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Aflatoxins Biochemistry and Molecular Biology - Biotechnological Approaches for Control in Crops

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1. Introduction

Fungi play a very important, but yet mostly unexplored role. Their widespread occurrence on land and in marine life makes them a challenge and a risk for humans (Bräse *et al.,* 2009). Fungi are ingenious producers of complex natural products which show a broad range of biological activities (Bohnert *et al.,* 2010). However, a specific characteristic is the production of toxins. Mycotoxins (from "*myco*" fungus and toxin), are nonvolatile, relatively low-molecular weight, fungal secondary metabolic products (Bräse *et al.,* 2009). The most agriculturally important micotoxins are aflatoxins (AF) which are a group of highly toxic metabolites, studied primarly because of their negative effects on human health. Aflatoxins belong to a group of difuranocumarinic derivatives structurally related, and are produced meanly by fungi of genus *Aspergillus* spp. Its production depends on many factors such as substrate, temperature, pH, relative humidity and the presence of other fungi. It has been identified 18 types of aflatoxins; the most frequent in foods are B_1, B_2, G_1, G_2, M_1, and M_2 (Bhatnagar *et al.,* 2002). These secondary metabolites contaminate a number of oilseed crops during growth of the fungus and this can result in severe negative economic and health impacts (Cary *et al.,* 2009). The higher levels of aflatoxins have been found in cotton and maize seeds, peanuts, and nuts. In grains like wheat, rice, rye or barley the presence of aflatoxins is less frequent. Mycotoxins may also occur in conjugated form, either soluble (masked mycotoxins) or incorporated into/ associated with/attached to macromolecules (bound mycotoxins). These conjugated mycotoxins can emerge after metabolization by living plants, fungi and mammals or after food processing. Awareness of such altered forms of mycotoxins is increasing, but reliable analytical methods, measurement standards, occurrence, and toxicity data are still lacking (Berthiller *et al.,* 2009). A variety of studies has been conducted in order to understand the process of crop contamination by aflatoxins. Mycotoxins are dangerous metabolites that are often carcinogenic, and they represent a serious threat to both animal and human health (Reverberi *et al.,* 2010). Mycotoxins are considered secondary metabolites because

they are not necessary for fungal growth and are simply a product of primary metabolic processes. The functions of mycotoxins have not clearly established, but they are believed to play a role in eliminating other microorganisms competing in the same environment (Bräse *et al.,* 2009). The biosynthesis and regulation of these toxins represent one of the most studied areas of all the fungal secondary metabolites. Much of the information obtained on the AF biosynthetic genes and regulation of AF biosynthesis was obtained through studies using *A. flavus* and *A. parasiticus* and also the model fungus *Aspergillus nidulans* that produces sterigmatocystin (ST), the penultimate precursor to AF. Further studies in *A. nidulans* and *A. flavus* and also of the fungus-host plant interaction have identified a number of genetic factors that link secondary metabolism and morphological differentiation processes in *A. flavus* as well as filamentous fungi in general (Cary *et al.,* 2009). Recent investigations of the molecular mechanism of AF biosynthesis showed that the genes required for biosynthesis are in a 70 kb gene cluster. These genes encode for the proteins required in the oxidative and regulatory steps in the aflatoxins byosinthesis. A positive regulatory gene, *aflR*, coding for a sequence-specific, zincfinger DNA-binding protein is located in the cluster and is required for transcriptional activation of most, if not all, of the aflatoxin structural genes. Some of the genes in the cluster also encode other enzymes such as cytochrome P450-type monooxygenases, dehydrogenases, methyltransferases, and polyketide and fatty acid synthases (Bhatnagar *et al.,* 2003). The application of genomic DNA sequencing and functional genomics, powerful technologies that allow scientists to study a whole set of genes in an organism, is one of the most exciting developments in aflatoxin research (Yu *et al.,* 2004; Bennett *et al.,* 2007). Moreover, the rapid development of high throughput sequencing made it possible in genetic research to advance from single gene cloning to whole genome sequencing. Tremendous advances have also been made in understanding the genetics of four non-aflatoxigenic *Aspergillus* species, *A. oryzae, A. sojae, A. niger and A. fumiga*tus. Currently, the whole genome sequencing and/or Expressed Sequence Tag (EST) projects for *A. flavus* have been completed (Bhatnagar *et al.,* 2006). The characterization of genes involved in aflatoxin formation affords the opportunity to examine the mechanism of molecular regulation of the aflatoxin biosynthetic pathway, particularly during the interaction between aflatoxin-producing fungi and plants (Bhatnagar *et al.,* 2003).Aflatoxin contamination in crops is a worldwide food safety concern due that are compound carcinogenic highly and mutagenic in animals and human (Yin *et al.,* 2008). Therefore their management in agricultural (pre-harvest, harvest and post-harvest) is of importance vital, so quantity in food and feed is closely monitored and regulated in most countries for example, in the European Union has a maximum level of 2 ng/g for B1 and 4 ng/g for total aflatoxins in crops (van Egmond and Jonker, 2004).

2. Occurrence of mycotoxins

Mycotoxins occur in many varieties of fungi. Several mycotoxins are unique to one species, but most mycotoxins are produced by more than one species. The most important mycotoxins are aflatoxins, ochratoxins, deoxynivalenol (DON), searalenone, fumonisin, T-2 toxin, and T-2 like toxins. However, food borne mycotoxins likely to be of greatest significance in tropical developing countries are the fumonisins and aflatoxins (Kumar *et al*., 2008; Muthomi *et al*., 2009). Aflatoxins are carcinogenic secondary metabolites produced by several species of *Aspergillus* section *Flavi*, including *Aspergillus flavus* Link,

Aspergillus parasiticus Speare, and *Aspergillius nominus* Kutzman, Horn, and Hesseltime. The fungus forms sclerotia which allow it to survive in soil for extended periods of time (Schneiddeger & Payne, 2003). Conditions such as high temperatures and moisture, unseasonal rains during harvest and flash floods lead to fungal proliferation and production of mycotoxins (Bhat & Vasanthi, 2003). About 4.5 billion people in developing countries are chronically exposed to aflatoxin and the CODEX recommended sanitary and phytosanitary standards set for aflatoxins adversely affect grain trade in developing countries (Gebrehiwet *et al.*, 2007). Concerns for human and livestock health have led several countries to constantly monitor and regulate aflatoxin contamination of agricultural commodities (Wang & Tang, 2005). Since the discovery of aflatoxins in the early 1960s, many studies have been conducted to assess the occurrence and to describe the ecology of aflatoxin-producing fungi in natural and agricultural environments. *Aspergillus flavus* is the most abundant aflatoxin-producing species associated with corn (Abbas *et al.*, 2004*a*). While aflatoxins occur mostly in maize and groundnuts, the prevalence of fumonisins in maize is 100% (Wagacha & Muthomi, 2008). Mycotoxins have negative impact on human health, animal productivity and trade (Wagacha & Muthomi, 2008; Wu, 2006). Aflatoxin B_1 is the most toxic and is associated with liver cancer and immune suppression (Sheppard, 2008). Exposure to large doses (> 6000 mg) of aflatoxin may cause acute toxicity with lethal effect, whereas exposure to small doses for prolonged periods is carcinogenic (Groopmann & Kensler, 1999). There may be an interaction between chronic mycotoxins exposure and malnutrition, immune-suppression, impaired growth, and diseases such as malaria and HIV/AIDS (Williams *et al.,* 2004). Mycotoxin poisoning may be compounded by the co-ocurrence of aflatoxins with other mycotoxins such as fumonisins, zearalenone and deoxynivalenol (Kimanya *et al.*, 2008; Pietri *et al.*, 2009).

However, the presence of mycotoxins in food is often overlooked due to public ignorance about their existence, lack of regulatory mechanisms, dumping of food products and the introduction of contaminated commodities into the human food chain during chronic food shortage due to drought, wars, political, and economic instability. The largest mycotoxin-poisoning epidemic in the last decade occurred in Kenya in 2004. Aflatoxin poisoning was associated with eating home grown maize stored under damp conditions (Lewis et al., 2005). Acute aflatoxin poisoning has continued to occur severally in Eastern and Central provinces of Kenya (CDC, 2004). In the 2004 aflatoxin-poisoning outbreak, the concentrations of aflatoxin B1 in maize was high as 4,400 ppb, which is 220 times greater than the 20 ppb regulatory limit. The outbreak covered more than seven districts and resulted in 317 case-patients and 125 deaths (Lewis *et al.*, 2005). The association of mycotoxins with human and animal health is not a recent phenomenon; for example, in the past, ergotism was suspected of being a toxicosis resulting from these toxic fungal metabolites. Nowadays, more is known regarding this family of compounds. Mycotoxins were considered as a storage phenomenon whereby grains becoming moldy during storage allowed for the production of these secondary metabolites proven to be toxic when consumed by man and other animals. Subsequently, aflatoxins and mycotoxins of several kinds were found to be formed during development of crop plants in the field. The determination of which of the many known mycotoxins are significant can be based upon their frequency of occurrence and/or the severity of the disease that they produce, especially if they are known to be carcinogenic. The diseases (mycotoxicoses) caused by these mycotoxins are quite varied and involve a wide range of susceptible animal species

including humans. Most of these diseases occur after consumption of mycotoxin contaminated grain or products made from such grains but other routes of exposure exist. The diagnosis of mycotoxicoses may prove to be difficult because of the similarity of signs of disease to those caused by other agents. Therefore, diagnosis of a mycotoxicoses is dependent upon adequate testing for mycotoxins involving sampling, sample preparation and analysis (Richard, 2007).

2.1 Toxicology of mycotoxins

Mycotoxins primarly occur in the mycelium of the toxigenic moulds and may also be found in the spores of these organisms and cause a toxic response, termed a mycotoxicoses, when ingested by higher vertebrates and other animals (Bennett & Klich, 2003). These secondary metabolites are synthesized during the end of the exponential phase of growth and appear to have no biological significance with respect to mould growth/development or competitiveness. All moulds are not toxigenic and while some mycotoxins are produced by only a limited number of species, others may be produced a relatively large range from several genera (Hussein & Brasel, 2001). The toxic effect of mycotoxin ingestion in both humans and animals depends on a number of factors including intake levels, duration of exposure, toxin species, mechanisms of action, metabolism, and defense mechanisms (Galvano *et al.*, 2001). Consumption of mycotoxin-contaminated food or feed does however lead to the induction of teratogenic, carcinogenic, oestrogenic, neurotoxic, and immunosuppressive effect in humans and/or animals (Atroshi *et al.*, 2002). The mycotoxins of most significance from both a public health and agronomic perspective include the aflatoxins, trichotecenes, fumonisins, ocharotoxin A (OTA), patulin, tremorgenic toxins, and ergot alkaloids (Papp *et al.*, 2002).

