CHAPTER II

THEORY AND LITERATURE REVIEW

Aflatoxins are a group of toxins having similar molecular structures. The toxin was first discovered in 1960s when there were mass deaths from liver disease of turkey in England followed by deaths of farm animals. More than 100,000 turkeys died within a few months (Carlile et al., 2001). Scientists first called the new disease "Turkey X disease" because they did not know its cause. It was finally established that all birds affected had been fed with feed prepared with contaminated groundnut meal. Examination of the incriminated groundnut meal revealed the presence of mold (Moore-Landecker, 1996). The main microbial contaminant of the groundnut meal was identified to be *Aspergillus flavus* and the toxin was named aflatoxin. Since then, aflatoxin is considered as one of the most harmful mycotoxins in the world

2.1 Economic significance of aflatoxin contamination

Aflatoxin contamination of foods and feeds is a serious worldwide problem (Cleveland et al., 1997, and Bhatnagar et al., 2002) resulting either from improper storage of commodities or preharvest contamination in corn, peanuts, cottonseed and tree nuts, especially during drought years. The worldwide extent of contamination of commodities is not totally understood often because of reluctance to report its occurrence (Yu et al., 2002). Aflatoxin contamination in food for human consumption as well as in feed for livestock has been found in many geographically diverse regions of the world. Such contamination has resulted in serious food safety and economic implications for the agriculture industry. Because of the health concern (Cullen and

Newberne, 1994), regulatory guidelines of 20 parts aflatoxin per billion parts of food or feed substrate (ppb) is the maximum allowable limit imposed by the U.S. Food and Drug Administration for consumption and for interstate shipment of foods and feeds. In some European countries aflatoxin levels are regulated below five ppb. Aflatoxin contamination has been a chronic problem in some parts of USA including in Arizona cotton growing areas and Southeast USA peanut farming regions. However, sporadic severe outbreaks of aflatoxin contamination have occurred in the Midwest USA cornbelt in 1977, 1980 and 1988. The total costs associated with aflatoxin contamination in corn, both in the private and public sectors, have been estimated to be over $200 million in bad years. Aflatoxins have been shown to be immunosuppressive, mutagenic, teratogenic and hepatocarcinogenic in experimental animals. The mode of action, metabolism and biosynthesis of aflatoxins has been extensively studied (Yu et al., 2002). The chemical binding of the liver cytochrome P450-activated aflatoxin B1 forms adducts with guanidine residues in DNA that ultimately can cause liver cancer in certain animals. An association of hepatocellular carcinoma and dietary exposure to aflatoxins was established from patients living in high-risk areas of Kenya, Mozambique, Swaziland, Thailand, People's Republic of China, Philippines, and the Transkei of South Africa (Chen et al., 1996, Wogan, 1991, and Yu et al., 2002). The chemistry, biochemistry and molecular biology and synthesis of aflatoxins B1 and B2 have been investigated in significant detail. Since aflatoxin B1 (AFB1) is the most toxic of this group of toxins, extensive research has been done on its synthesis, toxicity and biological effects (Bhatnagar et al., 2002). Major aflatoxins are aflatoxin B1, B2, G1 and G2. *A. flavus* typically produces B1 and B2 (Diener *et al.* 1987). B1 is normally predominant in amount and is usually quantified because it is the most carcinogenic and the only member which is regulated

by the FDA. There are different ways to analyze aflatoxin quantatively or qualitatively.

2.2 *Aspergillus* section *Flavi*; Major source of aflatoxin producing fungi

THE GENUS *ASPERGILLUS*

The genus *Aspergillus*, a member of the phylum Ascomycota, includes over 185 known species. Several members of *Aspergillus* section *Flavi* produce aflatoxin. These includes *Aspergillus flavus* and *Aspergillus parasiticus*, as well as several less common taxa including *Aspergillus nomius, A. tamarii, A. pseudotamarii, A. minisclerotigenes* and *A. bombycis* (Klich et al., 1988; Cotty et al., 1994b). *Aspergillus* species classified outside of section *Flavi* can also produce aflatoxins. For example, *Aspergillus ochraceoroseus* from section *Ochraceorosei*, SCRR 1468, morphological resembling members of section *Circumdati*, and the ascomycete *Emericella astellata* and *E. venezuelensis* (*Aspergillus* section Nidulantes, Cary et al., 2005) also produce aflatoxin. The group of aflatoxin producing species is more complex than previously thought.

Aspergillus section *Flavi* including *A. flavus*, belongs to the genus *Aspergillus*. It is the second most common species after *A. fumigatus*. Colonies of *A. flavus* grow rapidly and the diameter will reach 6-7 cm in 10-14 days. The color of colony is initially yellow, and turns into yellow green or olive green. The old colony appears dark green. The shape is smooth and some have radial wrinkles. The reverse phase of colony is colorless or sandy beige. A differential medium called aspergillus flavus and parasiticus agar (AFPA) for screening and identified *A. flavus* species has been designed (Govrama and Bullerman, 1995). *A. flavus* can be identified on these medium by production of typical yellow to olive green spores and a bright orange

reverse (Raper and Fennell, 1965). Conidiophores are long (400-800 μ) and are often rough just beneath the globose vesicles (25-45 μ); phialides arise circumferentially and are biserate (two layered) or sometimes uniserate (single layered); the shape of conidial heads vary from columnar to radiate and globose; the arrangement of phialides on the vesicle dictates the shape of the conidial head. Vesicles are elongated in shape and varies with the composition of the substrate. The diameter varies from 10 to 65 μm (Raper and Fennell, 1965). Conidia from *A. flavus* isolates are smooth to slightly roughened and produce brown sclerotia (Klich, 2002).

