basal medium was depressed by orange and lemon oils at a concentration of 1.6% through 10 days incubation [Lillehoj & Zuber, 1974; Bullerman et al., 1977]. The essential oils of anise, caraway and fennel showed inhibitory effects on the four tested fungi, Aspergillus flavus, Aspergillus parasiticus, Aspergillus ochraceus and Fusarium moniliforme, at various concentrations [Soliman and Badeaa, 2002]. The inhibitory effect of the oil is proportional to its concentration, and the anise essential oil has more inhibitory effect than the other two members of the Umbellifereae family, caraway and fennel. The antifungal activity of these oils can be assigned to their active components, such as anithole. Caraway and spearmint belong to different families but they contain carfone as a main component of their essential oils, which may be responsible for their antifungal activity. The essential oils of members of the Labiateae family have inhibitory effects on the Aspergillus flavus, Aspergillus parasiicus, Aspergillus ochraceous and Fusarium moniliforme. Thyme oil was more toxic to the four pathogenic fungi than spearmint and basil (two members of the Labiateae family). The antifungal activity of thyme, spearmint and basil was also demonstrated by Montes-Belmont and Carvajall (1998) and Basilico and Basilico (1999) on the toxigenic fungi Aspergillus flavus, Aspergillus parasiticus, Aspergillus ochraceus, Aspergillus fumigatus and Fusarium spp. The antifungal activity of mint, basil and thyme on some pathogenic fungi, including Aspergillus flavus and Aspergillus parasiticus has also been reported which suggested their inhibitory effects on the sporulation of fungi and aflatoxin production [Ela et al., 1996; Inouye et al., 1998; Inouye et al., 2000; El-Maraghy, 1995; Dube et al., 1989]. These effects could be related to several components known to have biological activities, such as α-pinene, β-pinene in thyme, basil and spearmint, respectively. In addition, thyme oil contained thymol and p-cymene as the most prevalent components. The major substances for basil oil were ocimene and methyl chavecol. The essential oils extracted from some medicinal plants belonging to the family Compositeae have fungistatic activity against all toxigenic fungi. The fungistatic effects of chamomile and hazanbul essential oils, marigold and quyssum ghafath essential oil (family: Rosaceae, Species: agrimonia eupatoria) on the growth of Aspergillus flavus, A. parasiticus, Aspergillus ochraceus and Fusarium moniliforme showed differential effects on growth of these fungi. In a similar study with essential oil of cinnamon it was shown that this oil can completely inhibit the growth of the test fungi. The three components of cinnamon that have been identified as the agents active against moulds are cinnamic aldehyde [Bullerman, 1974], O-methoxycinnamaldehyde [Morozumi, 1978] and carfone [Dwividi and Dubey, 1993]. Many previous studies had verified cinnamon oil as a fungistatic agent against many toxigenic fungi and proved its highly fungicidal activity [Sinha et al., 1993]. Morover, the effect of essential oils (thyme, cinnamon, anise and spearmint) in inhibition of toxin production in inoculated wheat further confirm the applications of essential oils [Soliman and Badeaa, 2002]. White wood, cinnamon and lavender significantly inhibited the growth of Aspergillus flavus IMI 242684 [Thanaboripat et al, 2007]. The major constituents of white wood oil was found to be monoterpene compounds such as terpinolene (24.74%) and γ-terpinene (22.84%) [Brophy et al., 2002]. There has been speculation on the contribution of the terpene fraction of the oils to their antimicrobial activity [Conner, 1993]. A number of compounds and substances have

been found to effectively inhibit fungal growth and aflatoxin production, while others have stimulatory properties [Zaika & Buchanan, 1987]. It is worth mentioning that low concentrations of test compounds often stimulate fungal growth and/or toxin production, while higher concentrations may completely inhibit the fungal growth. For instance, clove oil at 50 and 100 µg/ml and cinnamon oil at 50 µg/ml stimulated the growth of Aspergillus flavus in liquid media whereas higher concentrations reduced the mycelial growth. Essential oils from different sources such as those extracted from cinnamon (Cinnamomum zeylanicum), peppermint (Mentha piperita), basil (Ocimum basillicum), origanum (Origanum vulgare), the flavoring herb epazote (Teloxys ambrosioides), clove (Syzygium aromaticum) and thyme (Thymus vulgaris) were also proved to completely inhibit Aspergillus flavus growth on maize kernels.