3. Aflatoxins

Aflatoxin was initially identified as toxic after investigations of the death of 100,000 turkeys in the United Kingdom in 1960 (Blout, 1961). This prompted a major revolution in mycotoxin research resulting in intensive testing of mycotoxins in any moldy products. Since then several *Aspergilli* have been identified as capable of producing aflatoxins. The two most agriculturally important species are *Aspergillus flavus* and *A. parasiticus*, which are found throughout the world, being present in both the soil and the air (Abbas *et al.*, 2005). When conidia (spores) encounter a suitable nutrient source and favorable environmental conditions (hot and dry conditions) the fungus rapidly colonizes and produces aflatoxin (Payne, 1992). Contamination of agricultural commodities by aflatoxin is a serious problem due to the substantial health effect it has on humans and animals. The use of agrochemicals (fungicides), timely irrigation, and alternate cropping systems have independently shown limited success in preventing aflatoxin contamination. Integration of these tactics will be required to manage such a difficult problem (Cleveland *et al.*, 2003). A more recent and promising technology is the use of non toxigenic strains of Aspergillus as biocontrol agents. However, to maximize this methodology and to prevent the colonization of multiple crops by *A. flavus* and related species (*A. parasiticus* and *A. nominus*), it is critical that a complete understanding of the ecology of these unique fungi be developed (Abbas *et al.*, 2009). Aflatoxins are toxic compounds chemically related to bisfuranocoumarin that are produced by *A. flavus* and *A. parasiticus* strains (Abbas *et al.*,

2004b). These two aflatoxigenic species have been frequently studied due to their impact on agricultural commodities and their devastating effects on livestock. The name aflatoxin comes from the genus *Aspergillus* which is where the letter "a" in aflatoxin is derived and "fla" from the species name *flavus*. In agricultural grains the fungi *A. flavus* and *A. parasiticus* are capable of producing four major aflatoxins (AfB1, AfB2, AfG1, and AfG2). *A. flavus* tipically produces only the B toxins (Abbas *et al*., 2004b). Corn and cottonseed are typically contaminated with the aflatoxin B1, produced after colonization by *A. flavus* (Klich, 1986). *A. parasiticus* is more prevalent in peanuts than any other crop; however, it is typically outcompeted by *A. flavus* when the two fungi are both present (Horn *et al*., 1995). These fungi are ubiquitous in the environment, being readily isolated from plants, air, soil, and insects (Wicklow *et al*., 2003). Soil populations of *A. flavus* in soils under maize cultivation can range from 200 to >300,000 colony-forming units (CFU) g^{-1}soil (Zablotowicz *et al*., 2007) and can constitute from ≤0.2% to ≤8% of the culturable soil fungi population. The major soil property associated with maintaining soil populations of *A. flavus* is soil organic matter. Higher populations of A. flavus are maintained in the soil surface of no-till compared to conventional-till soils (Zablotowicz *et al*., 2007). The presence of Aspergillus species in dust can compromise individuals with elevated allergies to the fungus or its products (Benndorf et al., 2008). Of more concern is the colonization of certain food and feed crops (corn, cottonseed, peanuts, and some tree nuts) by the fungus, where it may produce a high concentration of these chemical compounds, specifically aflatoxin, to cause them to be considered contaminated and unfit for their intended use (Abbas *et al*., 2009). When suitable environmental conditions arise, sclerotia and conidia germinate into mycelia that produce numerous conidiophores and release conidia into the air that can be available for colonizing plants. Although *A. flavus* colonizes a plant structure, it doesn´t necessarily produce aflatoxin to excessive levels. In this manner, *A. flavus* is an opportunistic pathogen in a similar context to the opportunistic human pathogens *Pseudomonas fluorescens* and *Burkholderia capacia*. These bacteria may colonize in low levels in compromised individuals, such as burn patients or the immunocompromised, and become pathogenic. In the same context, healthy plant tissues are less prone to be extensively colonized by *A. flavus*. However, under heat stress and moisture deficit, corn reproductive structures are readily susceptible to high levels of aflatoxin contamination, (O'Brian *et al*., 2007). Therefore, inoculum potential modified by life cycle of the fungus is as critical as the environment and the host. The *A. flavus* life cycle can be divided into two major phases: the colonization of plant residues in the soil, and the infection of crop tissues, including grain and seeds of actively growing plant tissues. At the beginning of the growing season, usually in spring and sometimes at the end of winter, when sclerotia are exposed to the soil surface, they quickly germinate and form new conidial inoculum. This new inoculum will be vectored by insects and carried by the wind to begin the colonization and infection of the freshly planted crops (Horn, 2007). During the growing season, infected plant tissues can serve as sources of secondary conidial inoculum, which colonize new non-infected plant tissues (Fig.1). Despite our understanding of how the initial and secondary inocula occur for plant infection, little information is available about the saprotrophic activities of these fungi in soil. Recently, Accinelli *et al*. (2008) confirmed the presence of A. flavus in the soil actively synthesizing aflatoxins. However, not all *A. flavus* and *A. parasiticus* isolates produce aflatoxins (Abbas *et al*., 2004b).

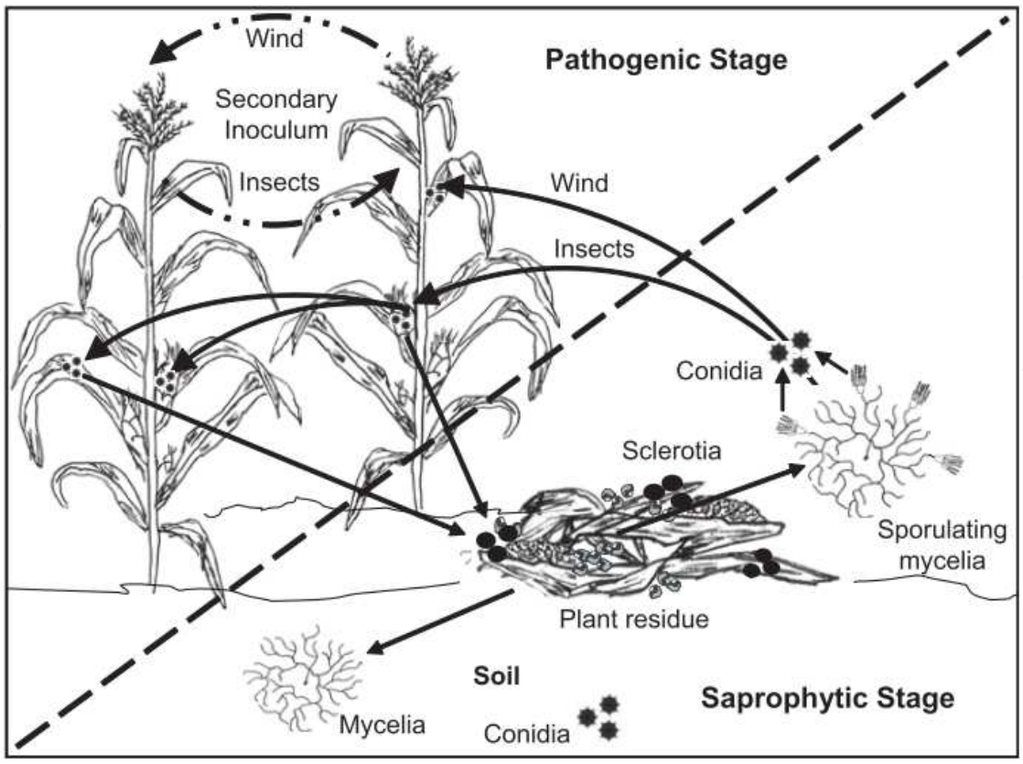

Fig. 1. Life cycle of *A. flavus* in a corn cropping system (Abbas *et al*., 2009)

Fungi are classified as nonaflatoxigenic if they do not produce aflatoxins but produce other toxins. If fungi produce no toxins at all, they are classified as nontoxigenic. Generally, in any environment, the frequency of aflatoxigenic isolates can range from 50% to 80% (Abbas *et al*., 2004a). The relative distribution of aflatoxigenic versus nonaflatoxigenic isolates is modulated by many factors including plant species present, soil composition, cropping history, crop management, and environment conditions, including rain fall and temperature (Abbas *et al*., 2004b). Each of these factors can reduce the levels of *A. flavus*, for example, noncultivated fields near cultivated land are observed to have very low populations of *A. flavus* (Horn, 2007). Similarly, the frequency of drought is a factor in populations of fungi, with significant drops in soil populations of *A. flavus* after several years without drought. The conidia remain dormant in soil and only germinate when nutrient sources are present (Zablotowicz *et al*., 2007). The behavior of Aspergilli structures in soil needs to be investigated and evaluated thoroughly, especially in agricultural soils, due to the fungal structures serving as the primary inoculum resulting in aflatoxin contamination in agricultural commodities (Abbas *et al*., 2009).

3.1 Biosynthesis

Aflatoxins the most carcinogenic substances known to date have gained much interest among organic chemists since the elucidation of their structure by Buchi and co-workers in 1963. Even though numerous syntheses of racemic aflatoxins were reported in the following years , it took 40 years for the first enantioselective total synthesis of (-)-aflatoxin B_1 and B_2 to be published by Trost *et al*. (2003), their approach resembles in part (construction of the DE ring system) the first total synthesis of (±)-aflatoxin by Buchi *et al*. (1967). The biosynthetic pathway in *A. flavus*

consists of approximately 23 enzymatic reactions and at least 15 intermediates (reviewed in Bhatnagar *et al*., 2006; Bräse *et al*., 2009) encoded by 25 identified genes clustered within a 70-kb DNA region on chromosome III (Bhatnagar *et al*., 2006a; Cary & Ehrlich, 2006; Smith *et al*., 2007; Cary & Calvo, 2008). The initial substrate acetate is used to generate polyketides with the first stable pathway intermediate being anthraquinone norsolorinic acid (NOR) (Bennett *et al*., 1997). This is followed by anthraquinones, xanthones, and ultimately aflatoxins synthesis (Yu *et al*., 2004). Few regulators of this process have been identified (Cary & Calvo, 2008), and a general model based on Aspergillus has recently been reviewed by Georgianna & Payne (2009) (Fig. 2). In addition to pathway-specific regulators, production of aflatoxins is also under the control of a number of global regulatory networks that respond to environmental and nutritional cues. These include responses to nutritional factors such as carbon and nitrogen sources and environmental factors such as pH, light, oxidative stress, and temperature. Nitrogen source plays an important role in aflatoxin biosynthesis (Bhatnagar *et al.,* 1986). In general, nitrate inhibits aflatoxin production, while ammonium salts are conducive (Cary & Calvo, 2008). Ammonium acetate does not have any significant impact on the level of OTA-related pks (the gene encoding for a polyketide synthase) expression. Nevertheless, this compound does lead to an increase in OTA production (Abbas *et al*., 2009). Some aminoacids as proline, asparagines, and tryptophane significantly increase the biosynthesis of aflatoxins B1 and G1 in *A. parasiticus* (Payne and Hagler, 1983). Tryptophane acts by up-regulating aflatoxin gene expression in *A. parasiticus* and down-regulating it in *A. flavus*. Some nitrogen sources can also be non-conducive for OTA production in A. ochraceus, and their inhibitory effect is probably exerted at the transcriptional level (O'Callaghan *et al*., 2006). The influence of carbon sources on aflatoxins and OTA biosynthesis has been studied for decades and it has produce contradictory results (Abbas *et al*., 2009). Aflatoxin biosynthesis is induced by simple sugars such as glucose and sucrose that are present or generated by fungal hydrolytic enzymes during invasion of seed tissues (Cary & Calvo, 2008). A key factor determining whether a carbon source can support aflatoxin production and fungal growth is its availability to both hexose monophosphate and glycolitic pathways. This finding was confirmed by the identification of a set of genes including *enoA* and *pbcA* genes, both these genes are up-regulated in response to sucrose supplementation (Price *et al*., 2006). The addition of different simple sugars may have opposite effects on OTA synthesis depending on the culture media used. Nevertheless, lactose exhibited a significant enhancing effect on OTA biosynthesis both in restrictive and conducive media, whilst glucose can show a repressive effect on OTA synthesis (Abbas *et al*., 2009). This negative effect may be partially explained by the involvement of *CreA*, the regulator of the carbon repression system which also acts as a controller of the secondary metabolism in many fungal species (Roze *et al*., 2004). Other environmental factors, such as temperature, water activity and pH, strongly influence mycotoxin biosynthesis. Some examples have been provided for OTA and aflatoxins biosynthesis (Ramirez *et al*., 2006; Ribeiro *et al*., 2006). The optimal temperature for production of aflatoxins is approximately 30°C (Boller & Schroeder, 1974). The establishment of temperature as an important component of infection by A. flavus and subsequent aflatoxin contamination has been clearly demonstrated under controlled greenhouse conditions (Payne *et al*., 1988). Some efforts to illustrate a relationship between temperature and aflatoxin contamination were unsuccesfull (Stoloff and Lillehoj, 1981). The reason for this phenomenon can be traced to the finding that a detectable relationship exists only during years when amounts of contamination are high (McMillian *et al*., 1985). Conclusions of this work were that high temperatures do significantly contribute to the contamination process and the ultimate

amount of aflatoxin which is produced. Naturally, nothing can be done to control ambient temperatures, but it is possible to avoid their full impact during the later stages of kernel filling by early planting (Abbas *et al.*, 2009). Relative humidity above 86% also promotes colonization and aflatoxin production in the field (Plasencia, 2004).

