

BIOCONTROL OF *Aspergillus flavus* AND AFLATOXIN PRODUCTION

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ABSTRACT

Aflatoxins are potent carcinogenic and mutagenic metabolites mainly produced by the fungal species *Aspergillus flavus* and *A. parasiticus*. These species can contaminate several food commodities including cereals, peanuts and crops. This article is a review on the morphological and biological characters of *A. flavus*, the genetic research on aflatoxin biosynthetic pathway and biocontrol research on *A. flavus* and aflatoxin production.

Keywords: *Aspergillus flavus*; Aflatoxins; Biocontrol

1. INTRODUCTION

Aspergillus flavus and the closely related subspecies *parasiticus* have a world-wide distribution and normally occur as saprophytes in soil and on many kinds of decaying organic matter [1]. These fungi readily colonize several important crops such as corn, cottonseed, peanuts, and tree nuts. Most *A. flavus* and *A. parasiticus* can produce polypeptide-derived secondary metabolites called aflatoxins, which are highly toxic, mutagenic, and carcinogenic to animals [2, 3]. They may also be involved to some degree in primary liver cancer in humans [4]. Possible cases of aflatoxicoses in humans have been reported in many countries in Southeast Asia and Africa [5]. Aflatoxins have been implicated in hepatocellular carcinoma, acute hepatitis, Reye's syndrome, cirrhosis in malnourished children, and kwashiorkor [6, 7].

Concern for human and animal health has led to regulatory limitations on the quantity of aflatoxins permitted in foods and feeds throughout most of the world. According to WHO and FAO regulation the content of aflatoxin B (AFB) should be less than 0.5 µg/kg in milk [8]. American Food and Pharmacology Administration stipulated the content of AFB should not exceed 20 ppm in edible food and 30 ppm in animal feed. The permissible content of AFB is also regulated in China: 20µg/kg for corn, peanut core, peanut oil, 10µg/kg for rice, edible oil, and for other food 5µg/kg [9].

Morphological and biological characters of *Aspergillus flavus* subsp. *parasiticus*

Aspergillus flavus belongs to the genus *Aspergillus*. It is the second most common species after *Aspergillus 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 is colorless or sandy beige. A differential medium called *A. flavus* and *parasiticus* agar (AFPA) for screening and identifying *A. flavus* species has been designed [10, 11, 12]. *A. flavus* and *A. parasiticus* can be identified on this medium by production of typical

yellow to olive green spores and a bright orange reverse [13]. More details can be observed under scanning-electron microscope: Conidiophores are long (400-800 μ) and are often rough just beneath the globose vesicles (25-45 μ); phialides arise circumferentially and are biserial (two layered) or sometimes uniserial (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 μ m [13, 14]. Conidia from *A. flavus* isolates are smooth to slightly roughened, while conidia from *A. parasiticus* were rough. Certain species produce brown sclerotia.

Aspergillus flavus species are present in soil and contaminate a wide variety of agricultural products in the field, storage areas, processing plants and during distribution. *Aspergillus flavus*, *A. flavus* subsp. *parasiticus*, *A. nomius*, *A. tamaris* and *A. bombycis* are the only molds that have so far been shown to produce aflatoxins [15, 16]. *Aspergillus flavus* strains range from nontoxic to those that produce aflatoxins B₁ and B₂, (AFB₁ and AFB₂) whereas *A. flavus* subsp. *parasiticus* produces aflatoxins B₁, B₂, G₁, G₂, (AFB₁, AFB₂, AFG₁, and AFG₂) and nonaflatoxigenic isolates are rare in nature. *A. flavus* subsp. *parasiticus* tends to be more stable in producing aflatoxins than *A. flavus* [17, 18].

Production of aflatoxin

Aflatoxins are a group of toxins having similar molecular structures. The toxin was first discovered in 1960 when there were mass deaths from liver disease of turkeys in England followed by deaths of other farm animals. More than 100,000 turkeys died within a few months [19]. 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 mould [20]. 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.

