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A Review on Biological Control of Aflatoxin Crop Contamination

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ABSTRACT

Aflatoxins are mycotoxins with highly toxic and carcinogenic properties produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius.* These fungi are frequently found in foodstuffs and animal feeds. Aflatoxin contamination of crops compromises the safety of food and feed supplies and causes significant economic losses each year. Of the many research approaches being studied to reduce and, ultimately, eliminate aflatoxin contamination, biological control is one of the more promising, particularly for the near-term. Numerous organisms have been tested for biological control of aflatoxin contamination including bacteria, yeasts, and nontoxigenic strains of the causal organisms, *A. flavus* and *A. parasiticus*. Most of the field successes to date have been achieved by applying certain nontoxigenic strains of *A. flavus* and *A. parasiticus* to soil of susceptible crops, such as peanuts, cotton, and corn. The applied strains occupy the same niche as the naturally-occurring toxigenic strains and competitively exclude them when crops are susceptible to infection. Various formulations have been used to apply the nontoxigenic strains to soil, but the most effective methods have been to combine the desired strain with a carrier/substrate, such as a small grain. This was done either by minimally growing the desired strain on sterilized grain or by coating the surface of the grain with conidia of the strain. After application to the field and uptake of moisture, the fungus completely colonizes the grain, and abundant sporulation provides inoculum levels sufficient to achieve a competitive advantage for the nontoxigenic strain. In several years of field studies, particularly with peanuts and cotton, significant reductions in aflatoxin contamination in the range of 70-90% have been achieved consistently. In the present paper we propose biological control of aflatoxin crop contamination.

Keywords: Aflatoxin, *A. flavus*, *A. parasiticus* and Biological control.

INTRODUCTION

Mycotoxins are toxic metabolites produced by fungi. According to WHO, about 25% of world's food is contaminated by mycotoxins. Fungi belonging to *Aspergillus, Penicillium* and *Fusarium* species are responsible for causing mycotoxins of agro-economic importance. Aflatoxin, Ochratoxins, Trichothecenes, Zearalenone and Fumonisins are mycotoxins of greatest agricultural concerns. However, in recent years scientific investigations have revealed the wider economic, toxicological and public health importance of certain mould species. For example, it is now known that in some circumstances mould-infected foods can be associated with serious toxicity, and sometimes death.

The consumption of these mycotoxin-contaminated feedstuffs by animals leads to adverse effect on animal health and the effects are more serious in monogastric animals depending on the species and the susceptibility to toxins within the species. Ruminants, however, are considered generally more resistant to adverse effects of mycotoxins. This assumption is based on the findings that rumen microbiota has the biotransformation ability of mycotoxins to less toxic or non toxic metabolites. However, this is not applicable to all mycotoxins and the impact of mycotoxins in ruminant animals also depends upon age, breed, sex, dose level and immune status of individual animal. The carryover of toxins from animal food may have severe consequences on human health.

Mycotoxicosis is difficult to diagnose, because few signs of poisoning is produced. The impact of mycotoxins upon animals extends beyond their obvious effect in producing death in a wide variety of animals. The economic impact of lowered productivity, reduced weight gain, reduced feed efficiency, damage to body organs, interference in reproduction is many times greater than that of immediate mortality and morbidity. Potential threats of cancer induced by mycotoxins in feeds and human foods along with the unknown effects of these mycotoxins are coupled to the universal concern about health risk. Consumption of some mycotoxins at levels does not cause overt clinical mycotoxicosis but may suppress immune function and lower resistance to diseases. Thus, effective measures for detoxification of mycotoxins are essential for the improved production and productivity of livestock.

Aflatoxin contamination of crops compromises the safety of food and feed supplies and causes significant economic losses each year. Aflatoxin contamination can occur when crops are infected with aflatoxin-producing strains of *A. flavus* and *A. parasiticus,* molds that are relatively abundant in agricultural soils. One promising strategy to prevent

aflatoxin contamination is biological control that is achieved by applying a strain of *A. flavus* to soil that cannot produce aflatoxin. When a competitive, nontoxigenic strain of *A.flavus* is applied to soil, it competitively excludes the toxigenic strains and preferentially infects the susceptible crop, such as peanuts. But even though the crop is infected, aflatoxin contamination does not result because the infecting strain cannot produce the toxin. Various formulations have been used to apply the nontoxigenic strain to soil, but the most cost-effective is a small grain, such as barley, coated with spores of the nontoxigenic strain. One such product has been given the trade name afla-guard(R), and it is being marketed commercially for use on peanuts. Another product, *A. flavus* AF-36, is made by fermentation of the nontoxigenic strain on wheat, and it has been approved for use on cotton. Use of these products has produced significant reductions in aflatoxin contamination in peanuts and cottonseed.

This paper focuses on different mycotoxins of agricultural importance, significant reductions in aflatoxin contamination particularly with peanuts and cotton. A method for biological control of aflatoxin contamination of crops has been developed that is based on competitive exclusion. A large population of a nonaflatoxigenic strain of *A. flavus* and/or *A. parasiticus* is established in the soil where they compete with aflatoxigenic strains that are naturally present when crops become susceptible to invasion. In field plot experiments, application of various nonaflatoxigenic isolates of *A.flavus* and *A. parasiticus* to soil has effectively reduced aflatoxin concentrations in peanuts, cottonseed, and corn. In initial peanut studies, efficacy was demonstrated with a nonaflatoxigenic strain of *A. parasiticus* (NRRL 13539), which was applied to soil as a suspension of homogenized liquid cultures. Although the combination of species has been effective for biocontrol of aflatoxin contamination, it would be preferable to use only one isolate in a commercial biocontrol formulation. Therefore, the objective of the current study was to determine the efficacy of nontoxigenic strains of *A. flavus* and *A. parasiticus* applied separately and in combination on aflatoxin contamination of peanuts.

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 [1] 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. subtilis* and *P. solanacearum* isolated from the non-rhizophere of maize soil were also able to inhibit aflatoxin accumulation [2]. 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 [3].

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 [4,5]. 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 [3, 6, 7]. 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 [3].

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 [8] 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 [9]. 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 [10]. 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 [11]. 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 [12]. 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 [13]. In Australia, application of nontoxigenic strains could reduce aflatoxin formation in peanuts by 95% [14]. 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 [15]. 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 [3]. 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% [16]. 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 [14].

Optional time for application of non toxigenic 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 [14]. 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 [15, 16, 3]. 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 days 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 [14]. 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), [17] reported that spore production of AF36 was reduced significantly when AF36 product was exposed to sixherbicides, 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.

CONCLUSIONS

Of the many research approaches being studied to reduce and, ultimately, eliminate aflatoxin contamination, biological control is one of the more promising, particularly for the near-term. In field plot experiments, application of various nonaflatoxigenic isolates of *A.flavus* and *A. parasiticus* to soil has effectively reduced aflatoxin concentrations in peanuts, cottonseed, and corn. The combination of species has been effective for biocontrol of aflatoxin contamination, it would be preferable to use only one isolate in a commercial biocontrol 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. 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. The data indicate that there was a strong relationship between inoculum rate and effectiveness of biocontrol agent in reducing aflatoxin contamination. The results indicate that application of nontoxigenic strains to soil should be delayed until soil temperature reaches at least 20°C. The application of herbicides may adversely affect the growth of the nontoxigenic *Aspergillus* strains. Laboratory tests showed that in 9 days of inoculation, herbicides paraquat and trifluralin did not inhibit *Aspergillus* growth until the concentration reached to 5 times the recommended level.

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