The concentration of essential oils used for fungal inhibition studies varies depending on different factors. For instance, five different oils namely; geraniol, nerol, citronellol (aliphatic oils), cinnamaldehyde (aromatic aldehyde) and thymol (phenolic ketone), used at concentration of 100 ppm could completely suppress growth of *Aspergillus flavus* and consequently prevented aflatoxin synthesis in liquid medium. The hydrosols of anise, cumin, fennel, mint, picking herb, oregano, savory and thyme showed a strong inhibitory effect on mycelial growth of *Aspergillus parasiticus* NRRL 2999 [Sinha et al., 1993].

Inhibition of *Aspergillus parasiticus* growth and its aflatoxin production in presence of the essential oils extracted from two varieties of thyme i.e. Thymus eriocalyx and Thymus x-porlock were studied. The oils from the above plants were found to be strongly fungicidal and inhibitory to aflatoxin production. The oils analyzed by GC and GC/MS lead to identification of 18 and 19 components in Thymus eriocalyx and Thymus x-porlock oils respectively. The profile of the oil components from Thymus eriocalyx was similar to that of Thymus x-porlock in almost all the compounds but at different concentrations. The major components of Thymus eriocalyx and Thymus x-porlock oils were thymol β-phellandrene and cis-sabinene hydroxide respectively. Thymus eriocalyx oil exerted higher antifungal as well as antitoxic effects than that of Thymus x-porlock. This difference in antifungal and aflatoxin inhibition efficacy of thymus essential oils may be attributable to the oil compositions. Thymus eriocalyx oil contains higher (more than 2-fold) thymol than T. x-porlock oil [Rasooli et al., 2006b].

Growth of *Aspergillus parasiticus* NRRL 2999 was also reported to be completely inhibited by thyme (wild), thyme (black), oregano and savory extracts at the 2% level in Czapek-Dox Agar [Ozcan, 1998]. Inhibition of growth in phytopathogenic fungi such as *Rhizoctonia solani, Pythium ultimum* var. *ultimum, Fusarium solani and Colletotrichum lindemuthianum* inhibition was reported to be associated with the degeneration of fungal hyphae after treatment with Thymus vulgaris L., Lavandula R.C., and Mentha piperita L. essential oils with the oil of thyme being more effective than that of lavender or mint [Zambonelli et al., 1996].

The effectiveness of *Thymus kotschyanus* and *Zataria multiflora* Boiss. on the growth of the *Aspergillus parasiticus* strain and aflatoxin production are probably due to major substances such as thymol and carvacrol showing antifungal effects [Pinto et al., 2006] and completely suppressing aflatoxin synthesis [Mahmoud, 1994]. It is well known that a phenolic-OH group is very reactive and can easily form hydrogen bonds with the active sites of enzymes [Farag et al., 1989; Rasooli et al., 2009]. Based on the antifungal potential of essential oils derived from *Thymus vulgaris* L., *Thymus tosevii* L., *Mentha spicata* L., and *Mentha piperita* L.

(Labiatae) it has been suggested that these products could be used as natural preservatives and fungicides [Sokovic et al., 2009].

It appears that carvone has better antifungal properties because of its high water solubility. One of the reasons for lower antifungal activity of *Mentha piperita* essential oil could be due to the large amount of menthyl acetate, which is probably responsible for causing a decrease in antifungal properties [Griffin et al., 2000; Sokovic et al., 2009].

