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Review:

Biological control of aflatoxin contamination of crops*

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Abstract: Aflatoxins produced primarily by two closely related fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, are mutagenic and carcinogenic in animals and humans. Of many approaches investigated to manage aflatoxin contamination, biological control method has shown great promise. Numerous organisms, including bacteria, yeasts and nontoxigenic fungal strains of *A. flavus* and *A. parasiticus*, have been tested for their ability in controlling aflatoxin contamination. Great successes in reducing aflatoxin contamination have been achieved by application of nontoxigenic strains of *A. flavus* and *A. parasiticus* in fields of cotton, peanut, maize and pistachio. The nontoxigenic strains applied to soil occupy the same niches as the natural occurring toxigenic strains. They, therefore, are capable of competing and displacing toxigenic strains. In this paper, we review recent development in biological control of aflatoxin contamination.

Key words: Aflatoxin, *Aspergillus*, Biocontrol, Food safety
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INTRODUCTION

Aflatoxin contamination of crops is a worldwide food safety concern. Aflatoxins refer to a group of four mycotoxins (B1, B2, G1 and G2) produced primarily by two closely related fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Strains of *A. flavus* show a great variation in their ability to produce aflatoxins. Toxigenic strains of *A. flavus* typically produce only two aflatoxins, B1 and B2, but most strains of *A. parasiticus* could produce all the four toxins (Dorner, 2004). Since aflatoxins are potential carcinogens, their quantity in food and feed is closely monitored and regulated in most countries. For example, the European Union has a maximum level of 2 μg/kg for B1 and 4 μg/kg for total aflatoxins in crops (van Egmond and Jonker, 2004).

Many strategies, including biological control, control of insect pest, development of resistant cultivar, have been investigated to manage aflatoxins in crops. Among them, biological control appears to be the most promising approach for control of aflatoxin in both pre- and post-harvested crops. Different organisms, including bacteria, yeasts and nontoxigenic *Aspergillus* fungi, have been tested for their ability in the control of aflatoxin contamination. This paper reviews recent development on this topic.

POTENTIAL BIOCONTROL AGENTS USED FOR MANAGEMENT OF AFLATOXIN CONTAMINATION

Bacteria

Several bacterial species, such as *Bacillus subtilis*, *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. Palumbo *et al*.(2006) reported that a number of *Bacillus*, *Pseudomonas*, *Ralstonia* and *Burkholderia* strains isolated from California almond samples could completely inhibit *A. flavus* growth. Several strains of *B.*

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subtilis and *P. solanacearum* isolated from the non-rhizophere of maize soil were also able to inhibit aflatoxin accumulation (Nesci *et al*., 2005). In most cases, although these strains were highly effective against aflatoxin production and fungal growth under laboratory conditions, they do not give good efficacies in fields because it is difficult to bring the bacterial cells to the *Aspergillus* infection sites on commodities under field conditions (Dorner, 2004).

Yeasts

Some saprophytic yeast species (such as *Candida krusei* and *Pichia anomala*) have shown promise as biocontrol agents against *A. flavus*. Similar to bacterial agents, these yeast strains were able to inhibit *Aspergillus* growth greatly in laboratory conditions (Hua *et al*., 1999; Masoud and Kaltoft, 2006). Although they were considered to be potential biocontrol agents for management of aflatoxins, further field experiments are necessary to test their efficacies in reducing aflatoxin contamination under field conditions.

Nontoxigenic *Aspergillus* strains

Greatest successes to date in biological control of aflatoxin contamination in both pre- and post-harvest crops have been achieved through application of competitive nontoxigenic strains of *A. flavus* and/or *A. parasiticus*. In many field experiments, particularly with peanut and cotton, significant reductions in aflatoxin contamination in the range of 70%~90% have been observed consistently by the use of nontoxigenic *Aspergillus* strains (Dorner, 2004; Pitt and Hocking, 2006; Dorner, 2008). Recently, two products of nontoxigenic strains have received U.S. Environmental Protection Agency (EPA) registration as biopesticides to control aflatoxin contamination in cotton and peanuts in several states of USA (Dorner, 2004).