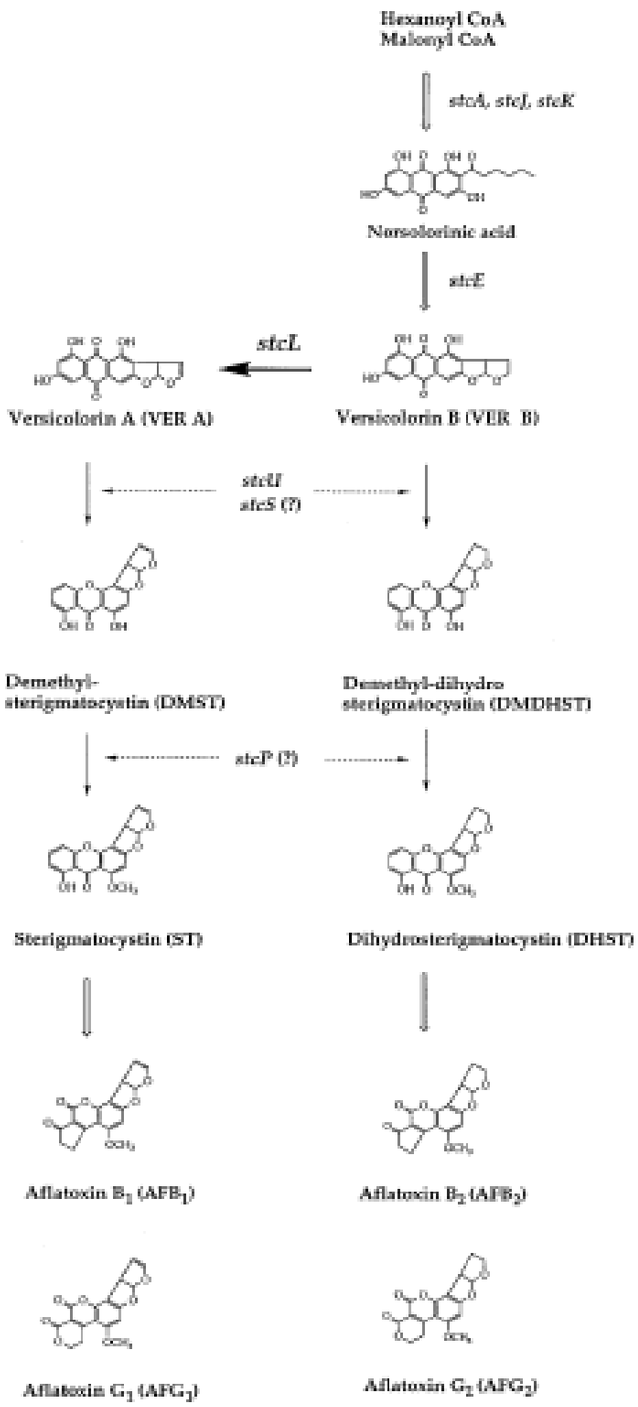

Fig. 2. AF/ST biosynthetic pathway in *Aspergillus spp.* (Kelkar *et al.*, 1997)

Aflatoxin production, in general, is greatest in acidic medium and tends to decrease as the pH of the medium increases (Keller *et al*., 1997). Response to changes in pH is regulated by the globally acting transcription factor PacC, which is posttranslationally modified by a pH-sensing protease (Tillburn *et al*., 1995). PacC binding sites indentified in the promoters of aflatoxins biosynthetic genes could be involved in negative regulation of aflatoxins biosynthesis during growth at alkaline pH (Ehrlich et al., 2002). Fungal development also appears to respond to changes in pH as sclerotial production was found to be reduced by 50% at pH 4.0 or less while aflatoxins production was at its maximal (Cotty, 1988). According to Georgianna and Payne (2009), only temperature has a greater influence on aflatoxin biosynthesis than pH. pH values lower than 4.0 are needed for aflatoxin production, and generally, the lower the pH value, the higher is the toxin synthesis (Klich, 2007).

In addition to temperature, water activity, and pH, the application of suboptimal concentrations of fungicides can boost mycotoxin biosynthesis (Schmidt-Heydt *et al*., 2007; D'Mello *et al*., 1998). A more appropriate general strategy is therefore to investigate natural products within the crop which confer resistance to *Aspergillus* colonization and growth, and/or aflatoxin biosynthesis. Two classes of protective natural factors exist in nature: phytoalexins, inducible metabolites, formed after invasion *de novo*, e.g. by activation of latent enzyme systems; phytoanticipins, constitutive metabolites, present *in situ*, either in the active form or easily generated from a precursor. Since phytoalexins are produced only in response to fungal attack, it is obvious that their presence would lag behind the infection and levels capable of suppressing aflatoxin would be difficult to regulate. In contrast, phytoanticipins are always present and such factors offer the potential for enhancement through breeding and selection of more resistant cultivar, or even genetic manipulation to introduce or enhance their levels. Once such compounds have been identified, it is only necessary to ensure that they are present in large enough quantities and in tissues from which fungal growth and aflatoxin deposition must be excluded (Campbell *et al*., 2003). Currently available methods of removing aflatoxins from tree nuts after contamination are impractical and expensive (Scott, 1998). There is a need to design new and environmentally safe methods of reducing infection by aflatoxigenic aspergilla and to inhibit aflatoxin biosynthesis.

3.2 Genetics of aflatoxin biosynthesis

Cloning of genes involved in aflatoxin biosynthesis is the key to understanding the molecular biology of the pathway (Trail et al., 1995). There are 21 enzymatic steps required for aflatoxin biosynthesis and the genes for these enzymes have been cloned (Bhatnagar et al., 2003). Molecular research has targeted the genetics, biosynthesis, and regulation of aflatoxin formation in *A. flavus and A. parasiticus*. Aflatoxins are biosynthesized by a type II polyketide synthase and it has been known for a long time that the first stable step in the biosynthetic pathway is the norsolorinic acid, an anthraquinone (Bennett et al., 1997). A complex series of post-polyketide synthase steps follow, yielding a series of increasingly toxigenic anthraquinone and difurocoumarin metabolites (Trail et al., 1995). Sterigmatocystin (ST) is a late metabolite in the aflatoxin pathway and is also produced as a final biosynthetic product by a number of species. It is now known that ST and aflatoxins share almost identical biochemical pathways (Bhatnager et al., 2003). Aflatoxin (AF) was one of the first fungal secondary metabolites shown to have all its biosynthetic genes organized within a DNA cluster (Fig. 3). These genes, along with the pathway specific regulatory genes

aflR and *aflS*, reside within a 70 kb DNA cluster near the telomere of chromosome 3 (Sweeney et al., 1999; Georgianna and Payne, 2009). Research on *A. flavus, A. parasiticus and A. nidulans* has led to our current understanding of the enzymatic steps in the AF biosynthetic pathway, as well as the genetic organization of the biosynthetic cluster. *A. nidulans* does not produce AF but has all of the genes and enzymatic steps preceding the production of ST. The AF and ST pathways appear to have a common biosynthetic scheme up to the formation of ST, and thus information gained from both pathways has been used to study AF regulation (Georgianna & Payne, 2009). The biosynthetic and regulatory genes required for ST production in *A. nidulans* are homologous to those required for aflatoxin production in *A. flavus* and *A. parasiticus* and they also are clustered. The physical order of the genes in the cluster largely coincides with the sequential enzymatic steps of the pathway and both gene organization and structure are conserved within *A. favus* and *A. parasiticus* (Sweeney *et al*., 1999; Bhatnagar *et al.,* 2006). Of the 25 genes identified in the pathway, only four (*norA, norB, aflT*, and *ordB*) have yet to have the function of their protein product determined experimentally. Only one of these genes, *aflR*, appears to encode a transcription factor (Bhatnagar *et al.,* 2006, 2003). The expression of the structural genes in both aflatoxin and ST biosynthesis is regulated by a regulatory gene, *aflR*, which encodes a GAL4-type C6 zinc binuclear DNA-binding protein (Bhatnagar *et al.,* 2003). This gene is located in the cluster and is required for transcriptional activation of most, if not all, of the aflatoxin structural genes. Adjacent to and divergently transcribed from the *aflR* gene is *aflJ*. This gene is also involved in the regulation of the aflatoxin gene cluster because no aflatoxin pathway intermediates are produced when it is disrupted. The gene product of *aflJ* has no sequence homology with any other genes or proteins present in databases. It interacts with *aflR* but not with the structural genes of the pathway. It has been speculated that *aflJ* is an *aflR* coactivator (Yu *et al*., 2002; Bennett *et al.,* 2007). The function of most of the aflatoxin gene products has been deduced either by genetic or biochemical means (Bhatnagar *et al.,* 2006). Two of the genes of the ST gene cluster in *A. nidulans, stcJ* and *stcK*, encode the K- and L-subunit of a fatty acid synthase (FAS) which is specific for the formation of the hexanoate starter of ST. Disrupted *stcJ/stcK* mutants do not synthesise ST, but retain the ability to do it when provided with hexanoic acid (Sweeney *et al*., 1999). The protein set requested for ST/AF transduction regulatory pathways includes: FlbA, an RGS (regulator of G-protein signaling) protein; FluG, an early acting development regulator; FadA, the alpha subunit of a heterotrimeric G-protein; and PkaA, encoding the catalytic subunit of protein kinase A. When FadA is activated following the signal "perception" both directly and indirectly it is able to inhibit AflR activity. FlbA whose activation is dependent on FluG, suppresses FadA and triggers AflR activation (Reverberi *et al*., 2010)

3.2.1 The pathway specific regulator gene

Two genes, *aflR* and *aflS*, located divergently adjacent to each other within the AF cluster are involved in the regulation of AF/ST gene expression. The gene *aflR* encodes a sequence-specific DNA-binding binuclear zinc cluster (Zn(II)2*Cys*6) protein, required for transcriptional activation of most, if not all, of the structural genes (Georgianna and Payne, 2009). It was first cloned from an A. flavus cosmid library by showing that it could restore aflatoxin-producing ability to a mutant blocked in all steps of aflatoxin biosynthesis. An increase in the copy number of *aflR* somehow altered normal regulation of aflatoxin biosynthesis (Bhatnagar *et al.,* 2003). The aflR locus has been compared among isolates of AF producers such as *A. parasiticus* and *A. flavus*. These comparisons revealed differences in