A. flavus that produce sclerotia can be divided into two types of strains, L and S, on the basis of physiological, morphological, and genetic criteria (Cotty, 1989 and Bayman and Cotty, 1993). Under adverse conditions such as dry and poor nutrition, the mycelium congregates to form resistant structures called sclerotia (Yu et al., 2005). Sclerotia are pigmented, compacted aggregates of hyphae, which resist unfavorable environmental conditions and are capable of remaining dormant for long periods (Wicklow et al., 1983; Cotty, 1988; Rollins et al., 1998). The fungus overwinters either as mycelium in plant debris and litter on the soil, on insects or as sclerotia in the soil (Diener et al., 1987). When the growth conditions are favorable the sclerotia either germinate to produce additional hyphae or they produce conidia (asexual spores), which can be further dispersed in the soil and air (Bennett et al., 1986; Cotty, 1988). The fungus mostly exists in the form of mycelium or asexual conidia spores. S strain isolates produce numerous small sclerotia (<400 μm in diameter) and fewer conidia than L strain isolates. Typical L strains of *A. flavus* may produce only B aflatoxins or no aflatoxins at all. S strain isolates, reported to date, produce large quantities of aflatoxins B and/or G (Bayman and Cotty, 1991).

A. flavus has no sexual stage; however, some of its variability can be examined by sorting isolates into vegetative compatibility groups (VCGs). VCGs have been associated with many morphological and physiological features, such as sclerotia size, mycotoxin production, and spatial distribution (Papa, 1986, Bayman and Cotty, 1991). Heterokaryon formation can only occur between strains with identical alleles at each of several loci governing vegetative compatibility (Novas and Cabral, 2002). *Aspergillus* strains produce no clear signs of anastomosis or antagonism when they meet as wild strains; therefore, complementary nitrate nonutilizing (*nit*) mutants are commonly used to identify compatible isolates (Bayman and Cotty, 1991). Once identified, complementary mutants are paired. Complementation is indicated by a zone of dense, wild type growth and heavy sporulation at the intersection of the two colonies due to hyphal anastomosis and nutritional complementation in the heterokaryon. Heterokaryons can form only between *nit* mutants of vegetative compatible isolates. Thus, isolates that are vegetative compatible belong to the same VCG (Bayman and Cotty, 1991)..

A. flavus species are present in soil and contaminate a wide variety of agricultural products in the field, storage areas, processing plants and during distribution. *A. flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, and *A. bombycis* are the only molds that have abilities to produce aflatoxins (Kurtman et al., 1987). *A. flavus* strains range from nontoxigenic to those that produce aflatoxins B_1 and B_2, (AFB_1 and AFB_2) whereas *A.* parasiticus and *A. nomius* produce aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) and nonaflatoxigenic isolates are rare in nature (Dorner et al.,1989).

The fungi of *Aspergillus* section *Flavi* are one of the most abundant and widely distributed soil-borne molds and can be found anywhere on earth (Yu et al.,

2005). *A. flavus* is a saprophytic fungus that is capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, animal fodder, cotton, compost piles, dead insects and animal carcasses, outdoor and indoor air environments, stored grains, and even on live humans and animals (Klich, 1998). The life cycle in agriculture fields can be divided into two stages: (1) colonization of plant debris in soil and (2) invasion of seeds and grain in actively growing crop plants (Horn, 2007). Soil serves as a reservoir for primary inoculum of *A. flavus* and *A. parasiticus* (Horn et al., 1995; Payne, 1998). *A. parasiticus* appears to be more adapted to a soil environment, being prominent in peanuts, whereas *A. flavus* seems adapted to the aerial and foliar environment, being dominant in corn, cottonseed, and treenuts (Diener et al., 1987).

2.3 Morphological studies of aflatoxin producing fungi (*A. flavus*, *A. parasiticus* and *A. nomius*)

An important group of foodborne fungi are the aflatoxin producers: *A. flavus*, *A. parasiticus* and more recently *A. nomius*. Isolates of these are maintained in all the major world biological resource centres, which are used extensively for reference and as verified isolates for mycotoxin and other research. The veracity of isolate species names associated with such collections is rarely if ever questioned. Contemporary diagnosis of the two species (*A. flavus* and *A. parasiticus*) is based on the descriptions and keys of Raper and Fennell (1965). The primary separation being the presence of metulae and phialides (biseriate conidial head) for *A. flavus* and phialides only (uniseriate conidial head) for *A. parasiticus*. In the key for *A. parasiticus,* the words "strictly uniseriate" replace the former terms of "usually" or "mostly uniseriate" as used in previous keys (Thom and Church, 1926). Examination of a large number of *A.*

parasiticus isolates (Kozakiewicz, 1995) has shown that up to 10% of conidial heads in an *A. parasiticus* colony can have metulae and phialides (biseriate). Furthermore, not all *A. flavus* isolates consistently produce metulae (Klich and Pitt, 1988). Conidial wall ornamentation is now regarded as the primary diagnostic character for separation of these two species. Conidia of *A. flavus* have relatively thin walls which are finely to moderately roughen. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose. Scanning Electron Micoroscopy (SEM) micrographs clearly show these ornamentation differences. Furthermore, once SEM micrographs have been studied and compared, then with practice these differences become apparent using light microscopy. Additionally, there are a few selected media, which may be employed to help less trained mycologist: (i) *Aspergillus* differentiation agar (AFPA); (ii) coconut cream agar (CCA) and (iii) Czapek Dox agar (CZ). AFPA is a selective identification medium for the detection of *A. flavus* group strains (Pitt et al., 1983). With this method is possible to distinguish these species from other *Aspergillus* based on the development of orange colour on the reverse of the plates. The CCA is used to detect aflatoxin producer strains. The production of aflatoxin is detected by a blue fluorescence when exposed to a UV-light (Davis et al., 1987). When grown on CZ, colonies taxonomically between the two species can also be separated. Those of *A. flavus* being yellow-green and those of *A. parasiticus* a distinctly darker green, referred to as near ivy green.