This strategy is 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. In the late 1980s, Cotty (1990) tested nontoxigenic *A. flavus* strains for their ability in reducing aflatoxin contamination of cottonseed. Results from greenhouse experiments showed that six of seven nontoxigenic strains significantly reduced the amount of aflatoxin produced by the toxigenic strains in cottonseed when they were co-inoculated with toxigenic strains, and that the strain AF36 was the most effective in reducing aflatoxin contamination (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. 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 aflatoxin concentrations were significantly reduced, compared to inoculation with the toxigenic strain alone (Brown *et al*., 1991).

Except for the strain AF36, other nontoxigenic strains of *A. flavus* and *A. parasiticus* have also shown effective in reducing aflatoxin contamination of crops. *A. flavus* NRRL21882, a natural strain isolated from peanut in Georgia, has been tested in fields for more than 10 years. Several field experiments have shown that this strain was very effective in controlling aflatoxin contamination in both pre- and post-harvest peanuts. For example, in 1999, peanuts in field plots were treated with nontoxigenic strains of *A. flavus* (NRRL21882) and *A. parasiticus* (NRRL21369) at 67 d after planting. At harvest, peanuts were contaminated with aflatoxins averaging 516.8 μg/kg in the untreated plots, but 54.1 μg/kg in the nontoxigenic treatments. After storage, aflatoxins in non-field-treated peanuts averaged 9145.1 μg/kg compared with 374.2 μg/kg for that in field-treated peanuts. These results indicate that field application of the nontoxigenic strains had a carry-over effect and reduced aflatoxin contamination that occurred in storage (Dorner and Cole, 2002). Recently, a commercial biopesticide product (called afla-guard) has been developed based on the *A. flavus* strain NRRL21882. This strain is the active ingredient in an EPA-registered biopesticide afla-guard. Additionally, the nontoxigenic *A. flavus* strains CT3 and K49 have been tested in the USA and showed good efficacies in reduction of aflatoxin contamination in corn (Abbas *et al*., 2006).

Since applications of nontoxigenic *Aspergillus* strains have shown a great success in controlling aflatoxin contamination in the USA, similar studies were also conducted in several other countries. In Africa, nontoxigenic strain BN30 was very effective in reducing the amount of toxin produced in maize when co-inoculated with the highly toxigenic S-strain (Cardwell and Henry, 2004). In Australia, application of nontoxigenic strains could reduce aflatoxin formation in peanuts by 95% (Pitt and Hocking, 2006). In China, we have recently screened one highly competitive strain AF051 from more than 30 nontoxigenic strains of *A. flavus*. Field tests showed that this strain reduced naturally *Aspergillus* populations by up to 99% in the soil of peanut fields. These results indicate that applications of nontoxigenic strains could be used in different agro-ecozones for the control of aflatoxin contamination.

FACTORS AFFECTING NONTOXIGENIC *ASPERGILLUS* SPP. IN REDUCING AFLATOXIN CONTAMINATION

Formulation

The formulation is the combination of competitive strain and carrier/substrate. In initial studies with peanuts, although direct application of suspension of homogenized culture of nontoxigenic *A. parasiticus* to emerged plants or directly to the soil surface prior to planting was very effective in significant reduction of aflatoxin contamination, it was too expensive to apply this formulation for large-scale fields (Dorner *et al*., 1992). Later, solid-substrates, such as a small grain, wheat and rice, were used to produce the biocontrol formulation. In this process, after the grains were sterilized and inoculated with a conidial suspension of the nontoxigenic strain, they were incubated with agitation in order to prevent clumping and inhibit fungal sporulation. After the incubation was completed, the grains were dried at 50 °C, and then stored at 5 °C until use (Dorner, 2004). When the fermented grains are applied to the field, the nontoxigenic strains resume growth and produce numerous conidia on the surface of the grains. Those conidia are then dispersed in the soil and compete with naturally toxigenic strains. Development of a dominant population of the competitive nontoxigenic strain at the time when the crop is susceptible to be infected by *Aspergillus* spp. is critical for reducing aflatoxin contamination.