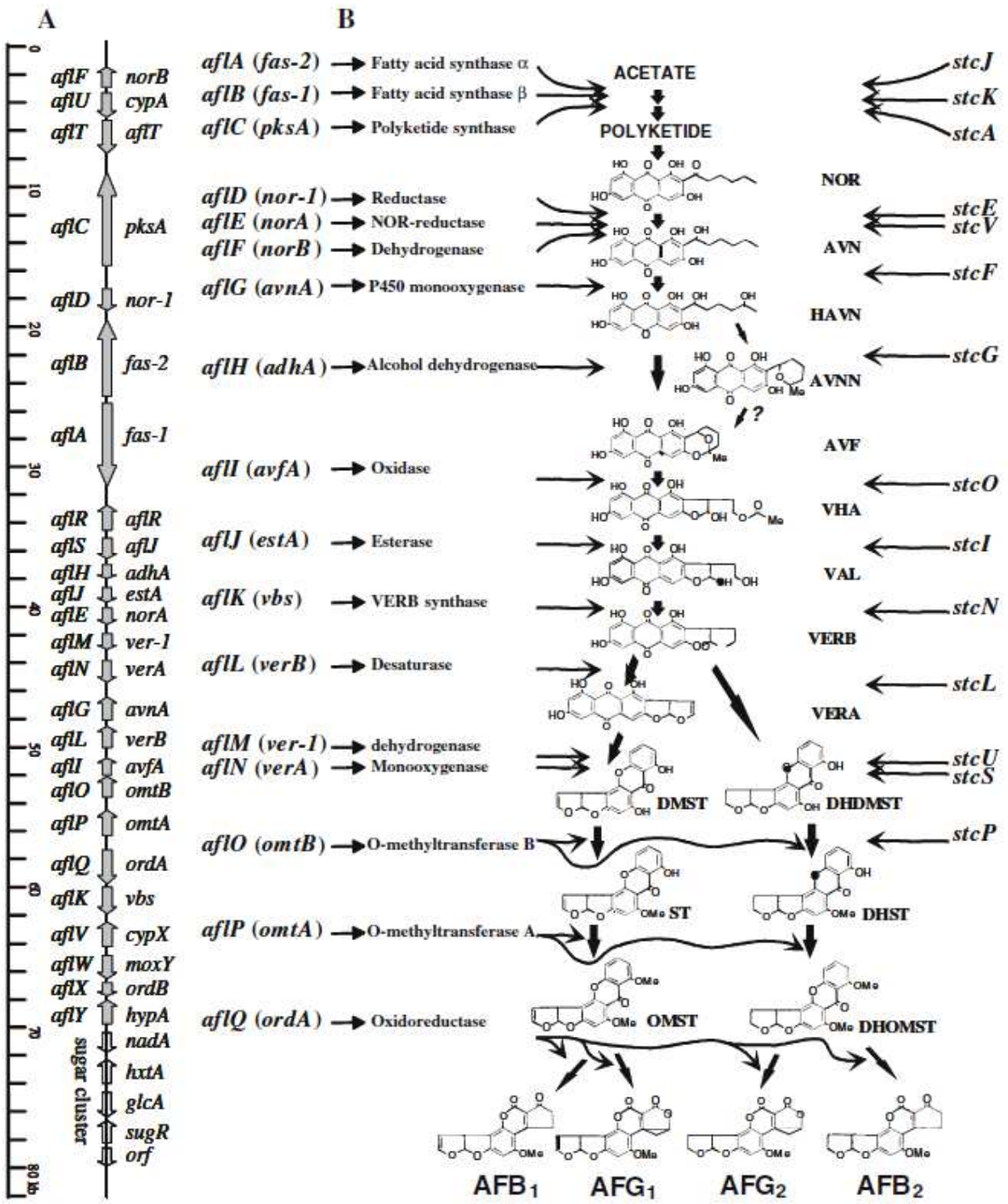

Fig. 3. The gene cluster responsible for aflatoxins biosynthesis in *A. flavus* and *A. parasiticus*. A) Clustered genes (arrows indicate the direction of gene transcription) and B) the AF biosynthetic pathway (Bhatnagar *et al*., 2006).

many promoter regulatory elements such as PacC and AreA binding sites. The *aflR* gene is also found in *A. nidulans* and *A. fumigatus*. Despite clear differences in the sequence of AflR between A. nidulans and A. flavus, function is conserved. AflR from *A. flavus* is able to drive expression of the ST cluster in an A. nidulans *aflR* deletion strain (Carbone *et al.,* 2007; Georgianna and Payne, 2009). AflR binds to the palindromic motif 5′-TCGN5CGA-3′ (also called AflR binding motif) in the promoter region of aflatoxin structural genes in *A. parasiticus*, *A. flavus*, and *A. nidulans*. The promoter regions of the majority of aflatoxin genes have at least one 5′-TCGN5CGA-3′ binding site within 200 bp of the translation start site, though some putative binding sites have been identified further upstream. AflR probably binds to its recognition site as a dimer. The gene, *aflR* may be self-regulated, as well as, under the influence of negative regulators. Upstream elements may be involved in negative regulation of *aflR* promoter activity. When *aflR* is disrupted, no structural gene transcript can be detected. Introduction of an additional copy leads to overproduction of aflatoxin biosynthetic pathway intermediates (Fernandes et al., 1998; Bennett et al., 2007).

Electrophoretic mobility shift assays (EMSA) have been used to thoroughly examine promoters for AflR binding in 11 different genes from the AF cluster, with three of these genes having sites that deviate from the predicted AflR binding motif, and an additional three AF genes for which AflR binding sites could not be demonstrated. Among these genes are *aflE, aflC, aflJ, aflM, aflK, aflQ, aflP, aflR*, and *aflG*. All of these genes have predicted sites and demonstrate some degree of AflR binding in EMSA assays. Moreover, they were differentially expressed between WT and the *DaflR* mutant, suggesting that AflR is required to activate their expression (Price et al., 2006; Georginna and Payne, 2009). Aflatoxins biosynthesis is also regulated by *aflS* (formerly *aflJ*), a gene that resides next to *aflR*. The genes *aflS* and *aflR* are divergently transcribed, but have independent promoters. The intergenic region between them, however, is short and it is possible that they share binding sites for transcription factors or other regulatory elements (Ehrlich and Cotty, 2002; Georgianna and Payne, 2009). The roles of AflR and AflS were examined by studying the expression of pathway genes in transformants of *A. flavus* strain 649-1 that received the respective genes individually. Strain 649-1 lacks the entire AF biosynthetic cluster but has the necessary upstream regulatory elements to drive the transcription of *aflR* (Du et al., 2007). These studies showed that AflR is sufficient to initiate gene transcription of early, mid, and late genes in the pathway, and that AflS enhances the transcription of early and mid aflatoxin pathway genes. Moreover, the induced expression of *A. flavus aflR* in *A. nidulans*, under conditions in which ST biosynthesis is normally suppressed, resulted in activation of genes in the ST biosynthetic pathway. These studies demonstrated that *aflR* function is conserved in widely different *Aspergillus* spp (Bhatnagar et al., 2003). Roles for AflS have been suggested to be as diverse as aiding in transport of pathway intermediates to the interaction of AflS with AflR for altered AF pathway transcription. The observation that AflS binds to AflR argues that AflS modulates aflatoxin expression through its interaction with AflR (Chang, 2003; Georgianna and Payne, 2009). Metabolite feeding studies showed that a functional *aflR* allele is required for accumulation of NOR, the first stable intermediate in the aflatoxin biosynthetic pathway. When this gene was disrupted, the fungi were incapable of aflatoxin metabolite production or transcription of nor-1, but otherwise grew normally (Bhatnagar et al., 2003). In addition to the binding sites for AflR, there are binding sites within the cluster for other transcriptional factors that may play important roles in transcriptional regulation of the AF cluster. A novel cAMP-response element, CRE1, site has been studied specifically in the *aflD* (nor-1) promoter of *A. parasiticus* (Georgianna and Payne, 2009).

3.2.2 Aflatoxins and fungal development

The association between fungal morphological development and secondary metabolism, including aflatoxin production, has been observed for many years (Calvo et al., 2002). The environmental conditions required for secondary metabolism and sporulation are similar, and both processes occur at about the same time (Reiss, 1982; Bennett et al., 2007). A number of studies have identified a genetic connection between aflatoxin/sterigmatocystin biosynthesis and fungal development. In *Aspergillus*, several observations linked a fluffy phenotype to loss of AF/ST production. The available well characterized fluffy mutants in *A. nidulans* were instrumental in the discovery of a signal transduction pathway regulating both conidiation and ST/AF biosynthesis. These mutants are deficient in ST formation (Weiser et al., 1994). Proteins identified as belonging to this signal transduction pathway include FlbA, an RGS (Regulator of G-protein Signaling) protein, FluG, an early acting development regulator, FadA, the alpha subunit of a heterotrimeric G-protein and PkaA, encoding the catalytic subunit of protein kinase A (Gerogianna and Payne, 2009). Furthermore, a possible transcription regulatory gene, *veA*, has been identified in *A. nidulans* and *A. parasiticus* and this gene controls both toxin production and sexual development. Both *A. nidulans* and *A. parasiticus veA* mutants fail to produce ST or aflatoxin. Moreover, *A. nidulans* and *A. parasiticus* do not produce cleistothecia (sexual fruiting bodies harboring ascospores) and sclerotia (asexual overwintering structures) respectively. Finally, a number of genetic loci were identified in *A. nidulans* mutants that resulted in loss of ST production but had normal developmental processes. Complementation studies with one of these mutants identified a gene called *laeA*. This gene encodes an enzyme with sequence similarity to methyltransferases and appears to be required for expression of ST. LaeA homologs have been found in a number of filamentous fungi and in all species examined, disruption of *laeA* resulted in loss of secondary metabolite production while overexpression of *laeA* results in hyperproduction of the secondary metabolite (Bhatnagar et al., 2006; Reverberi et al., 2010).