Essential oil-related inhibition in mycelial growth was observed to be associated with significantly decreased levels of aflatoxin production. Exposure of toxigenic Aspergillus parasiticus to neem leaf aqueous extract resulted in the inhibition of aflatoxin production not fungal growth, while exposue of fungus to essential oils from Thymus species caused inhibition in both fungal growth and aflatoxin synthesis [Rasooli & Razzaghi-Abyaneh 2004; Razzaghi-Abyaneh et al., 2005b]. Bhatnagar and McCormic (1988) have demonstrated that addition of neem leaf extract above 10% (v/v) effectively inhibited aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus*. Neem oil at 0.5% had moderate antifungal activity (84% reduction versus control). Neem oil at below 0.2%, neem seed cake at above 0.5–10% was moderate in preventing fungal growth i.e. 25–52%. A major feature of the neem leaf extracts is that when added to growth media did not affect the fungal growth, but it could essentially block aflatoxin biosynthesis at concentrations greater than 10%. These results were further confirmed in our laboratory by showing that different concentrations of aqueous neem leaf extract inhibited fungal growth and aflatoxin production by *Aspergillus parasiticus* (NRRL 2999). The inhibition of aflatoxin synthesis by neem extracts was found to be time- and dose-dependent [Ghorbanian et al.,2007]. The maximum inhibitory effect was 80-90% in the presence of 50% concentration that when compared with control samples were significant. Aflatoxin was at its lowest level (>90% inhibition) when the concentration of neem extract was adjusted to 50%. In this study the interference of neem components in aflatoxin biosynthesis pathways is not ruled out [Allameh et al. 2001]. These results are inconsistent with previous reports on existence of a positive correlation between aflatoxin activity and glutathione S-transferase activity in toxigenic strains of *Aspergillus* [Saxena et al., 1989]. In this connection it has been reported that feeding high level of neem seed cake (>10%) has adverse effects on palatability and performance of poultry [Gowda et al., 2004]. Antifungal effects of neem leaf extract also reported from South America against Crinipellis perniciosa and Phytophthora species causing Witches broom and Pot Not of cocoa (Ramos et al., 2007). Azadirachtin, Azadiradione, nimonol and epoxy azadradione were yielded from the organic extract of seeds and leaves of neem. Nimonol (82%) is likely to be a major active component of neem organic extract. Inhibition of seed-born infection by neem leaf extract has been reported earlier [Massum et al., 2009]. According to Moslem & El-Kholie (2009), the extracts prepared from neem leave and seed are effective as antifungal against all tested fungi namely, *Alternaria solani, Fusarium oxysporum, Rhizoctonia solani,* and *sclerotinia sclerotiorum*, but *Fusarium oxysporum* and *Rhizoctonia solani* were the most sensitive fungi. The essential oil of Ocimum gratissimum may be recommended as a plant based safe (nontoxic) food additive in protecting the spices from deteriorating fungi as well as from aflatoxin contamination. Methyl cinnamate (48%) and γ-terpinene (26%) were recorded the major components of the oil through GC-MS analysis. The biological activity of an essential oil is related to the presence of bioactive compounds, the proportions in which they are present and due to the interactions between different compounds of the oil (Burt, 2004). The oils with antioxidant properties may be recommended in enhancing shelf life of products such as spices [Prakash et al., 2011].