Inoculum rate

Inoculum rate is one of the important factors influencing the effectiveness of biocontrol agents. Several experiments have been conducted for determination of effects of inoculum rate of biocontrol agents on reduction of aflatoxin contamination in pre- and post-harvest peanuts. In the USA, when nontoxigenic *A. flavus* strain NRRL21368 and *A. parasiticus* strain NRRL6111 were applied at different rates in a peanut field in 1994, aflatoxin concentrations in total kernels were 337.6, 73.7, 34.8 and 33.3 μg/kg for the 0, 2, 10 and 50 g/m row treatments, respectively. For the same repeated treatments in 1995, aflatoxin concentrations in total kernels averaged 718.3, 184.4, 35.9 and 0.4 μg/kg. Compared with untreated controls, the 2, 10 and 50 g/m row treatments produced aflatoxin reduction by 74.3%, 95.0% and 99.9% (Dorner *et al*., 1998). The data indicate that there was a strong relationship between inoculum rate and effectiveness of biocontrol agent in reducing aflatoxin contamination. Additionally, a higher degree of control might be achieved when plots or fields were retreated with biocontrol agents in subsequent years. Similar results were also been obtained in Australia (Pitt and Hocking, 2006).

Optional time for application of nontoxigenic strain

Soil temperature can affect the growth and sporulation of the nontoxigenic fungus significantly. *A. flavus* germinates at temperatures below 10 °C on medium in the laboratory, but field experiments showed that establishment of biocontrol strains did not occur readily when soil temperature below 20 °C (Pitt and Hocking, 2006). The results indicate that application of nontoxigenic strains to soil should be delayed until soil temperature reaches at least 20 °C. In Arizona, USA, later April and early June are the suitable time for application of the nontoxigenic biocontrol agents. For most studies conducted in Georgia, the biocontrol agent NRRL21882 was applied between 50~70 d after planting of peanuts (Dorner *et al*., 1992; 1998; Dorner, 2004). It needs to point out that since the variation in environmental

conditions (including populations of *Aspergillus* spp. in soil) and in crop cultivars, the best time for application of nontoxigenic strains has to be optimized in each region.

Herbicide application

Herbicides are frequently applied in the fields where nontoxigenic strains are used. Therefore, the application of herbicides may adversely affect the growth of the nontoxigenic *Aspergillus* strains. Laboratory tests showed that in 9 d of inoculation, herbicides paraquat and trifluralin did not inhibit *Aspergillus* growth until the concentration reached to 5 times the recommended level. Sixteen days after the fungus was grown in the Czapek Yeast Extract agar amended with herbicide at up 10 times recommended concentration, the herbicides did not show obvious inhibition against *Aspergillus* growth (Pitt and Hocking, 2006). The results suggest that the use of pre-emergent herbicides did not have a long term effect on the biocontrol fungi. Interactions between herbicides and the nontoxigenic strain AF36 were also investigated recently. Garber and Cotty (2006) reported that spore production of AF36 was reduced significantly when AF36 product was exposed to six herbicides, Buctril, Bueno, Caparol, Gramoxone, Prowl and Roundup, at the recommended use rates, which indicated that nontoxigenic strains should be applied after all herbicide applications have completed.

MOLECULAR MECHANISMS OF THE LOSS OF AFLATOXIN PRODUCTION IN NONTOXIGENIC STRAINS

A. flavus and *A. parasiticus* have complex pathways in biosynthesis of aflatoxins. Enzymes and regulatory proteins for aflatoxin synthesis in these two fungi are encoded by more than 25 clustered genes in a 70-kb region (Yu *et al*., 2004; Ehrlich *et al*., 2005). Among these genes, *hexA*, *hexB* and *pksA* are larger than 5 kb, and encode fatty acid synthase (FAS) alpha (5.8 kb) subunit, FAS beta (5.1 kb) subunit, and polyketide synthase (6.6 kb), respectively. Except for these three genes, the average size of the other 22 genes is approximately 2 kb. At the 5′-end of this gene cluster, an approximate 2-kb DNA region without identifiable open reading frame (ORF) was located. This sequence presumably marks the end of this cluster. The 3′-end of this gene cluster is delineated with a well-defined sugar utilization gene cluster consisting of four genes (*hadA*, *hxtA*, *gleA* and *sugR*) (Yu *et al*., 2004; Ehrlich *et al*., 2005).