3.3 Economic impact of aflatoxins

Aspergillus spp. is a fungal that grows and produces aflatoxins in climes ubiquitous but is commonly found in warm and humid climates (Dohlman, 2003). Hence most commodities from tropical countries, especially peanut and maize, are likely to be easily contaminated with aflatoxins (Bley, 2009). Aflatoxin contamination of human and animal feeds poses serious health and economic risks worldwide (Bley, 2009). The economic impact of aflatoxin contamination is difficult to measure, but the following losses have been documented. In United States (US) from 1990 to 1996, litigation costs of $34 million from aflatoxin contamination occurred. In 1998, corn farmers lost $40 million as a result of aflatoxin contaminated grain (AMCE, 2010). The FAO estimates that 25% of the world food crops are affected by mycotoxins each year and constitute a loss at post-harvest (FAO, 1997). According to Cardwell et al (2004) aflatoxin contamination of agricultural crops causes annual losses of more than $750 million in Africa. Dohlman (2003) defined mycotoxin as toxic by-products of mould infestations affecting about one-quarter of global food and feed crop output. Newly in the US, it was reported that income losses due to AF contamination cost an average of more than US$100 million per year to US producers (Coulibaly et al., 2008). As of this date, the average direct loss to the US is estimated at $200 million annually for corn. Indirect losses because of contaminated byproducts, such as distillers' grain, compound these losses. Ultimately, all contribute to increased costs to consumers (AMCE, 2010). Jolly et al. (2009) also reveal that post-harvest losses of crops are greater than the

improvements made in primary production. In other hand, Otsuki et al (2001) has calculated that the European Union (EU) regulation on aflatoxins costs Africa $670 million each year in exports of cereals, dried fruit and nuts. But another study (World Bank, 2005) indicated that Otsuki et al. had overestimated the impact of the EU aflatoxin standard on Africa, and that the largest losses were incurred by Turkey, Brazil, and Iran. However, several studies have indicated that these costs may increase not only for Africa but for other countries that are suppliers of grains of the EU (Otsuki *et al*., 2001; Wu, 2004). This due to that the regulation on aflatoxins is among the strictest in the world, at 4 ng/g total aflatoxins for all foods except peanuts (15 ng/g). The EU regulation standards on aflatoxins are base in the ALARA principle (As Low As Reasonably Achievable) which has a strong potential impact on nations attempting to export foods that are susceptible to aflatoxins contamination into the EU (Wu, 2008). In the study of 2004, Wu estimated a $450 million annual loss to the U.S., China, Argentina, and sub-Saharan African peanut markets if the EU aflatoxin standard were adopted worldwide. Nevertheless, in other study realized in 2008, Wu also mentions that under certain conditions, export markets may actually benefit from the strict EU standard. These conditions include a consistently high-quality product, and a global scene that allows market shifts. Even lower-quality export markets can benefit from the strict EU standard, primarily by technology forcing. Nevertheless, if the above conditions are not met, export markets suffer from the strict EU standard. Recent studies have linked aflatoxins production in foods to environmental conditions, poor processing and lack of proper storage facilities in developing countries (Farombi, 2006; Hell et al., 2000; Kaaya and Kyamuhangire, 2006).

3.4 Control of aflatoxin contamination in crops

Mycotoxin contamination often is an additive process, beginning in the field and increasing during harvest, drying, and storage (Wilson and Abramson, 1992). Environmental conditions are extremely important in pre-harvest mycotoxin contamination of grain and oilseed crops. Aflatoxin generation is favored in years with above average temperature and below average rainfall (Wilson and Abramson, 1992). Fungal contamination both at pre-harvest and post-harvest is determined by a range of factors which can be classified into four main groups including: intrinsic nutritional factors, extrinsic factors, processing factors and implicit microbial factors (Sinha, 1995). The Fig. 4 summarises the factors which affect fungal colonization of stored grain (Megan and Aldred, 2008). Strategies to address the food safety and economic issues employ both pre-harvest and post harvest measures to reduce the risk of mycotoxin contamination in food and feed (Dorner, 2004). Pre-harvest control includes good cultural practices, biocontrol and development of resistant varieties of crops through new biotechnologies. The good cultural practices consist in planting adapted varieties, proper fertilization, weed control, and necessary irrigation as well as crop rotation, cropping pattern, and use of biopesticides as protective actions that reduce mycotoxin contamination of field crops. Among the strategies of biotechnology in the pre-harvest control is the development of transgenic plants resistant to fungal infection as well as crops capable of catabolism/interference with toxin production. Pre-harvest prevention especially through host resistance is probably the best and widely explored strategy for control of mycotoxins (Kumar and Kumari, 2010; Bhatnagar, 2010). Post-harvest control is based mainly eliminate or inactivate mycotoxins in grains and other commodities. Among the methods used in this control, are physical separation, detoxification, biological inactivation, chemical inactivation, and decreasing the bioavailability of mycotoxins to the host animal

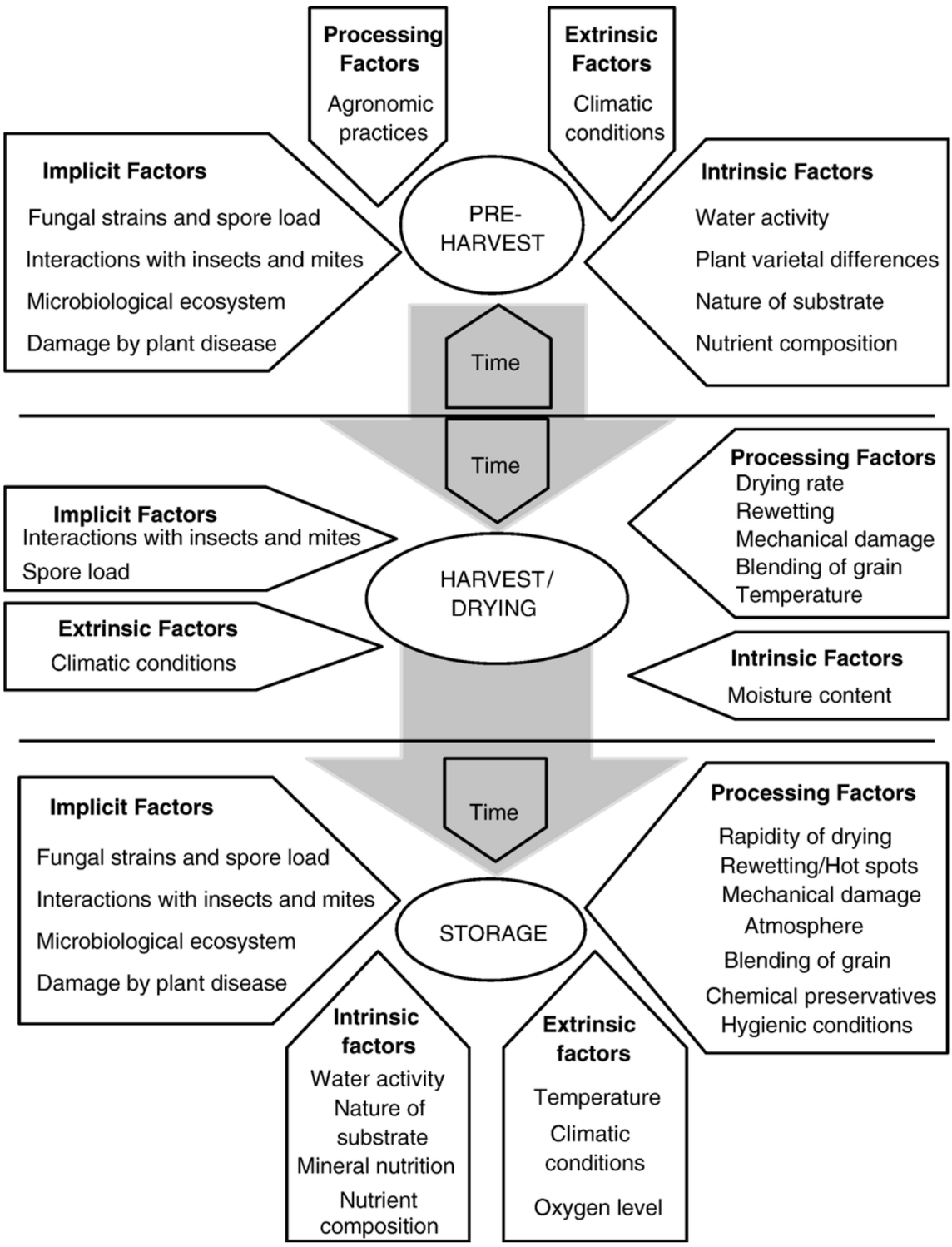

Fig. 4. Interaction between intrinsic and extrinsic factors in the food chain which influences mould spoilage and mycotoxin production in stored commodities (Magan *et al.*, 2004)

(Richard, J.L. et al., 2003). Because of the detrimental effects of mycotoxins, a number of strategies have been developed to help prevent the growth of mycotoxigenic fungi as well as to decontaminate and/or detoxify mycotoxin contaminated foods and animal feeds (Rustom, 1997). These strategies include: the prevention of mycotoxin contamination, detoxification of mycotoxins present in food and feed, as well as the inhibition of mycotoxin absorption in the gastrointestinal tract. Mycotoxin contamination may occur in the field before harvest, during harvesting, or during storage and processing. Thus methods can conveniently be divided into pre-harvest, harvesting and postharvest strategies (Heathcote & Hibbert, 1978). Whereas certain treatments have been found to reduce specific mycotoxin formation in different commodities, the complete elimination of mycotoxin contaminated commodities is currently not realistically achievable. Several codes of practice have been developed by Codex Alimentarius for the prevention and reduction of mycotoxins in cereals, peanuts, apple products, and raw materials. The elaboration and acceptance of a General Code of Practice by codex will provide uniform guidance for all countries to consider in attempting to control and manage contamination by various mycotoxins. In order for this practice to be effective, it will be necessary for the producers in each country to consider the general principles given in the Code, taking into account their local crops, climate, and agronomic practices, before attempting to implement provisions in the Code. The recommendations for the reduction of various mycotoxins in cereals are divided into two parts: recommended practices based on Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP); a complementary management system to consider in the future is the Hazard Analysis Critical Control Point (HACCP) (Codex Alimentarius Commission, 2002). Mycotoxins are secondary metabolites which are produced by several fungi mainly belonging to the genera: *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. While Aspergillus and Penicillium species are generally found as contaminants in food during dry and storage, Fusarium and Alternaria spp. can produce mycotoxins before or immediately after harvesting (Sweeney & Dobson, 1999). Up until now, approximately 400 secondary metabolites with toxigenic potential produced by more than 100 moulds, have been reported, with the Food and Agricultural Organization (FAO) estimating that as much as 25% of the world´s agricultural commodities are contaminated with mycotoxins leading to significant economic losses (Kabak *et al*., 2006).

Although *A. flavus* is readily isolated from diverse environmental samples, soil and plant tissues or residues are considered the natural habitat of this fungus (Jaime-Garcia & Cotty, 2004). Soil serves as a reservoir for primary inoculums for the infection of susceptible crops. Information concerning the soil ecology of *A. flavus* is consequently considered a prerequisite for developing effective measures to prevent and to control aflatoxin contamination of crops (Zablotowics *et al*., 2007). Soil and crop management practices and a number of environmental factors can influence the population size and spatial distribution of *A. flavus* in cultivated soils (Abbas *et al*., 2004*b*). The population size of A. flavus has been correlated with soil organic matter and nutritional status, with the most fertile soils containing the greatest concentration of aspergilli (Zablotowics *et al*., 2007). Subsequently, as more soils are managed under no tillage systems, a higher inoculums of this fungus may result, which could contribute to increased pre-harvest aflatoxin contamination of susceptible crops. It should be noted that the post-harvest control is a corrective method, and in this Chapter be addressed essentially biotechnological approaches that serve as preventive methods from emergency and development of *Aspergillus flavus* and consequently; inhibition synthesis of aflatoxins –a pre-harvest level–. Such approaches include of biologic control methods and use of elicitors.

3.4.1 Pre-harvest control strategies

It is well established that mycotoxin contamination of agricultural product can occur in the field as well as during storage (Wilkinson, 1999). Since phytopathogenic fungi such as *Fusarium* and *Alternaria* spp can produce mycotoxins before or immediately post harvesting several strategies have been developed including biological and cultural control practices to help mycotoxin contamination occurring in this way.

3.4.1.1 Prevention strategies in cereals

The main mycotoxin hazards associated with wheat pre-harvest in Europe are the toxins that are produced by fungi belonging to the genus *Fusarium* in the growing crop. Mycotoxins produced by these fungi include zearalenone (ZEN) as well as trichothecenes and include nivalenol (NIV), deoxynivalenol (DON) and T-2 toxin. *Fusarium* species are also responsible for a serious disease called *Fusarium* Head Blight (FHB), which can result in significant losses in both crop yield and quality. It is important to note that although *Fusarium* infection is generally considered to be a pre-harvest problem, it is possible for poor drying practices to lead to an increased susceptibility for storage mycotoxin contamination (Aldred & Magan, 2004).