Table 2.1 summarises the morphological differences between the two species.

Table 2.1 Morphological separation of *A. flavus* and *A. parasiticus*

Fungal species	Colony color	Seriation	Conidia	
			shape	texture
Aspergillus flavus	Yellow/green	b/u	gl/el	sm/fr
Aspergillus parasiticus	Ivy green	u/b	gl	r

u = uniseriate; b = biseriate

gl = globose; el = elliptical

sm = smooth; fr = finely roughened; r = rough

The situation is further complicated by the species *Aspergillus nomius* (Kurtzman et al., 1987). Morphologically, it resembles *A. flavus* but differs by the production of small bullet-shaped sclerotia; those in *A. flavus* being more globose. However, it is unclear whether fresh isolates of *A. nomius* always produce these distinctive sclerotia. In their absence only isoenzyme patterns and mycotoxin production provide reliable identification techniques. That is, for *A. nomius* the detection of aflatoxins B1, B2, G1, G2 (as does *A. parasiticus*), but without the detection of the secondary metabolite cyclopiazonic acid. *A. flavus* produces detectable aflatoxin B1 and B2 and cyclopiazonic acid only (Samson et al., 2006).

2.4 Major metabolites products from *Aspergillus* section *Flavi*

2.4.1 Primary metabolites: Extracellular enzymes and their application

Due to their high capacity for producing and secreting extracellular enzymes, *Aspergilli* play an important role in production of industrial enzymes (de Vries et al., 1999a, b; Lockington et al., 2002b). *Aspergillus* species are also important microorganisms in the fermented food industry and produce a variety of amylases and proteases (MacKenzie et al., 2000; Petersen et al ., 1999). *Aspergillus* species, especially GRAS-designated strains, produce and secrete a variety of industrial enzymes including amylases, glucoamylases, cellulases, pectinases, xylanases and other hemicellulases, and proteases. Enzymes degrading cellulose, hemicellulose, pectin, and other plant polysaccharides are typically complexes of a variety of enzymes having different substrate specificities with respect to the nature of the substrates and linkages they attack and also the locations of these linkages (endo- or exo-) within polysaccharides. Fungal α-amylases are used substantially in baking applications and to produce certain high maltose-containing starch syrups (Hata et al., 1991).

2.4.1.1 Amylolytic enzymes

amyloglucosidase, which catalyses exo hydrolytic cleavage of glucose monomers from starch, is used in combination with other amylases to maximize conversion of starch to glucose or fermentable sugars. Introduction of multiple gene copies of the genes *amyB*, *glaA*, and *agdA*, encoding industrial Taka-amylase, glucoamylase, and alpha-glucosidase production, respectively, in *A. oryzae* resulted in

enhanced expression of these enzymes (Hata et al., 1991; Minetoki et al., 1995; Tada et al., 1991).

2.4.1.2Xylanolytic enzymes

Aspergillus xylanases may be used in biobleaching (although xylanases from other sources may be more effective), but they do have applications in the bakingindustry to solubilize arabinoxylans to expand doughs and to increase animal feed conversion, and in vegetable processing and fruit juice clarification (Petersen et al., 1999).

2.4.1.3 Cellulolytic enzymes

All four classes of essential enzymes involved in biodegradation of cellulose, endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), exoglucanases, and beta-glucosidase (EC 3.2.1.21), have been found in *Aspergillus* species (Singh et al., 1990). Several different endoglucanases and beta-glucosidases, and an exoglucanase, have been characterized in *A. niger*, and the genes encoding a variety of *Aspergillus* cellulases have been characterized. The biodegradation of the xylan depends on endoxylanase (EC 3.2.1.8) and b-xylosidases (EC 3.2.1.37) and industrial *Aspergillus* strains, including *A. awamori, A. niger* and *A. oryzae*, produce multiple endoxylanase forms and one or more beta-xylosidases and the genes encoding a variety of each class of enzyme have been sequenced (Petersen et al., 1999).

2.4.2 Secondary metabolites

2.4.2.1 Cyclopiazonid acid (CPA)

Cyclopiazonid acid is a toxic indole tetramic acid (Figure 2.1) which was first isolated from cultures of *Penicillium cyclopium* in 1968 (Holzapfel, 1968), and subsequently found to be produced by *A. versicolor* (Ohmono et al., 1973) and *A.flavus* Link (Luke et al., 1977). Investigations on flavutoxin, a toxic nitrogen-containing metabolite of *A. flavus* (Kirksey and Cole, 1973), subsequently revealed that flavutoxin was a metal chelate-complex of cyclopiazonic acid (Gallagher et al., 1978).