The aflatoxin molecule contains a coumarin nucleus linked to a bifuran and either a pentanone, as in AFB₁ and the dihydro derivative AFB₂, or a six-member lactone, as in AFG₁ and its corresponding derivative AFG₂ [21]. These four compounds are separated by the color of their fluorescence under long-wave ultraviolet illumination (B = blue; G = green) [22]. The subscripts relate to their relative chromatographic mobility. Of the four, B₁ is found in highest concentrations followed by G₁ and G₂. *Aspergillus flavus* only produces B₁ and B₂ and *A. parasiticus* produces these same metabolites along with G₁ and G₂. Dutton and Heathcote characterized the hemiacetal derivatives of B₁ and G₁ that were designated B_{2a} and G_{2a} [23]. Two 4-hydroxylated derivatives of these last toxins have been found in peanuts and maize. These derivatives, AFM₁ and AFM₂, were first isolated from the milk of cows fed on aflatoxin-contaminated rations. Various other minor aflatoxins are produced by *A. flavus* in culture, and in the liver and probably other organs.

Aflatoxin production is the consequence of a combination of species, substrate, and environment. The factors affecting aflatoxin production can be divided into three categories: physical, nutritional, and biological factors. Physical factors include temperature, pH, moisture, light, aeration and level of atmospheric gases. Aflatoxins are produced only between temperatures of 12 and 42 °C, and the optimal temperature is 25 °C to 35 °C [24]. Aflatoxin production is particularly favored by very moist conditions. Maximum moisture content for aflatoxin production in corn kernels is 25 % at 30 °C and the minimum relative humidity for aflatoxin production varies between 83 % and 88 %. Presence of CO₂ and O₂ influences mold growth and aflatoxin production. A 20 % level of CO₂ in air depresses aflatoxin production and markedly depresses mold growth. Decreasing the O₂ concentration of air to 10 % depresses aflatoxin production, but only at O₂ levels of less than 1 % are growth and aflatoxin production completely inhibited [25]. Many researches 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 [26]. 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 are essential for aflatoxin biosynthesis, but a mixture of cadmium and iron depressed mold growth and hence aflatoxin production.

In order to devise effective methods for preventing aflatoxin contamination of feed, elucidation of the aflatoxin biosynthetic mechanism in *A. flavus* species is very important (Since the first gene was isolated and described in 1992 [27, 28], the last review on the molecular biology of aflatoxin biosynthesis, rapid and significant progress followed the discovery that genes involved in the pathways of aflatoxin biosynthesis has been investigated). The aflatoxin biosynthetic pathway consists of at least 18 multienzymatic conversion reactions initiated by polypeptide synthesis from acetate, a process similar to fatty acid synthesis. The generally accepted pathway for aflatoxin B₁ formation is as follows [29]: NOR → averantin → averufanin → averufin (AVF) → hydroxyversicolorone → versiconal hemiacetal acetate (VHA) → versicolorin A → sterigmatocystin (ST) → O-methylsterigmatocystin (OMST) → aflatoxin B₁. In aflatoxin biosynthesis, norsolorinic acid (NOR) is the first stable intermediate. The conversions of ST to OMST and OMST to aflatoxin, which represent the final steps of the pathway, are unique to the aflatoxin-producing fungi *A. flavus* and *A. parasiticus* [30, 31, 32]. Some of the 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 the *aflR* gene, which codes for a regulatory factor (AFLR) that activates the transcription of these pathway genes [28, 29, 33].

Studies reveal that all of the identified genes related to aflatoxin biosynthesis are located within a 75-kb DNA region in both *A. parasiticus* and *A. flavus*, and their relative positions in the clusters of both fungal species are similar [30]. On the other hand, several fungi (*Aspergillus nidulans*, *Bipolaris* spp., *Chaetomium* spp., *Farrowia* spp., *Monocillium* spp.) produce sterigmatocystin, a precursor of aflatoxins, and it has been suggested that pathways almost identical to the aflatoxin biosynthesis pathway may also be involved in sterigmatocystin biosynthesis; in fact, genes similar to the genes involved in aflatoxin biosynthesis have been isolated from *A. nidulans*, and it has been found that these genes are located in a 60-kb cluster in the *A. nidulans* genome [34, 35].