3.4.1.2 Resistant varieties and transgenics

Research has demonstrated that insecticides cannot be applied economically to control corn insects well enough to reduce aflatoxin to acceptable levels. The most successful approach has been the use of corn resistant to ear-feeding insects. Several authors have shown that *Bacillus thuringensis* (Bt)-transformed corn hybrids, which are resistant to ear-feeding insects, reduce aflatoxin contamination of the grain. The adoption of *Bt* corn hybrids has given producers crop with increased insect resistance, however these hybrids may only reduce aflatoxin contamination under certain circumstances. However, commercial production of these genetically modified hybrids is not allowed in some nations. Several sources of natural resistance to insects have been identified, and crosses between insect- and aflatoxin-resistant lines have shown potential to increase resistance to both insect damage and aflatoxin contamination (Williams *et al*., 2002). Ideally, management of aflatoxin contamination should begin with the employment of resistant genotypes as has been demonstrated by several U.S. breeding programs. In Mexico the wide genetic diversity of maize has not been fully exploited to identify resistance to aflatoxin contamination in breeding programs, thus impeding the reduction of aflatoxin levels in the field. Additional complications come from the fact that transgenic maize expressing insecticidal protein or any other trait to reduce aflatoxin is not viable in Mexico due to a government prohibition on the use of genetically modified maize (Plasencia, 2004). Four major genetically controlled components for which variability exist appear to be involved in determining the fate of *A. flavus*-grain interaction: 1) resistance to the infection process, 2) resistance to toxin production, 3) plant resistance to insect damage, and 4) tolerance to environmental stress (Widstrom, 1987). The latter two components have an indirect influence since their effects only reduce aflatoxin contamination but do not prevent it. Although differences among genotypes have been found, heritability of the trait appears to be low, and the genotype/environment interaction may often mask true differences among genotypes (Plasencia, 2004). There are many new and exciting pre-harvest prevention strategies being explored that involve new biotechnologies. These new approaches involve the design and production of plants that reduce the incidence of fungal infection, restrict the growth of toxigenic fungi, or prevent toxic accumulation. Biocontrols using non-toxigenic biocompetitive agents is also a

potentially useful strategy in corn. However, the possibility of recombination with toxigenic strains is a concern (Abdel-Wahhab & Kholif, 2008). The differences between crop species appear to differ between countries. This is probably due to the differences in genetic pool within each country's breeding program and the different environmental and agronomic conditions in which crops are cultivated (Edwards, 2004). With respect to genetic resistance to Aspergillus infection and subsequent aflatoxin production, since the early 1970s, much work has been done to identify genetically resistant crop genotypes in both laboratory and field based experiments to help control of aflatoxigenic mould growth and aflatoxin and aflatoxin biosynthesis (D'Mello *et al*., 1998). This has led to the identification of a number of well-characterized sources of both resistance of *Aspergillus flavus* infection and to aflatoxin production. These include kernel proteins such as a 14-kDa trypsin-inhibiting protein and others including globulin 1 and 2 and a 22-kDa zeamatin protein (Chen *et al*., 2001). Although the role of insects in fostering *Aspergillus* colonization of maize kernels is well documented, there is little evidence that transgenic corn expressing insecticidal proteins has a significant effect on reducing aflatoxin contamination. In contrast, several studies have reported a protective effect of Cry-type proteins in maize to *Fusarium* kernel rot and fumonisin accumulation (Dowd, 2003). Cry-type proteins constitute a family of insecticidal proteins from *Bacillus thuringensis*, whose genes have been incorporated into several crops to confer protection against insect pests. In corn, several hybrids expressing distinct Cry-type proteins have been developed and widely used in the U.S., Canada, Argentina, and other maize-producing countries (Plasencia, 2004). The distribution of aflatoxin in agricultural commodities has been fairly well characterized because of its importance to food supply. However, little is known on the occurrence and fate of aflatoxin in soil. Radiological assays conducted to assess the fate of aflatoxin B1 (AFB1) in soil indicated that a low level of mineralization of AFB1 to CO_2 was observed, with less than 1-8% mineralized in 120 days (Angle, 1986). Not surprisingly, several microorganisms have the potential to degrade aflatoxins, especially bacteria, e.g., *Flavobacterium* and *Mycobacterium* (Hormisch *et al*., 2004). In addition, *A. flavus* also is capable of degrading aflatoxins during later stages of mycelial growth in pure culture (Huyhn & Lloyd, 1984). In recent years, molecular techniques have increased the possibilities to characterize soil microbial ecology. While molecular methods have been extensively used for studying soil bacteria, these techniques have been applied to studying soil fungi, such as the biological control agents Colletotrichum coccodes (Dauch *et al*., 2003), Trichoderma (Weaver *et al*., 2005), and mycorrizal fungi (Ma *et al*., 2005). Amplification of specific DNA fragments using polymerase chain reaction (PCR) and specific gene probes is extremely sensitive and has the potential to detect the presence of *A. flavus* in agricultural commodities (Manonmani *et al*., 2005). Since all of the genes involved in the aflatoxin biosynthesis pathway have been identified and cloned (Yu *et al*., 2004*a*, 2004*b*), and the entire genome of *A. flavus* sequenced (Payne *et al*., 2006), molecular methods for the detection of Aspergillus should be fairly readily adapted by using biosynthetic pathway genes as probes, as evidenced by the recent work differentiating toxigenic and atoxigenic *A. flavus*-utilizing aflatoxin gene expression using the reverse transcription-polymerase chain reaction (RT-PCR) (Degola *et al*., 2007). Application of these molecular techniques to *A. flavus* soil ecology should greatly enhance our understanding of this fungus. *Aspergillus flavus* is commonly considered a saprophytic fungus; however, its ability to colonize growing crops and inflict economic damage clearly shows that it can and does function as an opportunistic pathogen. Despite the elucidation of many aspects influencing *A. flavus* ability to colonize crops and accumulate aflatoxins, its activity and potential to

produce aflatoxins in soil and in crop residues has remained unexplored (Accinelli *et al*., 2008). One interesting approach is the engineering of cereal plants to catabolize fumonisins *in situ*. Typically, these approaches require considerable research and development but have the potential of ultimately producing low cost and effective solutions to the mycotoxin problem in corn and other cereals. Thus this level of prevention is the most important and effective plan for reducing fungal growth and mycotoxin production.

3.4.1.3 Field management

Appropriate field management practices including crop rotation, soil cultivation, irrigation, and fertilization approaches are known to influence mycotoxin formation in the field. Crop rotation is important and focuses on breaking the chain production of infectious material, for example by using wheat/legume rotations. The use of maize in a rotation is to be avoided however, as maize is also susceptible to *Fusarium* infection and can lead to carry-over onto wheat via stubble/crop residues (Nicholson *et al*., 2003). Dill-Macky and Jones (2000) observed that FHB disease severity and DON contamination of grain was significantly different when the previous crop was maize, wheat, or soya bean; with the highest levels following maize and the lowest levels following soya bean. Soil cultivation can be divided into ploughing, where the top 10-30 cm of soil is inverted; minimum tillage, where the crop debris is mixed with the top 10-20 cm of soil; and no till, where seeds are directly drilled into the previous crop stubble with minimum disturbance to the soil structure. Ay crop husbandry that results in the removal, destruction, or burial of infected crop residues is likely to reduce the *Fusarium* inoculum for the following crop. Dill-Mackey and Jones (2000) reported that no till (direct drilling) after wheat or maize significantly increase DON contamination of the following wheat crop compared to ploughing, but no till had no effect when the previous crop was soya bean. Irrigation is also a valuable method of reducing plant stress in some growing situations. It is first necessary that all plants in the field have an adequate supply of water if irrigation is used. It is known that excess precipitation during anthesis makes conditions favorable for dissemination and infection by Fusarium spp., so irrigation during anthesis and during ripening of the crops, specifically wheat, barley, and rye, should be avoided. The soil must be tested to determine if there is need to apply fertilizer and soil conditioners to assure adequate soil pH and plant nutrition to avoid plant stress, especially during seed development. Fertilizer regimes may affect FHB incidence and severity either by altering the rate of residue decomposition, by creating a physiological stress on the host plant or by altering the crop canopy structure. Martin et al., (1991) observed the increasing N from 70 to 170 kg/ha significantly increased the incidence of *Fusarium* infection grain in wheat, barley, and triticale. Recent work by Lemmens *et al*., (2004) has shown that a significant increase in FHB intensity and DON contamination in the grain was observed with increasing a mineral N fertilizer from 0 to 80 kg/ha. This group concluded that in practical crop husbandry, FHB cannot be sufficiently controlled by only manipulating the N input.

3.4.1.4 Environmental conditions

Environmental conditions such as relative humidity and temperature are known to have an important effect on the onset of FHB. For example, it has been shown that moisture conditions at anthesis are critical in Fusarium infection of the ears (Aldred & Magan, 2004); while Lacey et al. (1999) have shown that Fusarium infection in the UK is exacerbated by wet periods at a critical time in early flowering in the summer, which is the optimum window for susceptibility. Equally, there is evidence that droughed-damaged plants are

more susceptible to infection, so crop planting should be timed to avoid both high temperature and drought stress during the period of seed development and maturation. On the other hand, the planning of harvesting grain at low moisture content and fully maturity may be an important control point in the preventing of mycotoxin contamination, unless allowing the crop to continue to full maturity would subject it to extreme heat, rainfall or drought conditions. Delayed harvest of the grain already infected by Fusarium species is known to cause a significant increase in the mycotoxin content of the crop.

3.4.2 Biotechnological approaches

3.4.2.1 Biological control of aflatoxins

The first approach which we will discuss is the biological control, which is focuses in the use of living organisms to control pests (insects, weeds, diseases and disease vectors) in agriculture. The objective of the biologic control is to stimulate the colonization of antagonist organism on plant surfaces to reduce the inoculum of the pathogens (FAO, 2004). Different organisms, including bacteria, yeasts and nontoxigenic *Aspergillus* fungi, have been tested for their ability in the control of aflatoxin contamination (Yin et al., 2008). According to reported by Palumbo et al., (2006) several bacterial species as *Bacillus* spp., *Lactobacilli* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp., have shown the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus* spp. in laboratory experiments (Yin et al., 2008), the same effect was observed in strains of *B. subtilis* and *P. solanacearum* isolated from the non-rhizophere of maize soil were also able to inhibit aflatoxin accumulation (Nesci *et al*., 2005). In other experiments, is showed that *Bacillus subtilis* prevented aflatoxin contamination in corn in field tests when ears were inoculated with the bacterium 48 hours before inoculation with *A. flavus* (Cuero et al., 1991). However, no reduction in aflatoxin occurred when bacteria were inoculated 48 hours after inoculation with A. flavus. Bacillus subtilis (NK-330) did not inhibit aflatoxin contamination in peanuts when it was applied to pods prior to warehouse storage for 56 days (Smith et al., 1990). Saprophytic yeasts isolated from fruits of almond, pistachio, and walnut trees inhibited aflatoxin production by *A. flavus* in vitro (Hua et al., 1999; Masoud and Kaltoft, 2006). A strain of *Candida krusei* and a strain of *Pichia anomala* reduced aflatoxins production by 96% and 99%, respectively, in a Petri dish assay. Efforts are underway to apply these yeasts to almond and pistachio orchards to determine their potential for aflatoxin reduction under crop production conditions (Hua, 2002). Although they were considered to be potential biocontrol agents for management of aflatoxins, further field experiments are necessary to test their efficacies in reducing aflatoxins contamination under field conditions (Yin et al., 2008). Alternatively, a limited number of biocompetitive microorganisms have been shown for the management of *Fusarium* infections. Antagonistic bacteria and yeasts may also lead to reductions in pre-harvest mycotoxin contamination. For instance, *Bacillus subtilis* has been shown to reduce mycotoxin contamination by *F. verticilloides* during the endophytic growth phase. Similarly antagonistic yeasts such as *Cryptococcus nodaensis* have also been shown to inhibit various *Fusarium* species (Cleveland *et al*., 2003). Recent glasshouse studies by Diamond and Coke (2003) involving the pre-inoculation of wheat ears at anthesis, with the two non-host pathogens, *Phoma betae* and *Pytium ultimum* showed a reduction in disease development and severity caused by *F. culmorum*, *F. avenaceum*, *F. poae*, and *M. nivale*. *A. flavus* is not considered to be an aggressive invader of pre-harvest corn ear tissue. However, developing grain when damaged is easily contaminated by the pathogen (Diener *et al*.,