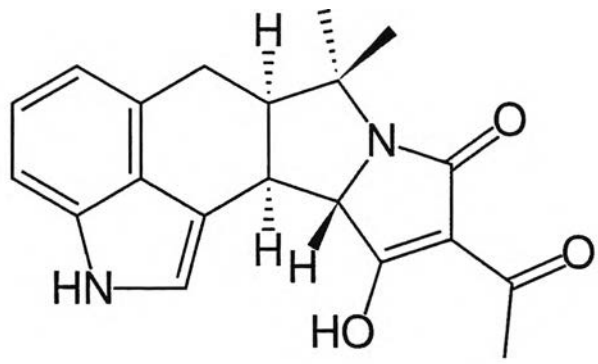

Figure 2.1 Cyclopiazonic acid (Gallagher et al., 1978).

2.4.2.2 Kojic acid

Kojic acid, another toxic metabolite, has been known since 1907, when it was first isolated by Saito from *A. oryzae* grown in steamed rice (Saito, 1907). Several species of *Aspergillus* produce this unusual toxin as do certain members of *Penicillium* and *Acetobacter* (Wilson, 1966). Yabuta (1924) defined its structure (Figure 2.2) in 1924. Significant quantities of this neurological poison have been detected in various foods, especially corn, that were experimentally contaminated

with *A. flavus* and other kojate-producing *Aspergilli* (Wilson and Wilson, 1962). Concentrations varying from 1.0 to 25.0 mg per gram of contaminated food material were attained. On the other hand, only trace amounts were detected in toxic-feed corn found heavily contaminated with *A. flavus* under natural conditions.

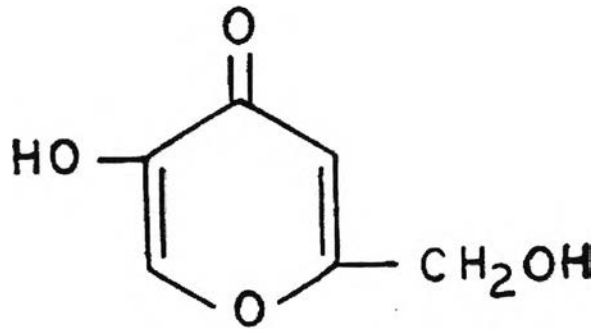

Figure 2.2 Kojic acid (Wilson, 1966)

2.4.2.3 Aflatoxin

Aflatoxins were first identified in the early 1960s and compose a family of toxic compounds (Wild et al., 2002). Aflatoxin B1 is predominant and the most toxic and potent hepato-carcinogenic natural compound ever characterized (Squire, 1981; Bhatnagar et al., 2001). The conditions favoring formation of the aflatoxins have been described, as has their metabolism, toxicity, DNA adduct formation, mutagenic, and carcinogenic activity (Eaton et al., 1994). The immunosuppressive properties of aflatoxin B1, particularly on cell-mediated immunity, have been demonstrated in various animal models (Ali et al., 1994; Neiger et al., 1994; Pestka et al., 1994). A major metabolic of aflatoxin B1 is aflatoxin M1 which is usually excreted in the milk and urine of dairy cattle and other mammalian species that have consumed aflatoxin contaminated food or feed (Gourama et al., 1995)

The aflatoxin molecule contains a coumarin nucleus linked to a bifuran and either a pentanone, as in AFB_1 and dihydro derivative AFB_2, or a six number lactone, as in AFG_1, and its corresponding derivative AFG_2 (Figure 2.3) (Sanz et al.,1989). These four compounds are separated by the color of their fluorescence under long wave untraviolet illumination (B = blue; G = green). *A. flavus* only producs B_1 and B_2 and *A. parasiticus* produces these same metabolites along with G_1 and G_2. The derivatives AFM_1 and AFM_2 were first isolated from the milk of cows fed on aflatoxin contaminated rations (Dutton and Heathcote, 1966).

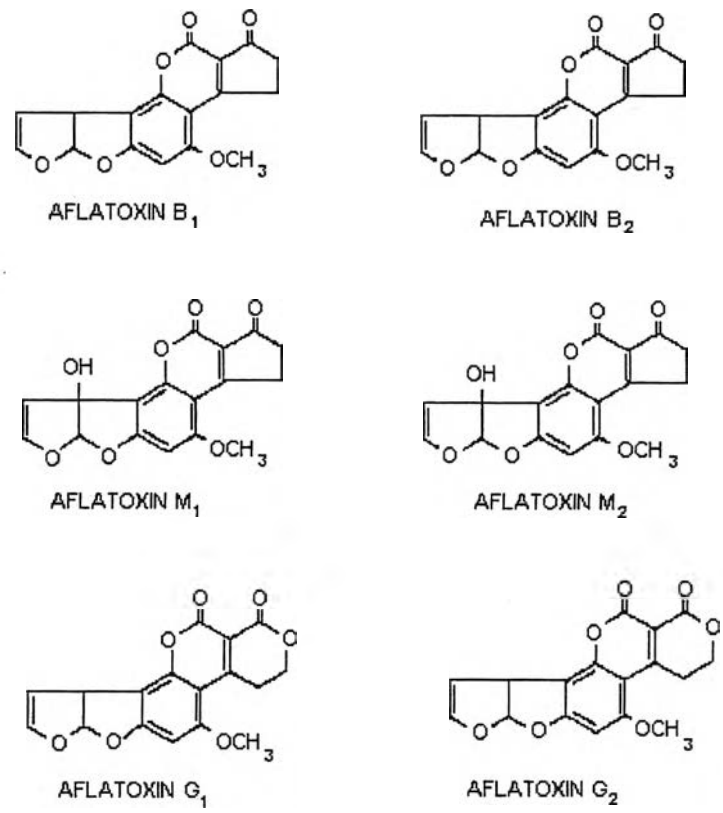

Figure 2.3 The structure of aflatoxins: B1, B2, G1, G2 (http://www.food-info.net/images/aflatoxins.jpg)