Sinha et al. (1993) showed that cinnamon and clove essential oils were effective against aflatoxin formation in maize grain by *Aspergillus flavus* after 10 days under favorable conditions of mycotoxin production. It has also been shown that 500 µgg-1 of boldus, poleo, clove, anise, and mountain thyme were necessary to reduce growth rate and aflatoxin production in high pecentage (85–100%) in maize meal extract agar (MMEA) [Bluma et al., 2008b]. Also, the essential oils of Pimpinella anisum L. (anise), Pëumus boldus Mol (boldus), Hedeoma multiflora Benth (mountain thyme), Syzygium aromaticum L. (clove), and Lippia turbinate var. integrifolia (griseb) (poleo) have been shown to have significant inhibitory effect on lag phase, growth rate and aflatoxin B1 accumulation by *Aspergillus* section Flavi isolates in sterile maize grain at different water activity conditions. Only the highest concentration of the oils (3000 µgg-1) showed the ability to maintain antifungal activity [Bluma et al., 2008]. The effects of clove essential oil and its principal component, eugenol on growth and mycotoxin production by some toxigenic fungal genera such as *Aspergillus spp.*, *Penicillium spp.* and *Fusarium spp.* had been reported [Bullerman et al. 1977; Velluti et al., 2003, Velluti et al., 2004; Bluma et al., 2008].
The complete inhibition of mycelial growth of *Aspergillus flavus* and aflatoxin B production on rice grains can be assigned to eugenol extracted from clove which was effective at a concentration of 2.4 mg/g [Reddy et al., 2007]. Although clove oil is a good antifungal compound, cost is a major criterion for considering its inclusion in animal feeds [Gowda et al., 2004]. The seed extract of Ajowan (Trachyspermum ammi (L.) Sprague ex Turrill) showed the degradation of aflatoxin G1 (up to 65%). The dialyzed Trachyspermum ammi extract was more effective than the crude extract, capable of degrading >90% of the toxin. The aflatoxin detoxifying activity of the Trachyspermum ammi extract was drastically reduced upon boiling at 100 ˚C for 10 min. Significant levels of degradation of other aflatoxins viz., aflatoxin B1 (61%), aflatoxin B2 (54%) and aflatoxin G2 (46%) by the dialyzed Trachyspermum ammi extract was also observed. Time course study of aflatoxin G1 detoxification by dialyzed Trachyspermum ammi extract showed that more than 78% degradation occurred within 6 h and 91% degradation occurred 24 h after incubation [Velazhahan et al., 2010]. Other plant extracts namely, Syzigium aromaticum, Allium sativum, Curcuma longa, Ocimum sanctum, Annona squamosa, Azadirachta indica (Neem), Allium cepa, Eucalyptus terticornis, and Pongamia glaberima are among the list of the plant extracts in inhibiting both fungal growth and aflatoxin production by *Aspergillus* [Reddy et al., 2009]. In this conection, Haciseferogullary et al. (2005) reported the effect of garlic and onion extract on the mycoflora of pepper, cinnamon and rosemary and reported the effectiveness of garlic extract up to 0.25% (v/v) to inhibit the *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus ochraceus, Aspergillus terreus, Penicillium chrysogenum, Penicillium puberulum, Penicillium citrinum, Penicillium corylophilum, Rhizopus stolonifer, Stachybotrys chartarum, Eurotium chevalieri and Emericella nidulans* growth. [Reddy et al., 2009]. Extract of garlic exhibited anti-fungal effects at all levels 0.1, 0.2, 0.5 and 1%. A maximum 84% reduction in toxin production occurred at the 1% level, but significant reductions in spore counts were recorded at all levels. The anti-fungal properties of garlic were also reported by Garcia and Garcia (1988) and Kshemkalyani et al. (1990).
Bilgrami et al. (1992) recorded up to a 60% reduction in aflatoxin production with onion extract supplementation by *Aspergillus flavus* in liquid SMKY medium and in maize grains. A lacrimatory factor (Thio propanol-S-oxide) in onion extract has a sporicidal effect on *Aspergillus parasiticus* [Sharma et al., 1981]. The anti-aflatoxigenic activity of Rosemary

(Rosmarinus officinalis L.) is probably due to borneol and other phenolics in the terpene fraction. In rosemary a group of terpenes (borneol, camphore, 1,8 cineole, α-pinene, camphone, verbenonone and bornyl acetate) were reported to be responsible [Davidson and Naidu; 2000; Rasooli et al., 2008; Jiang et al., 2011]. Antimicrobial activities of such extracts are mostly attributable to the presence of phenolic compounds such as thymol, and to hydrocarbons like γ-terpinene and p-Cymene with Limonenev being more active than p-Cymene [Dorman and Deans, 2000; Rasooli et al., 2007].