1987). The association between insect damage and fungal infection of corn ears was first recognized by Riley (1882) reported molds appearing on corn-ear tips soon after being infested with insect larvae. Garman and Jewett (1914) reported that in years with high insect populations, the incidence of moldy ears in field corn increased. Efforts to determine the specific role of insects in the *A. flavus* infection process increased dramatically when aflatoxin was recognized as a health concern, leading to recognition that ear feeding insects (e.g., corn earworm, *Helicoverpa zea*; European corn borer, *Ostrinia nubilalis*; fall armyworm, *Spodoptera frugiperda*; western bean cutworm, *Striacosta albicosta*; and southwestern corn borer, *Diatraea grandiosella*) can increase aflatoxin levels in pre-harvest corn (Catangui & Berg, 2006). The difficulty in establishing the relationship between insect damage and aflatoxin incidence is in part due to A. flavus ability to colonize silks, infect kernels, and produce aflatoxins in developing ears under insect-free conditions (Jones et al., 1980), and in part due to unknown factors that result in conflicting information (Abbas *et al.*, 2009). Because the relationship between insect damage to corn ears and aflatoxin is heavily influenced by environmental conditions, success in managing aflatoxin contamination via insect control has been highly variable. The greatest success to date regarding biological control of aflatoxins contamination in the field has been achieved through competitive exclusion by applying on aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* to soil of developing crops. These strains are typically referred to as atoxigenic or nontoxigenic, but those designations are often used with reference to production of aflatoxins only (Dorner, 2004). According to Yin *et al*., (2008) the use of non-toxigenic *Aspergillus s*trains is a strategy based on the application of nontoxigenic strains to competitively exclude naturally toxigenic strains in the same niche and compete for crop substrates. Thus, for competitive exclusion to be effective, the biocontrol nontoxigenic strains must be predominant in the agricultural environments when the crops are susceptible to be infected by the toxigenic strains (Cole and Cotty, 1990; Cotty, 1994; Dorner, 2004). For this to work, the applied strains must occupy the same niche as the naturally occurring toxigenic strains and compete for crop substrates (Dorner, 2004). Two primary factors exist that determine the effectiveness of this strategy. First, the applied strain(s) must be truly competitive and dominant relative to the toxigenic strains that are already present. Second, the formulation used to apply the competing strain(s) must be effective in delivering the necessary quantity of conidia to achieve a competitive advantage. In addition, the timing of that application is crucial for ensuring that the necessary competitive level is present when the threat of crop infection is greatest (Cotty, 1989; Dorner, 2004). Should be noted, that not only species of *Aspergillus* used for biological control are capable of producing aflatoxins, but also a variety of other toxins and toxic precursors to aflatoxins including cyclopiazonic acid, sterigmatocystin and related compounds, and the versicolorins (Cole and Cox, 1981). In the research realized by Cotty (1990) in greenhouse, demonstrated the ability of seven non-aflatoxigenic strains of *A. flavus* to reduce aflatoxins contamination of cottonseed when were co-inoculated with toxigenic strains. Six of these strains show significantly reduced the amount of aflatoxins produced in cottonseed by the toxigenic strain. Strain 36 (AF36) produced the largest reduction in aflatoxin under these conditions and it was Biological Control of Aflatoxin Contamination of Crops 429 subsequently shown to reduce aflatoxin contamination of cottonseed in the field when applied on colonized wheat seed (Cotty, 1994). This strain has been registered on cotton for control of aflatoxin contamination of cottonseed in Arizona, USA. It is also on a schedule for registration on pistachio in California. Additionally, this biocontrol agent was also tested for

control of aflatoxin in corn (Cotty, 1996). When corn ears were either co-inoculated with AF36 and a toxigenic strain of *A. flavus* or inoculated with AF36 at 24 h prior to inoculation with the toxigenic strain, subsequent aflatoxins concentrations were significantly reduced, compared to inoculation with the toxigenic strain alone (Brown *et al*., 1991). Also have been demonstrated that other strains of *A. flavus* and *A. parasiticus* are capable of reduce aflatoxin contamination in crops; as is case of *A. flavus* NRRL 21882, a naturally occurring strain isolated from a peanut in Georgia in 1991, that has been used in diverse studies where has been verified its efficacy for reducing contamination in the field. This strain is the active ingredient in an EPA-registered biopesticide called afla-guard1. A color mutant of this strain, NRRL 21368, was used in several early studies and also found to be effective when used in conjunction with a color mutant of *A. parasiticus* (NRRL 21369) (Dorner et al., 1998, 1999b). Atoxigenic strain technology based provides an opportunity to reduce the overall risk of contamina-tion during all phases of aflatoxin contamination including in the field during crop development, in storage or at any other time after harvest until the mature crop is eventually utilized. Atoxigenic strains are but one example of how improved knowledge of both the contamination process and the etiologic agents can result in improved methods for limiting human exposure to aflatoxins.

3.4.2.2 Chemical agents and use of elicitors to aflatoxin inhibition

Another factor which is known to increase the susceptibility of agricultural commodities to toxigenic mould is injury due to insect, bird, or rodent damage (Smith *et al*., 1994). Insect damage and fungal infection must be controlled in the vicinity of the crop by proper use of registered insecticides, fungicides, and other appropriate practices within an integrated pest management control. Part of the integrated control of FHB in wheat production involves the use of fungicides, but this introduces a complication as far as trichothecenes are concerned as there is evidence that under certain conditions, fungicide use may actually stimulate toxin production. This raises particular concerns, since circumstances may arise where the obvious manifestations of FHB are reduced or even eliminated and yet high levels of mycotoxins may be present. Clearly grain affected in this way cannot be identified by visual inspection for signs of FHB (e.g., pink grains) and, in fact, cannot be identified until a specific mycotoxin analysis is carried out (Simpson *et al*., 2001). Early investigation in vitro indicates that the fungicide chlobenthiazone is highly effective in inhibiting aflatoxin biosynthesis by cultures of *A. flavus*; however, aflatoxin synthesis by *A. parasiticus* was, in fact, stimulated by the fungicide (Wheeler, 1991). Various surfactants, including some used in pesticide formulations, reduced aflatoxin biosynthesis by >96% (Rodriguez & Mahoney, 1994). Use of natural oils from thyme (Kumar *et al*., 2008), and other herbs has also been studied and shown to repress aflatoxin in certain crops in Asia. The herbicide glufosinate has been reported as having antifungal activity against certain phytopathogenic fungi in vitro (Uchimiya *et al*., 1993) and has shown activity in reducing infection of corn kernels in vitro (Tubajika & Damann, 2002). Higher levels of aflatoxin were observed in glyphosate-resistant corn compared with traditional corn hybrids. Thus, effects of glyphosate on in vitro growth of *A. flavus* in pure culture and on native soil populations were examined, finding that high levels of glyphosate (> 5mM) were required for inhibition. In addition, application of greater amounts was found to have no effect on *A. flavus* populations. Interestingly, *A. flavus* when grown on glyphosate water agar media, produced 20% of aflatoxin produced on water agar without glyphosate (Abbas *et al*., 2009). Research carried out on fungicide use in terms of FHB and mycotoxin development has produced very interesting results. In

particular, fungicides in common use have been shown to have differential effects against toxin-forming Fusarium species and related non-toxing-forming pathogens such as *Microdochium nivale* on ears (Simpson *et al*., 2001). The outcome of the use of fungicides seems to depend on the fungal species present, and the effect that the particular fungicide has on these species. For example, in recent work commissioned by the Home Grown Cereal Authority, in an experimental situation where *Fusarium culmorum* and *M. nivale* where both present, the use of azoxystrobin showed a significant reduction in disease levels while increasing the levels of DON present in grain. This was believed to be the result of selective inhibition of *M. nivale* by azoxystrobin. *M. nivale* is a natural competitor of toxin-forming *Fusarium* species, particularly *F. culmorum*. Removal of *M. nivale* by the fungicide probably allowed development of the toxigenic species in its place with concomitant increase in toxin formation. This is an important finding as it indicates that the impact of the fungicide is not directly related to mycotoxin production. It follows from these findings that where FHB is caused by *Fusarium* species in the absence of *Microdochium*, disease development is associated with higher levels of toxin (Magan *et al.,* 2002). Ioos *et al*. (2005) also carried out a screen on the efficacy of fungicides, azeoxystrobin, metconazole, and tebuconazole at anthesis against *Fusarium* spp., *M. nivale* and on years on naturally infected fields of soft wheat, durum wheat, and barley. The infection levels of *F. graminearum*, *F. culmorum*, and *M, nivale* were significantly reduced by the application *Fusarium* mycotoxin concentration over three of fungicides, with tebuconazole and metconazole effectively controlling the Fusarium spp., but they had little effect on M. nivale. Although this conclusion concurs with Simpson *et al*. (2001) for tebuconazole, their benefits were apparently seasonal-with tebuconazole controlling these fungi in 2001, while having little effect in 200 and 2002. The second approach involves the application of elicitors in crops susceptible to *A. flavus*, with the aim of protecting the plant of subsequent aflatoxins contamination. This because that the elicitors are capable molecules from activating multiple reactions defense that are induced and agrouped both histological level of physical barrier as a biochemist with the de novo synthesis of proteins associated with pathogenicity (PR), in the absence of the pathogen. Besides serves as aguide of intracellular events that end in activation of signal transduction cascades and hormonal pathways, triggering the induced resistance (IR) and consequently activation of plant immunity to ivironmental stresses (Riveros, 2001; Odjacova and Hadjiivanova, 2001; Garcia-Brugger et al., 2006; Bent and Mackey, 2007; Holopainen et al., 2009; Mejía-Teniente et al., 2011). Between the elicitors that have been investigated for more control of aflatoxin contamination in crops of commercial interest is the jasmonic acid (JA) and related compounds, as well as ethylene (ET). One factor influencing the production of aflatoxin is the presence of high levels of oxidized fatty acids such as fatty acid hydroperoxides, which can form in plant material either preharvest under stress or postharvest under improper storage conditions, correlates with high levels of aflatoxin production (Goodrich-Tanrikulu et al., 1995). Fatty acid hydroperoxides can be formed by autooxidation, or enzymically by lipoxygenases acting on a-linoleic and a-linolenic acids (Vick, 1993). These hydroperoxides stimulate the formation of aflatoxins by *A. flavus* and *A. parasiticus* (Fabbri et al., 1983; Fanelli and Fabbri, 1989). Degradation of the hydroperoxides by later steps in the plant lipoxygenase pathway leads to multiple byproducts, depending on the polyunsaturated fatty acid substrate, the positional specificity of the lipoxygenase, and the activities of enzymes catalysing the subsequent steps. The jasmonic acid (JA) is α-linolenic acid metabolite, via lipoxygenase and hydroperoxide dehydratase, is jasmonic acid (JA) (Vick, 1993). JA and closely related compounds, such as its methyl ester, MeJA, are