2.5 Biosynthesis of aflatoxin

The aflatoxin biosynthetic pathway consists of at least 18 multienzymatic conversion reactions initiated by polypeptide synthesis from acetate (Skory et al., 1992), a process similar to fatty acid synthesis. The conversions of sterigmatocystin to O-methylsterigmatocystin and O-methylsterigmatocystin to aflatoxin, which represent the final steps of the pathway, are unique to the aflatoxin producing fungi *A. flavus* and *A. parasiticus* (Motomura et al., 1999, Prieto and Woloshuk, 1997). Some of these enzymes involved in aflatoxin biosynthesis have been characterized, and their respective genes have been cloned. These include the *pksA*, *pksL1*, *fas1A*, *nor-1*, *norA*, *avf1*, *vbs*, *ver1*, *stcP*, *omtA*, *ord1*, *avnA*, and *aflR* gene, which codes for a regulatory factor that activates the transcription of these pathway gene (Prieto and Woloshuk, 1997).

Attempts to decipher the aflatoxin biosynthetic pathway began with the discovery of the structure of these toxins. However, the major biochemical steps and thecorresponding genetic components of AFB1 biosynthesis have been elucidated only in the last decade at a molecular level. Several previous reviews have described the biochemistry and genetics of aflatoxin formation (Bhatnagar and Cleveland, 1990). Various studies have determined that aflatoxins are synthesized in two stages from malonyl CoA, first with the formation of hexanoyl CoA, followed by the formation of a decaketide anthraquinone (Payne and Brown, 1998). A series of highly organized oxidation-reduction reactions then allows formation of aflatoxin (Buchanan and Lewis, 1984). The currently accepted scheme (Figure 2.4) for aflatoxin biosynthesis is: hexanoyl CoA precursor —> norsolorinic acid, NOR —> averantin, AVN —> hydroxyaverantin, HAVN —> averufin, AVF —> hydroxyversicolorone,

HVN—> versiconal hemiacetal acetate, VHA —> versiconal, VAL —> versicolorin B, VERB —> versicolorin A, VERA

—> demethyl-sterigmatocystin, DMST —> sterigmatocystin, ST —>

O -methylsterigmatocystin, OMST—> aflatoxin B1, AFB1 and aflatoxin G1, AFG1 (Figure 2.4).

A branch point in the pathway has been established, following VHA production, leading to different structural forms of aflatoxins B2 and G2 (Cotty, 1988; and Detroy et al., 1973) A number of metabolic grids may provide alternate pathways to aflatoxins (Dutton, 1988; and Payne and Brown, 1998). Several specific enzyme activities associated with precursor conversions in the aflatoxin pathway have been partially purified (Hicks et al., 1997) (Figure 2.4, identified enzymes enclosed in boxes); whereas others such as methyltransferases (Holden, 1962; Payne and Brown, 1998; and Hsieh and Mateles, 1970) have been purified to homogeneity. Several other enzymes, which are involved in aflatoxin biosynthesis such as a reductase and a cyclase (Kale et al., 1994), have also been purified from *A. parasiticus.* A desaturase which converts VERA to VERB has been found in cell-free fungal extracts (Yabe, 1988). Matsushima et al (1994) have purified and characterized two versiconal hemiacetal acetate reductases involved in toxin synthesis, whereas Kusumoto and Hsieh (1996) purified to homogeneity an esterase that converts VHA to versiconal. Bhatnagar et al. Chatterjee and Townsend (1996) and Bhatnagar et al (1991) demonstrated that in the later stages of AFB1 and AFB2 synthesis, independent reactions and formation of different chemical precursors are catalyzed by common enzyme systems (Yabe et al., 1988; and Yu et al., 2002).

