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Biocontrol of Aflatoxins in Corn using Atoxigenic *Aspergillus flavus:* Review

Chepsergon Jane[1], E. K. Kiprop[2], Dr. L. A. Mwamburi[3]

[1]School of Science, P.O. Box 1125-30100, Eldoret- Kenya

[2]Professor, University of Eldoret, P.O. Box 1125-30100, Eldoret- Kenya

[3]University of Eldoret, P.O. Box 1125-30100, Eldoret- Kenya

Abstract: ***Mycotoxins are low-molecular-weight natural products formed as secondary metabolites by filamentous fungi. While aflatoxins are a group of four mycotoxins (B1, B2, G1 and G2)) that are primarily produced by two closely related fungi,* Aspergillus flavus *and* Aspergillus parasitica. *Since these toxins are mutagenic and carcinogenic, over 100 countries have imposed regulations for levels of aflatoxin both in feeds and foods. Physical and biological factors affect the production of aflatoxins in both pre- and post-harvest corn. Corn is a major crop associated with high economic risks and considerable safety concerns for aflatoxin contamination yet, it is one of the most utilized cereals in the world. Several control methods of aflatoxin production in corn have been devised. However, most of these methods are expensive and not environmentally friendly. Therefore, there is interest in developing a biological control method that has been characterized as; effective, environmentally friendly, cost- effective and innovative means of reducing aflatoxins levels in crops. Utmost achievements to date in biological control of aflatoxin contamination have been attained through the use of competitive nontoxigenic strains of AspergiIlus fungi. The working principle is that when the atoxigenic AspergiIlus fungi is introduced at the right time, a shift of strain profile from toxigenic to atoxigenic will take place. Moreover, atoxigenic strains competitively exclude the toxigenic strains in the field, which gives a carryover advantage in storage. Microbiological procedures, DNA, and field-based methodologies are the powerful techniques used in selecting atoxigenic strains from the environment. Efficacy of these atoxigenic A. flavus strains (AF36, K49, NRRL 21882, La3279, F3W4 and K54 have been reported with highest reduction of aflatoxins being 99.3%. Inoculation method, inoculum rate and Optional time for application of nontoxigenic strain are factors affecting the efficacy of atoxigenic Aspergillus flavus. Molecular studies reveal that the loss of aflatoxin production by atoxigenic Aspergillus strains is via mutation. Global climate change has being reported has a major challenge in utilizing Aspergillus flavus as a biocontrol agent in aflatoxin production in corn.***

Keywords: Aflatoxins, atoxigenic-*Aspergillus flavus*, corn and biocontrol

1. Introduction

Forgacs (1962) coined the term mycotoxins in the aftermath of an uncommon poultry catastrophe in England, during which approximately 100,000 turkey poults died. Thereafter, this mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* (aflatoxins).

It is difficult to define mycotoxins in a few words. Entirely mycotoxins are low-molecular-weight natural products formed as secondary metabolites by filamentous fungi. These metabolites institute a toxigenically and chemically assorted assemblage that is grouped together since they commonly cause disease and death in human beings and other vertebrates. Reddy et al. (2009) clearly defined the term mycotoxins as secondary metabolites naturally produced by molds (*Aspergillus, Fusarium* and *Penicillium*s pp.) that may contaminate agricultural commodities when environmental conditions are favorable. For these metabolites to be produced, fungi first gain entry into crops, synthesize the toxins which will be transmitted to the final food products. The contamination can occur in the field due to abiotic stress like drought or biotic stress like insect infestation or even in the store due to poor storage conditions.
On the other hand, aflatoxins are a group of four mycotoxins (B1, B2, G1 and G2)) that are primarily produced by two closely related fungi, Aspergillus flavus and Aspergillus parasiticus. Aflatoxins have been involved, to some extent, in main liver cancer in humans. Besides, they have been associated with hepatocellular carcinoma, acute hepatitis, Reye's syndrome and cirrhosis in malnourished children (Huwig et al., 2001). Several incidences of aflatoxicosis in humans have been reported in many countries including Southeast Asia and Africa. Furthermore, it is estimated that 4.5 billion people in the developing nations are chronically exposed to aflatoxins in their food, hence putting them at a risk of cancer related diseases (Li et al., 2001). As if that is not enough, Africa loses approximately US$450 through aflatoxins contaminated grain. United States federal guidelines for food and feed set a limit of 20 ng g^{-1} total aflatoxins, while the European Union guidelines are more, with a limit of 1.0 ng g^{-1} limit for B1 and 4.0-ng g^{-1} limit for total aflatoxins (van Egmond & Jonker, 2004).