endogenous plant growth regulators both higher and lower plants (Staswick, 1992; Sembdner and Parthier, 1993). JA and MeJ are two well-characterized plant growth regulators that exert a vast variety of biological activities in plants as the activation of defense responses (for review see Sembdener and Parthier, 1993). Among the diverse plant defense mechanisms, recent findings have demonstrated that low-concentrations of JA or MeJ induce protein inhibitors (Farmer and Ryan, 1992), thionin (Andresen et al., 1992; Epple et al., 1995) and several plant defense enzymes such as PAL (Gundlach et al., 1992), LOX (Bell and Mullet, 1991) and chalcone synthase (Creelman et al., 1992). MeJ is volatile suggesting its action could be exerted in gaseous form, similar to the plant hormone, ethylene. Goodrich-Tanrikulu et al., (1995) reporting the effect of MeJA on aflatoxins production and growth of *Aspergillus flavus* in vitro. They Found that at concentrations MeJA of 10-3-10-8 M in the growth medium was inhibited aflatoxin production, by as much as 96%. Besides that when cultures were exposed to MeJA vapour similarly was inhibited aflatoxin production, observing that the amount of aflatoxin produced depended on the timing of the exposure. MeJA treatment also delayed spore germination and was inhibited the production of a mycelial pigment. These fungal responses resemble plant jasmonate responses. In other hand, Zeringue (2002) carried out a series of experiments where artificially wounded 22–27-day old developing cotton bolls were initially inoculated with, (1) a cell-free, hot water-soluble mycelial extract (CFME) of an atoxigenic strain of Aspergillus flavus or with, (2) chitosan lactate (CHL) or with, (3) CFME or CHL and then exposed to gaseous methyl jasmonate (MJ) or, (4) exposed to MJ alone. The results indicated a two- or three-fold increase in the production of the phytoalexins when gaseous MJ was added in combination to the CFME or the CHL elicitors. While the effects of aflatoxin B1 production after the developing cotton bolls pretreated with CFME, CHL or with CFME–MJ, CHL–MJ or only with MJ, showed a lower aflatoxin (Table 2, taken of Zeringue, 2002). All pretreatments resulted in some degree of aflatoxin B1 inhibition in the seeds underlying the treatment. CFME pretreatment resulted in a 88% inhibition of aflatoxin B1 and CHL resulted in a 64% inhibition (Table 2). CFME–MJ boll treatment resulted in the maximum aflatoxin B1 inhibition (95%) compared to CHL–MJ (75%). These series of experiments demonstrate a correlation between increased phytoalexin induction with a decreased aflatoxin B1 formation under the influence of volatile MJ in combination with selected elicitors. Phytoalexins are synthesized and accumulated at the site of microbial infection or as shown in this study localized at the site of the placement of elicitors (carpel discs). Besides, these results demonstrate an added inducement of phytoalexins and aflatoxin B1 inhibition produced by MJ treatment in combination with elicitors. This inducement is perhaps produced by an added signal/signals that activates other secondary pathways that either enhance the concentrations of the demonstrated phytoalexins or inhibit aflatoxin B1 biosynthesis or both. These results further demonstrate the innate, natural defense responses of the cotton plant and its ability to defend itself upon microbial attack, with the possibility to extrapolate to other seeds (Zeringue, 2002).

3.4.3 Harvest management

For cereals, harvest is the first stage in the production chain where moisture management becomes the dominant control measure in the prevention of mycotoxin development. Since the moisture content may vary considerably within the same field, the control of moisture in several spots of each load of the harvested grain during the harvesting operation is very important. Another equally important control measure is an effective assessment of the crop

for the presence of disease such as FHB. This should be accompanied by an efficient strategy for separation of the diseased material from healthy grain. There is evidence that fungal infection can be minimized by avoiding the mechanical damage to the grain and by avoiding contact with soil at this stage.

3.4.4 Post-harvest management

Post-harvest strategies are important in the prevention of mycotoxin contamination and include improved drying and storage conditions, together with the use of natural and chemical agents, as well as irradiation.

3.4.4.1 Improving of drying and storage conditions

In cereals, mycotoxigenic fungal growth can arise in storage as a result of moisture variability within the grain itself or as a result of moisture migration results from the cooling of grains located near the interface with the wall of the storage container/silo (Topal *et al*., 1999). Thus control of adequate aeration and periodical monitoring of the moisture content of silos plays an important role in the restriction of mycotoxin contamination during the storage period (Heathcote & Hibbert, 1978). The moisture level in stored crops is one of the most critical factors in the growth of mycotoxigenic moulds and in mycotoxin production (Abramson, 1998), and is one of the main reasons for mycotoxin problems in grain produced in developing countries. Cereal grains are particularly susceptible to grow by Aspergilli in storage environments. The main toxigenic species are *A. flavus* and *A. parasiticus* for aflatoxins, and *Penicillium verrucosum* is the main producers in cereals for OTA (Lund & Frisvad, 2003), while *A. ochraceus* is tipically associated with coffee, grapes, and species, aflatoxins can be produced at a_w values ranging from 0.95 to 0.99 with a minimum a_w value of 0.82 for *A. flavus*, while the minimum a_w for OTA production is 0.80 (Sweeney & Dobson, 1998). It has been reported that *A. flavus* will not invade grain and oilseeds when their moisture contents are in equilibrium with a relative humidity of 70% or less. The moisture content of wheat at this relative humidity is about 15%, and around 14% for maize, but it is lower for seeds containing more oil, approximately 7% and 10% for peanuts and cottonseeds, respectively (Heathcote & Hibbert, 1978), while *A. parasiticus* has been reported to produce aflatoxins at 14% moisture content in wheat grains after 3 months of storage (Atalla *et al*., 2003). The second critical factor influencing post-harvest mould growth and mycotoxin production is temperature. Both the main aflatoxin producing Aspergillus strains *A. flavus* and *A. parasiticus* can grow in the temperature range from 10-12°C to 42-43°C, with an optimum in the 32 to 33°C range, with several studies highlighting the relatively high incidence of mycotoxins such as aflatoxins and ochratoxins in foods and feeds in tropical and subtropicals regions (Soufleros *et al*., 2003). The control of temperature of the stored grain at several fixed time intervals during storage may be important in determining mould growth. A temperature rise of 2-3°C may indicate mould growth or insect infestation. Until recently, little if any work has been carried out on monitoring how spoilage fungi interact with each other in the stored grain ecosystem, and the effect that this has on mycotoxin production. Magan *et al.* (2003), have shown that the system is in a state of dynamic flux with niche overlap altering in direct response to temperature and a_w levels. It appears that the fungi present tended to occupy separate niches, based on resources utilization, and this tendency increased with drier conditions. Initially, *A. flavus* and other *Aspergillus* spp. were considered exclusively storage fungi, and aflatoxin contamination was believed to be primarily a storage problem. This is very severe in many rural areas that lack of

infrastructure for drying and other appropriate storage conditions. Usually, corncobs are harvested at moisture contents that vary between 25-30% and are dried under the sunlight to reach 12-14% moisture content. Research has been conducted to determine the optimum temperature and moisture content of grains during storage to prevent Aspergillus spp. growth and aflatoxin production. In maize inoculated with *A. flavus* and stored at 27°C for 30 days with varying moisture contents, an association between moisture content and aflatoxin levels was established. At 16% moisture, aflatoxin levels reach 116 µg/kg while a 22% moisture 2166 µg/kg aflatoxin levels were obtained (Moreno-Martínez *et al.,* 2000). In this same study, the authors tested the protective effects of propionic acid salts (6.5-12.5 L/t) on fungal growth and aflatoxin production. All grains treated with ammonium, calcium, or sodium propionates yielded very low *Aspergillus flavus* growth and aflatoxin levels (2 - 5.6 µg/kg) at all moisture contents. It is well established that rapid crop drying may be useful in controlling aflatoxin contamination in storage and that in addition that crops containing different moisture values are not stored together. It is also well established that mould invasion is facilitated as a result of increased moisture levels of stored commodities. Moisture abuse can even occur in crops with very low moisture content. Another factor to bear in mind is the fact that if fungal growth does occur in storage, moisture will be released during metabolism, which will be released during metabolism, leading to the growth of other fungal species and to the production of mycotoxins such as OTA.

4. Detection of mycotoxins in food

Aflatoxigenic fungi can contaminate food commodities, including cereals, peanuts, spices and figs. Foods and feeds are especially susceptible to colonization by aflatoxigenic *Aspergillus* species in warm climates where they may produce aflatoxins at several stages in the food chain, i.e. either at pre-harvest, processing, transportation or storage (Ellis *et al*., 1991). The level of mold infestation and identification of the governing species are important indicators of raw material quality and predictors of the potential risk of mycotoxin occurrence (Shapira *et al*., 1996). Traditional methods for the identification and detection of these fungi in foods include culture in different media and morphological studies. This approach, however, is tim-consuming, laborious and requires special facilities and mycological expertise (Edwards et al., 2002). Moreover, these methods have a low degree of sensitivity and do not allow the specification of mycotoxigenic species (Zhao *et al.,* 2001). PCR-based methods that target DNA are considered a good alternative for rapid diagnosis due to their high specificity and sensitivity, and have been used for the detection of aflatoxigenic strains of *A. flavus* and *A. parasiticus* (Somashekar *et al*., 2004). However, as yet, none of these methods can reliably differentiate *A. flavus* from other species of the *A. flavus* group. In particular, *A. flavus* and *A. parasiticus* have different toxigenic profiles, *A. flavus* produces aflatoxin B1 (M1), B2, cyclopiazonic acid, aflatrem, 3-nitropropionic acid, sterigmatocystin, verdsicolorin A and aspetoxin, whereas *A. parasiticus* produced aflatoxin B1 (M1), B2, G1, G2 and versicolorin A. Another important fact is that *A. flavus* and *A. fumigatus* are responsible for 90% of the aspergillosis in human beings (González-Salgado *et al.,* 2008). It is evident that one fundamental solution to the problem of mycotoxins in food would be to ensure that no contamination of edible crops occurred during harvesting and storage. It is equally clear, however, that such a solution is virtually unattainable, and hence that the presence of mycotoxins in food will have to be accommodated. Three approaches to the problem are most widely encountered; one involves physico-chemical methods of

analysis, other relies on biological assays, and another one is microscopic examination. The former approach has found most widespread acceptance for routine purposes, but some authorities feel that a chemical diagnosis should be supported with some form of demonstration that the detected material is, in fact biologically toxic. The validity of this requirement is open to debate, but, for specific legal purposes, it may well become obligatory (Robinson, 1975).

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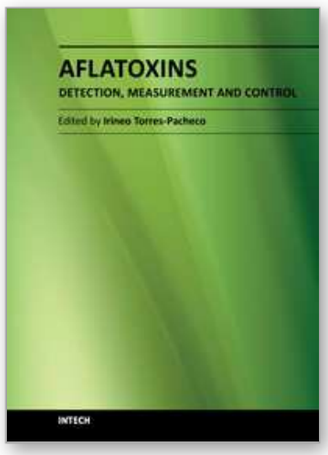

Aflatoxins - Detection, Measurement and Control

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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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