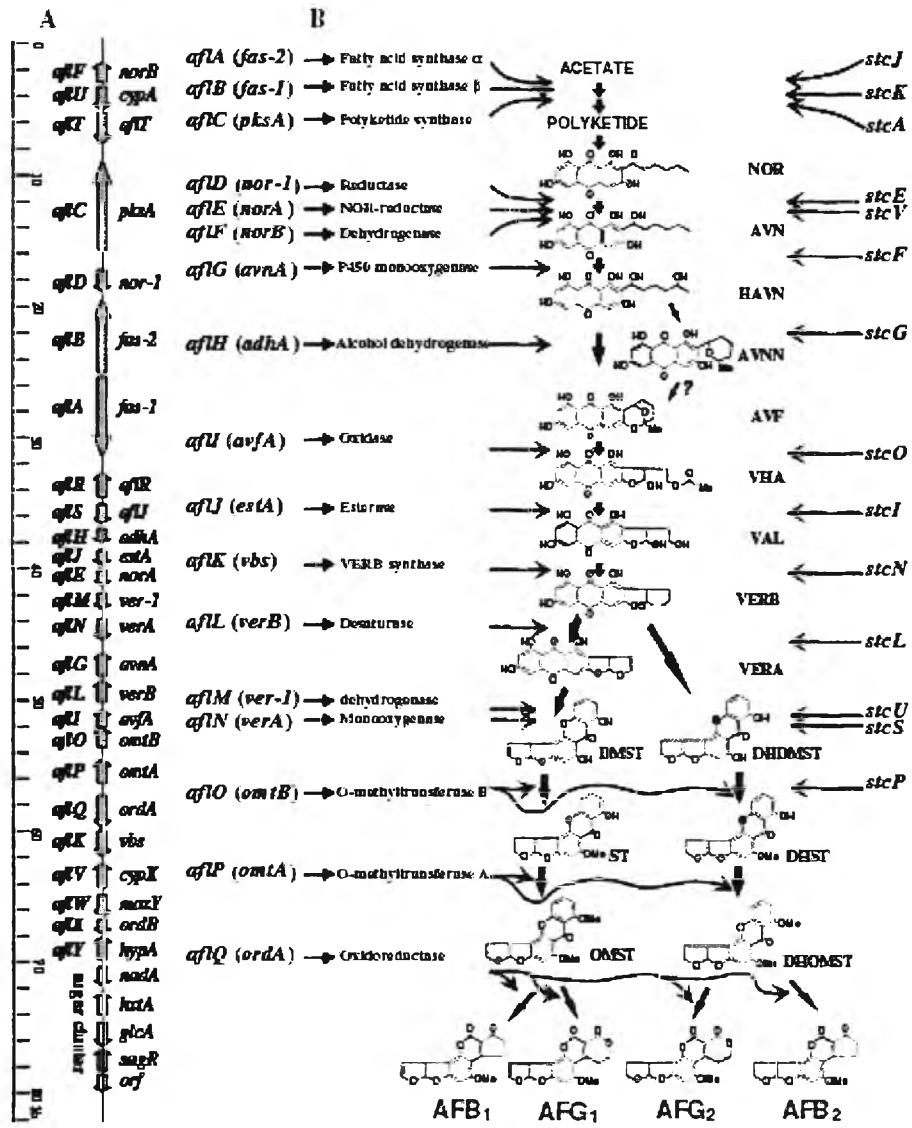

Figure 2.4 The gene cluster responsible for aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus*. (A) Clustered genes (arrows indicate the direction of gene transcription) and (B) the aflatoxin biosynthetic pathway. The ST biosynthetic pathway genes in *A. nidulans* are indicated at the right of panel B. Arrows in panel B connect the genes to the proteins they encode. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5ɪ-hydroxy-averantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorinA; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; and AFG2, aflatoxin G2 (Payne and Brown, 1998)

Evidence exists that secondary metabolism is associated with fungal developmental processes such as sporulation and sclerotia formation (Sekiguchi and Gaucher, 1977). It was observed that the environmental conditions required for secondary metabolism and for sporulation are similar. It was also reported that the spore formation and secondary metabolite formation occur at about the same time (Hicks et al., 1997). Certain compounds in *A. parasiticus* that exhibit the ability to inhibit sporulation have also been shown to inhibit aflatoxin formation (Reis, 1982; and Yu et al., 2002). Chemicals that inhibit polyamine biosynthesis in *A. parasiticus* and *A. nidulans* inhibit both sporulation and aflatoxin/sterigmatocystin biosynthesis. More evidence from mutant strains of *Aspergilli* demonstrated the relationship between aflatoxin formation and sporulation. Mutants that are deficient in sporulation were unable to produce aflatoxins (Bennett and Papa, 1988). Similar phenomenon has been reported in other fungi such as *Fusarium verticillioides* mutation in FCC1 gene that resulted in both reduced sporulation and reduced fumonisin B1 production. Since many environmental and nutritional factors affect aflatoxin formation, it is likely that one or more signal transduction pathways affect aflatoxin formation. Also, there appears to be a genetic connection between fungal development and toxin formation (Yu et al., 2002). A correlation between increased pool size of cAMP and aflatoxin production had been observed previously (Khan and Venkitasubramanian, 1986-1987). Sterigmatocystin production by *A. nidulans* appears to require inhibition of FadA-dependent signaling (Hicks et al., 1997). FadA is the alpha subunit of the *A. nidulans* heterotrimeric G-protein. When FadA is bound to GTP and in its active form, sterigmatocystin production (and sporulation) was repressed. However, in the presence of FlbA, the intrinsic GTPase, activity of FadA is stimulated, thereby leading to GTP hydrolysis, inactivation of FadA-dependent signaling, and stimulation

of sterigmatocysin production. A non-sporulating, "fluffy" mutant strain of *A. nidulans* was found to be deficient in sterigmatocystin formation (Wieser et al., 1994). Hicks et al. (1997) provided evidence that a G-protein signal transduction pathway mediated by protein kinase A regulates both aflatoxin/sterigmatocystin synthesis and sporulation. The G-protein signaling pathway involves the "fluffy" gene regulators FluG and FlbA, and the conidiation gene regulators BrlA, FadA and PkaA. A gene, *brlA*, in *A. nidulans* encodes a transcriptional regulator (BrlA) believed to activate developmental genes since mutation in *brlA* gene resulted in no conidiation (Clutterbuck, 1969). In the process of characterizing *A. nidulans* "fluffy" mutants, six loci were identified to be the results of recessive mutations in the fluffy genes *fluG, flbA, flbB, flbC, flbD*, and *flbE*. Two of these genes, *fluG* and *flbA* encoding protein factors FluG and FlbA, were found to be involved in the regulation of both asexual development (conidiation) and sterigmatocystin biosynthesis *in A. nidulans* (Yu et al., 2002). The *fluG* is involved in the synthesis of an extracellular diffusible factor that acts upstream of *flbA*. The *pkaA* gene encodes the catalytic subunit of a cyclic AMP (cAMP)-dependent protein kinase A (PKA), PkaA. Over expression of *pkaA* (PkaA) inhibits *brlA* and *aflR* (positive regulator of aflatoxin/sterigmatocystin genes) expression (Shimizu and Keller, 2001). The *fadA* encodes the alpha subunit of a heterotrimeric G-protein, FadA. A domain of the FlbA, the regulator of the G-protein signaling (RGS) is presumably to inhibit FadA (Yu et al., 2002). In the overall scheme of the proposed G-protein signaling pathway, the FadA and PkaA favor vegetative growth and inhibit conidiation and aflatoxin/sterigmatocystin production; while FluG and FlbA inhibit FadA and PkaA function and promote conidiation and aflatoxin/sterigmatocystin biosynthesis. This G-protein signaling pathway involving