Aflatoxin analysis

Molds and aflatoxins occur in an extremely heterogeneous fashion in food commodities. It is thus crucial that sampling is carried out in a way that ensures that the analytical sample is truly representative of the consignment. Failure to do this may invalidate the subsequent analysis [36, 37]. Analytical methods used are based on TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography) or ELISA (enzyme - linked immunosorbent assay). Extraction with aqueous acetonitrile or methanol, followed by clean-up of the extract solutions using immunoaffinity columns, provides sensitive and selective results for a wide range of foods and animal feed. Compared to these three methods [38, 39], TLC is the oldest of the chromatographic methods, requiring less advanced and expensive equipment than other chromatographic methods, and a little simpler. But the low accuracy has limited its use in research fields. HPLC is a chemistry-based tool for quantifying and analyzing the amount of a chemical compound within a mixture of chemicals. It is highly versatile and easily automated, for detection of target compounds. In addition, it is accurate, quick, and can determine many samples at the same time. However, expensive equipments and complex procedures restrict wide application. ELISA [36, 40] is a method developed recently for determining aflatoxin in food and other commodities. Since it is rapid, simple and highly sensitive, the method has become popular in analyzing for aflatoxin. However, due to the production of false positives, it is essential that positive results should be confirmed [41].

Biocontrol of aflatoxin producing fungi and aflatoxin production

Aflatoxin contamination of crops can be minimized by early harvest, prevention of insect damage, and proper storage [42]. However, even under careful management, unacceptable aflatoxin levels may occur from unpreventable insect damage to the developing crop or from exposure of the mature crop to moisture either prior to harvest, or during storage in modules, handling, transportation, or even use [43].

For many diseases, traditional chemical control methods are not always economical nor are they effective, and fumigation as well as other chemical control methods may have unwanted health, safety, and environmental risks. The antifungal abilities of some beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then. However, they are only now beginning to be used commercially.

Aflatoxins cannot be readily removed from contaminated foods by detoxification. Therefore, there is interest in developing a biological control method that can increase crop safety by decreasing toxin content and that is based on the displacement of toxigenic isolates using atoxigenic isolates of the same species. It has been reported that aflatoxin production is inhibited by lactic acid bacteria, *Bacillus subtilis* and many molds. This inhibition may result from many factors including competition for space and nutrients in general, competition for nutrients required for aflatoxin production but not for growth, and production of anti-aflatoxigenic metabolites by co-existing microorganisms.

Bacillus

Munimbazi and Bullerman [44] reported the influence of 6 *Bacillus pumilus* isolates on the growth of aflatoxin-producing molds and aflatoxin production using both simultaneous and deferred antagonism assays. Totally, percentages of inhibition of aflatoxin production ranged between 98.2 % and 99.0 %. Mycelium production was less inhibited with percentages of inhibition ranging between 34.4 % and 56.4 %. Bottone and Peluso [45] identified a compound produced by *B. pumilus* which could inhibit *Aspergillus* species. The active compound inhibited *Aspergillus* spore germination and aborted elongating hyphae, presumably by inducing a cell-wall lesion. Antifungal activity was stable in agar for a minimum of 8 days, and was resistant to Pronase degradation. Its molecular mass was determined by diffusion through dialysis membrane to be 500–3000 Da.

Bacillus subtilis, a bacterium isolated from groundnuts, was found to inhibit the growth of *A.flavus* in groundnuts. Sommartya *et al.* [46] showed that mixing *B. subtilis* with groundnuts could reduce the damage caused by *A. flavus*.

The inoculation of *A. flavus* spores into a culture of *Streptococcus lactis* in tryptone broth medium resulted in little or no aflatoxin accumulation even though the growth of the fungus was not hindered [47]. The drop in pH and reduced nutrient levels in the medium as a result of the *S. lactis* growth were not the cause of the observed inhibition. The inhibition was not eliminated by the addition of carbohydrate equal to the amount used by the bacterium before the inoculation with the fungus. Aflatoxin levels were also markedly reduced when *S. lactis* was inoculated into a growing *A. flavus* culture. In addition to inhibiting the synthesis of aflatoxin, *S. lactis* also degraded preformed toxin. *S. lactis* produced and excreted the inhibitor into the medium late in its growth phase. The inhibitor was a heat-stable low-molecular-weight compound. Wisman and Marth [45] also found that *S. lactis* had the ability to inhibit aflatoxin.

In the 1960s researchers screened over 1000 microorganisms for the ability to remove aflatoxin from solution [48]. Of these, bacterium *Flavobacterium aurantiacum* was found which could irreversibly remove aflatoxin from various foods. Many reports on this organism have appeared since then [49, 50, 51]. Line and Bracket [51, 52] demonstrated that the bacterium actually metabolized the toxin to water-soluble degradation products and CO₂. They concluded that the mechanism of detoxifying aflatoxin was mineralization.