Aflatoxin biosynthesis can be affected by various genetic and environmental factors. A positive regulatory gene, *aflR*, encoding a sequence-specific zincfinger DNA-binding protein, is required for transcriptional activation of most, if not all, of the aflatoxin structural genes (Bhatnagar *et al*., 2006). A second regulatory gene *aflJ*, adjacent to the *aflR* gene, has been shown to be associated with expression of *pksA*, *nor1*, *ver1* and *omtA* in the aflatoxin cluster (Chang, 2003). Additionally, chromosomal location and some global transcription factors (such as those mediating nitrogen, carbon and pH regulation) also affect expression of aflatoxin structural genes. But exact functions of these factors are not clear at this time. Excluding these genetic factors, carbon and nitrogen sources, pH, temperature, water activity and plant metabolites also influence aflatoxin synthesis significantly. Effects of these environmental factors on aflatoxin biosynthesis have been well discussed in several other literature reviews (Payne and Brown, 1998; Ehrlich *et al*., 2005; Bhatnagar *et al*., 2006).

Although aflatoxins may be involved in the processes of fungal development and in the protection of *Aspergillus* fungi against soil microbial or insect competitors, they do not appear to be essential for the *Aspergillus* fungal growth and survival, and nontoxigenic strains are equally able to invade susceptible crop species (Cotty, 1989; Ehrlich *et al*., 2005). Thus, some of these nontoxigenic strains can competitively exclude aflatoxin producers from plant tissues, subsequently reducing aflatoxin contamination.

The molecular mechanisms responsible for the loss of aflatoxin production in *Aspergillus* spp. have been investigated extensively. DNA sequence analysis of aflatoxin synthesis gene cluster showed that many nontoxigenic strains had point mutation or deletion in the aflatoxin gene cluster. In the biocontrol agent AF36, the G at nt591 in the polyketide synthase gene in toxigenic strains was replaced by an A in the AF36, which is predicted to introduce a stop codon at the amino acid position 176 in this gene. This nu-

cleotide change introduces a premature stop codon into the coding sequence, thereby preventing enzyme production and aflatoxin accumulation (Ehrlich and Cotty, 2004).

There is a different scenario for the biocontrol agent NRRL21882. This strain has a deletion of the entire aflatoxin gene cluster from the *hexA* coding region in the sugar utilization gene cluster to the telomeric region (Chang *et al*., 2005). Analysis of DNA sequence of aflatoxin gene cluster for 38 nontoxigenic strains of *A. flavus* showed that deletions in the aflatoxin gene cluster among *A. flavus* strains are very common. The 38 nontoxigenic strains had 8 different deletion patterns in the gene cluster (Chang *et al*., 2005). Recently, we have found two new deletion patterns in the nontoxigenic strains of *A. flavus* collected from China (Yin *et al*., 2008). These results indicate that the deletion patterns in aflatoxin gene cluster appear to be diverse in nontoxigenic *Aspergillus* strains.

More recently, when analyzing 134 nontoxigenic strains of *A. flavus* using quadruplex polymerase chain reaction (PCR)-based assay, Criseo *et al*.(2008) found that 36.5% of the strains showed DNA fragments that correspond to the complete set of genes as found in toxigenic *A. flavus*. The results indicate that some nontoxigenic *A. flavus* strains may have the complete aflatoxin gene cluster. The nontoxigenicity might be due to defects on various molecular levels such as post transcriptional level and/or protein level, but the exact mechanisms are unknown yet.

CONCLUSION

Many organisms have been investigated for their potentials in the reduction of aflatoxin contamination of crops. The most successful biological control approach to date is the application of nontoxigenic strains of *A. flavus* and *A. parasiticus* to soils where they competitively exclude naturally toxigenic strains. For competitive exclusion to be effective, the nontoxigenic strains must be predominant in the agricultural environments when the crops are susceptible to be infected by the toxigenic strains. Three primary factors, therefore, affect the effectiveness of this strategy. First, the biocontrol strains must be truly competitive against the toxigenic strains in soil. Second, the formulation of biocontrol strains must be able to delivery and disperse conidia of biocontrol agents effectively in soil. Finally, the suitable timing of application of biocontrol agents is crucial for ensuring that the nontoxigenic strains reach to high population levels when the threat of crop infection is the greatest.

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