FadA in the regulation of aflatoxin production may also exist in other *Aspergilli* such as *A. parasiticus* (Yu et al., 2002).

2.6 Environmental and nutritional factors affecting aflatoxin biosynthesis

Factors affecting aflatoxin production can be divided into three categories including physical, nutritional, and biological factors. Physical factors include temperature, pH, moisture, light, aeration, and level of atmospheric gases. Aflatoxins are produced only between temperature of 10 and 42 ^{O}C, and the optimum temperature is between 25 to 35 ^{O}C (Diener and Davis, 1966). Many researchers have reported that initial pH did not significantly affect aflatoxin production, while other investigators have shown that weak acid pH resulted in higher aflatoxin production and markedly depressed mold growth (Jarvis, 1971). Either natural or laboratory media exert a strong effect on aflatoxin production. Generally, the preferred carbon sources for aflatoxin production are glucose, sucrose, or fructose. Zinc and manganese ions are essential for aflatoxin biosynthesis, but a mixture of cadmium and iron depressed mold growth and hence aflatoxin production.

2.6.1 Carbon sources and aflatoxins production

Unlike many secondary metabolites, aflatoxin biosynthesis is induced by the presence of simple carbohydrates, especially glucose, sucrose, fructose, and maltose, but not by complex carbohydrate sources such as peptone, lactose, sorbose, and oleic acid (Davis et al., 1967). Glucose analogs that are not readily metabolized fail to induce aflatoxin (abdollahi and Buchanan, 1981). Hsieh & Mateles (1970) examined glucose utilization by aflatoxin-producing fungi and concluded that aflatoxins are synthesized extramitochondrially from acetyl CoA derived from the catabolism of

simple carbohydrates. Llewellyn et al (1980) showed that sucrose concentrations of 3, 10, 20, and 30% supported both growth and sporulation, but that the 10% and 20% sucrose concentrations were most conducive to aflatoxin biosynthesis. Wiseman & Buchanan (1987) demonstrated that of replacement media containing 1mM, 10 mM, 100 mM, or 330 mM glucose, the minimum level of glucose required for induction of aflatoxin was 100 mM glucose. However, Woloshuk et al (1997), using a *ver-1*::GUS reporter construct, determined that the minimal concentration of glucose, maltose, or maltotriose that induced measurable GUS activity was 1 mM, while the concentration that produced as much GUS activity as the PMSG positive control ranged from 10–20 mM. The consensus of these data is that simple carbohydrates, which are readily metabolized by the glycolytic and pentose phosphate pathways, serve as the best substrates for aflatoxin induction.

2.6.2 Nitrogen sources and aflatoxins production

Nitrogen source has been shown to play an important role in aflatoxin biosynthesis. Eldridge (1964) obtained the highest yields of aflatoxin by including either aspartate or glutamate in the medium. Reddy et al (1971) described a synthetic low-salts medium, incorporating ammonium sulfate and asparagine, that was markedly superior for aflatoxin productions to two previously described synthetic media that contained only inorganic nitrogen sources. Other good nitrogen sources for aflatoxin production include alanine, glutamine, and proline (Payne and Brown, 1998; and Davis and Diener, 1967). Marsh (1987), using a resuspension technique, found aspartate to support the highest levels of aflatoxin. This effect was in part due to stimulation of initial growth, resulting in an earlier onset of aflatoxin production than that observed when other amino acids were used. The addition of amino acids to

cultures that have ceased growing but begun aflatoxin production does not elevate aflatoxin production.

2.6.3 pH and aflatoxins production

Keller et al (1977) found that growth of *A. parasiticus* in continuous culture results in a decrease in pH and an increase in aflatoxin production over time. They also found that transcription of the *ver-1* gene in *A. parasiticus* and the *stcU* gene in *A. nidulans* was greatest at pH 4.0 but undetectable at pH 8.0. It has previously been established that aflatoxin biosynthesis occurs optimally in the pH range of 3.5–5.5, and production decreases with increasing ambient pH (Detroy and Hesseltine, 1969). Keller et al (1977) concluded, however, that NA production was dependent on both pH and media. Greater NA was produced at pH 5 than pH 6, but at pH 5 considerably less NA was produced on nitrate containing medium than on ammonium-containing medium. Thus the data clearly show that pH is involved in the production of aflatoxin, but does not rule out other effects such as nitrogen and carbon sources. These authors suggested that pH regulation may also explain glucose induction of aflatoxin biosynthesis.