It has been reported that the chemical structures of the most abundant compounds in the essential oils is correlated with its antimicrobial activity. It seems possible that phenol components of essential oils may interfere with cell wall enzymes like chitin synthase/chitinase as well as with the α- and β-glucanases of the fungus [Adams et al., 1996]. Accordingly, the high content of phenol components may account for the high antifungal activity of oils [Adam et al., 1998]. Phenolics are secondary metabolites synthesized via phenylpropanoid biosynthetic pathway. These compounds are building blocks for cell wall structures, serving as defense against pathogens [Bluma et al., 2008a]. Also, the physical nature of essential oils, that is, low molecular weight combined with pronounced lipophilic tendencies allow them to penetrate cell membrane more quickly than other substances [Pawar & Thaker, 2007]. However, there is evidence that minor components have a critical part to play in antimicrobial activity, possibly by producing a synergic effect between other components [Burt, 2004]. The antimicrobial activity of essential oils or their constituents such as thymol, carvacrol and vanillin could act in different ways; (1) The result could be in the form of damage to the enzymatic cell system, including those associated with energy production and synthesis of structural compounds (2) denaturation of the enzymes responsible for spore germination or interference with the amino acid involved in germination [Nychas, 1995] and (3) irreversible damage in cell wall, cell membrane and cellular organelles. This was further confirmed when *Aspergillus parasiticus* and *Aspergillus flavus* were exposed to different essential oils (*Thymus eriocalyx* and *Thymus X-porlock*). The evidences presented here suggest that the essential oils could be safely used as preservative materials for certain food materials, particularly those which prevent fungal infections at relatively lower concentrations [Rasooli & Owlia, 2005]. The antiaflatoxigenic actions of essential oil may be related to inhibition of the ternary steps of aflatoxin biosynthesis involving lipid peroxidation and oxygenation [Alpsoy, 2010].It is clear that phenolic compounds inhibited one or more early rather than late steps in the aflatoxin B1 biosynthesis pathway. According to Farag et al. (1989) the presence of phenolic OH groups able to form hydrogen bonds with the active sites of target enzymes was thought to increase antimicrobial activity [Bluma et al., 2008a]. Natural products may regulate the cellular effects of aflatoxins and evidence suggests that aromatic organic compounds of spices can control the production of aflatoxins.

3.4 Biotechnological approaches for fighting aflatoxin-producing fungi

Molecular breeding of crops with an ability to degrade aflatoxins offers an alternative strategy for the management of aflatoxin contamination in agricultural commodities. Poppenberger et al., 2003 reported the isolation and characterization of a gene from Arabidopsis thaliana encoding a UDP-glycosyltransferase that is able to detoxify deoxynivalenol. Takahashi-Ando et al. [2004] isolated a zearalenone-detoxifying gene, zhd101, from Clonostachys rosea. These investigators further demonstrated that a recombinant Escherichia coli expressing zhd101 completely inactivated zearalenone and zearalenol within 1 h. It has been demonstrated that

transgenic maize plants expressing the detoxification gene, zhd101 showed reduced contamination by the mycotoxin, zearalenone in maize kernels [Igawa et al., 2007]. Recently studies have been focused on identification of the aflatoxin detoxification genes from Trachyspermum ammi and to transfer them into crop plants in order to develop transgenic resistance to aflatoxin contamination [Velazhahan et al., 2010].

4. Conclusions

Several technologies have been tested to reduce mycotoxin risk. Field management practices that increase yields may also prevent aflatoxin. They include use of resistant varieties, timely planting, fertilizer application, weed control, insect control and avoiding drought and nutritional stress. Other options to control the toxin causing fungi *Aspergillus flavus* contamination in the field are use of non-toxigenic fungi to competitively displace toxigenic fungi, and timely harvest. Post-harvest interventions that reduce mycotoxins are rapid and proper drying, sorting, cleaning, drying, smoking, post harvest insect control, and the use of botanicals or synthetic pesticides as storage protectant. Another approach is to reduce the frequent consumption of 'high risk' foods (especially maize and groundnut) by consuming a more varied diet, and diversifying into less risky staples like sorghum and millet. Chemo-preventive measures that can reduce mycotoxin effect include daily consumption of chlorophyllin or oltipraz and by incorporating hydrated sodium calcium alumino-silicates into the diet. Detoxification of aflatoxins is often achieved physically, chemically and microbiologically by incorporating pro-biotics or lactic acid bacteria into the diet. There is need for efficient monitoring and surveillance with cost-effective sampling and analytical methods. Sustaining public education and awareness can help to reduce aflatoxin contamination. Phytochemicals may successfully replace physical and chemical agents and provide an alternative method to protect agricultural commodities of nutritional significance from toxigenic fungi such as *Aspergillus flavus* and aflatoxin production.

5. References

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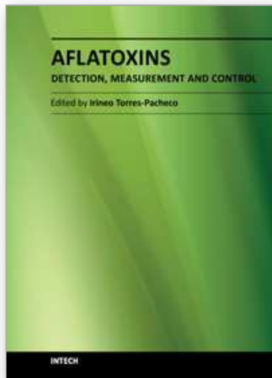

Aflatoxins - Detection, Measurement and Control
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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical prespective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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