Nontoxicogenic *A. flavus* and *A. parasiticus*

There is a great diversity of phenotypes of *A. flavus* in agriculture fields and the common occurrence of atoxicogenic strains [53, 54]. 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 atoxicogenic strains can be used to displace toxigenic strains [54, 55]. Cotty [56] tested the competitive ability of an atoxicogenic *A. flavus* strain to inhibit the aflatoxin contamination of developing cotton bolls. Competitive exclusion was found to contribute to the effect of the atoxicogenic 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 atoxicogenic strains of *A. flavus* is being developed for corn, cottonseed, peanuts, rice kernels and wheat seed [57, 58, 59]. Atoxicogenic isolate AF36 reduced aflatoxin B₁ content of bolls by 88 % to 99 % [60]. Boller and Schroeder [61] reported that *A. parasiticus* invaded stored rice rapidly but considerably smaller quantities of aflatoxins were produced when inoculated with *A. chevalieri* simultaneously at 100 % relative humidity. Reduction in aflatoxin B₁ ranged from 99 % at 25 °C, 00 % at 30 °C, to 95 % at 35 °C. No aflatoxins were detected at 85 % relative humidity. It could significantly reduce the number of sclerotia formed on locule surfaces and, in some cases, the percentage of seed containing sclerotia. Fermentation conditions of nontoxicogenic *A. flavus* for large-scale production have been investigated [62, 63]. Wheat seeds colonized with atoxicogenic *A. flavus* have been used in commercial trials [63].

***Trichoderma* spp.**

The filamentous fungus *Trichoderma* spp. has been investigated for more than twenty years for being a mycoparasite of plant pathogens and has been accepted as the most potent biological control agents for certain fungal plant diseases [64, 65]. Its mycoparasitism involves a complementary action of antibiosis, nutrient competition and cell wall degrading enzymes such as β -1,3-glucanases, proteases and chitinases. Since chitin is the major component of most fungal cell walls, a primary role has been attributed to chitinases in the biocontrol activity of *Trichoderma* [66, 67, 68]. Several hydrolases of *Trichoderma* were recently identified and purified. Some of genes encoding them were cloned and sequenced, and transformants were obtained which confirmed at overproduction of a single protease or chitinase resulted in better biocontrol agents. Although there have been numerous recent attempts to use *Trichoderma* spp. for experimental biological control, few researchers have studied the effect of *Trichoderma* spp. on *Aspergillus* species. Calistru and Mclean [69] reported that two isolates of *T. harzianum* and two of *T. viride* were capable of inhibiting the growth of *A. flavus*. A scan electron microscopical investigation of fungal interactions demonstrated no obvious hyphae penetration between *A. flavus* and *Trichoderma* spp., but the of morphological alteration of micro heads by *A. flavus* could be observed [69]. Their research showed that the culture filtrates of the *T. harzianum* and *T. viride* were inhibitory to *A. flavus* [70]. Another *T. viride* was found to inhibit production of aflatoxin B₁ (73.5 %) and aflatoxin G₁ (100 %) when cultured with *A. flavus* together [71].

Other biological control methods

Plant essential oil for controlling fungus in food preservation has been used for hundreds of years [72]. Morozumi isolated o-methoxycynamaldehyde from cinnamon and demonstrated this compound to be highly effective against *A. flavus* and *A. parasiticus* [73]. Montes-Belmont and Carvajal isolated 7 kinds of plant essential oils from 11 for protecting maize kernel against *A. flavus*. Cinnamon, peppermint, basil, origanum, the flavor herbs epazote, clove, and thyme caused total inhibition of fungal development on maize kernel and the optimal dosage varied from 3 % to 8 % [74].

Du *et al.* [75] used a green fluorescent protein (GFP) as a reporter to monitor gene expression and food colonization by *Aspergillus flavus*. These studies demonstrated that the

modified GFP encoded by pNuc'Em2 and pGAP33 was highly expressed in *A. flavus*. The intensity of fluorescence is sufficient to allow the visualization of a GFP-containing strain under a standard laboratory UV light. The use of these constructs in strains of *A. flavus* could facilitate the detection of the fungus in substrates such as soils or foods and be useful in screening corn genotypes for resistance to aflatoxin accumulation and making screening faster and more economical.

2. CONCLUSION

With the deeper understanding of the biosynthesis mechanism of aflatoxins, more biological control methods will spring up for eliminating aflatoxin contamination from animal feed and human food chains.

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