2.7 Isolation of aflatoxin producing fungi

Species of *Aspergillus* that produce aflatoxins are ubiquitous and easily isolated from nature. Soil samples are usually diluted with known volumes of sterile water, and soil suspensions are plated on agar media (''dilution plated'') to obtain quantitative estimates of the population density expressed as colony-forming units (CFUs)/g in soil. The same technique can be used for agricultural commodities by grinding the sample before suspending in water or by homogenizing the sample in

water with a blender (Dorner, 2002; Hartog and Notermans, 1988; Horn and Dorner, 1998; Klich et al.,1992). Alternatively, grains and seeds can be plated whole after surface sterilization to obtain the percentage infection. This technique does not measure the extent of fungal colonization of grains, and surface-sterilization techniques are not 100% effective (Andrews, 1996; Andrews et al., 1997; Sauer and Burroughs, 1986). Therefore, for precise population studies such as vegetative compatibility group (VCG) analysis (Horn and Greene, 1995), seeds must be individually surface-sterilized to avoid cross contamination during sterilization and the subsequent rinses. *A. flavus* and *A. parasiticus* are not fastidious in their nutritional requirements and will grow on nearly all commonly prepared media for fungi. Isolation of these fungi on agar media instead relies on their sensitivity to certain antibiotics relative to other fungi, their ability to growat relatively high temperatures ($37^{O}C$), and their tolerance of low moisture content in the growth medium. The most commonly used media for dilution plating contain the antibiotics dichloran and/or rose bengal for restricting fungal colony diameter (Cotty, 1994; Frisvad et al., 1992; King et al., 1979; Pitt, 1992). Antibiotics against bacteria are also added, and incubation at $37^{O}C$ inhibits the growth of many soil fungi that would interfere with the detection of aflatoxigenic species. Various formulations of dichloran-rose bengal medium permit the accurate identification of many *Aspergillus* species, including *A. flavus* and *A. parasiticus*, directly from the dilution plates (Horn and Dorner, 1998; Pitt, 1992). Other media rely on low water activity for selection of aflatoxigenic *Aspergillus* species. Sodium chloride is often used to adjust the water activity (Griffin et al., 1975, 2001). However, *A. flavus, A. parasiticus*, and other members of *Aspergillus* section *Flavi* cannot be identified to species in the presence of NaCl and must be subcultured to another medium such as Czapek agar for final

identification (Horn et al., 1995). When growing fungi in culture for aflatoxin production, single-spore isolates of *A. flavus* and *A. parasiticus* are necessary to ensure that mixed cultures do not compromise the results. Aflatoxigenic strains may be grown on solid substrates or in liquid media. Of the undefined media, various formulations of yeast extract-sucrose broth (YES) are most commonly used (Horn and Dorner, 1999; Wei and Jong, 1986). Large numbers of isolates can be examined by growing them in small vials of YES, manually macerating the mycelium, then adding chloroform and vortex mixing. The chloroform phase separates can be analyzed directly by TLC or HPLC without further cleanup (Horn and Dorner, 1999; Horn et al., 1996).

2.8 Biocontrol of aflatoxin producing strains with atoxigenic strains of *A. flavus*

Aflatoxin cannot be readily removed from contaminated foods by detoxification. Therefore, the use of a biological control to help decrease toxin content based on the displacement of toxigenic isolates using atoxigenic isolates of the same species has been developed. Moreover, it has been reported that aflatoxin production is inhibited by some of bacteria and molds including *Bacillus pumilus*, *Bacillus subtilis*, *Trichoderma* spp., and atoxigenic *A. flavus* (Munimbazi and Bullerman, 1998).

There is a great diversity of phenotypes of *A. flavus* in agricultural fields and the common occurrence of atoxigenic strains (Diener and Davis, 1966 and Cotty, 1989). Furthermore, toxigenicity is apparently unrelated to a strain's ability to colonize and/or infect living or dead plant tissues. These observations led to the finding that atoxigenic strains can be used to displace toxigenic strains (Cole and Cotty, 1990). Cotty and Bayman(1996) tested the competitive ability of an atoxigenic

A. flavus strain to inhibit the aflatoxin contamination of developing strain cotton bolls. Competitive exclusion was found to contribute to the effect of the atoxigenic strain on contamination, and the results also suggested that a second mechanism might also have been in effect. Now, biocontrol of aflatoxin producing strains with atoxigenic strains of *A. flavus* is being developed for corn, cottonseed, peanuts, rice kernels, and wheat seed (Brown et al., 1991, Bock and Cotty, 1999 and Cotty, 1994). Atoxigenic isolate AF36 reduced aflatoxin B1 content of bolls by 88% to 99% (Garber and Cotty, 1997). Boller and Sohroeder (1973) reported that *A. parasiticus* invaded stored rice rapidly but considerably smaller quantities of aflatoxins were produced when inocubated with *A. chevalieri* simultaneously at 100% relative humidity. Reduction in aflatoxin B1 ranged from 99% at 25 ^{O}C to 95% at 35 ^{O}C. No aflatoxins were detected at 85% relative humidity. It could significantly reduce the number of the sclerotia formed on locule surfaces and, in some cases, the percentage of seed containing sclerotia. Fermentation conditions of atoxigenic *A. flavus* for large scale production have been investigated (Bock and Cotty, 1999). Wheat seeds colonized with atoxigenic *A. flavus* have been used in commercial trials. The aim of this work is to determine the genus and related members of *Aspergillus* section *Flavi* isolated from corn fileds in Thailand and their ability to produce aflatoxins. The ability of atoxigenic isolates to intraspecifically inhibit aflatoxins synthesis by a toxigenic isolates of *A. flavus* will evaluated. This should provide potential isolates that could br tested in the field for biocontrol of aflatoxin contamination of corn.