2. Factors Affecting Aflatoxin Production

Aflatoxin production is determined by several factors. Normally, these factors have been classified into; physical and biological factors. Studies clearly show that, aflatoxins are produced between 25^0C – 28^0C temperature under acidic pH. Relative humidity between 83%-88% has been found to be appropriate and suitable level CO2 & O2 has also been reported to influence also the mold growth and aflatoxins production. (Bankoleand and Adebanjo, 2003). Research shows that 20% CO2 and 10% O2 in air reduce the aflatoxin

production. Present and absences of certain compounds and elements determine the production of aflatoxins. For example, Sugars like; glucose, sucrose or fructose, are the preferred carbon sources for aflatoxin production. Likewise, zinc and manganese are essential for aflatoxin biosynthesis. Nevertheless, according to a research done by Gilbert et al. (2002), a mixture of cadmium and iron lowers the mold growth and therefore aflatoxin production.

3. Why biocontrol of aflatoxins?

Aflatoxins have been reported to be mutagenic and carcinogenic in animals and humans. Dorner (2004) clearly elucidates that Toxigenic strains of *A. flavus* strictly produce two aflatoxins, B1 and B2, but then, most strains of *A. parasiticus* may perhaps produce all the four toxins (B1, B2, G1 and G2). Prior-harvest aflatoxin contamination has been recognized in crops such as maize for over 50 years. Corn is a major crop associated with high economic risks and considerable safety concerns for aflatoxin contamination (Robens and Cardwell, 2003).

Integrated aflatoxin management practices are recommended to reduce contamination in maize. Cotty(1991) demonstrates that aflatoxin contamination on crops can be reduced through; prevention of insect damage, harvesting crops early and practicing proper storage practices. Nevertheless, under proper management practices, unacceptable levels have been reported. This can be due to unpreventable damages to the crop, exposure of mature crops to moisture either in the before harvest, or during store, handling, or even transportation.

Development of resistant corn to aflatoxins contamination through breeding and transgenic has been investigated in the past. However, by the year 2008, commercially beneficial resistant crops had not been established (Brown et al., 2013). Chemical methods and fumigation have been employed. Although, they may pose unwanted health, safety and environmental risks, apart from not being economical and effective. It is worth mentioning that, aflatoxins can be eliminated from foods through detoxification, however, it is not a commonly used method because it is highly costly with complex procedures (Shetty and Jespersen, 2006). In that connection, there is interest in developing a biological control method that has been characterized as; effective, environmentally friendly, cost- effective and innovative means of reducing aflatoxins levels in crops. Several organisms like; bacteria, yeasts and nontoxigenic *Aspergillus* fungi, have been established for their capability in the control of aflatoxin contamination. However, utmost achievements to date in biological control of aflatoxin contamination in both pre- and post-harvest crops have been attained through the use of competitive nontoxigenic strains of *A*spergillus fungi. This paper therefore, reviews recent development on the use of *Aspergillus flavus* in controlling aflatoxin contamination in corn.

A*toxigenic A. flavus* as potential biocontrol agents for management of aflatoxin contamination in corn.

AspergiIlus spp. occur in nature in multifaceted communities consisting of varied genetic groups called vegetative compatibility groups, which differ in their capacity to produce aflatoxins, in that, some produce variable amounts of toxins and they are called toxigenic strains whereas others produce no toxins hence atoxigenic or nontoxigenic strains. Different locations host communities that differ in composition hence difference in aflatoxin-producing potential. Modifying the structures of fungal communities to favor the growth of atoxigenic strains can result in drastic reduction of aflatoxins because the chief causal agent of contamination is has been reduced (Mehl et al., 2012).

Aspergillus spp. as effective biocontrol agents operate under the following principles; When application of selected atoxigenic strains at suitable stages in crop development (just before local *Aspergillus* populations begin to increase), then the community configuration within the production area will shift from aflatoxin producers dominated area to one in which beneficial atoxigenic strains dictate. This leads to reduced crop aflatoxin contamination. This has been termed as shift of strain profile from toxigenic to atoxigenic. Also, atoxigenic strains competitively exclude the toxigenic strains in the field, which gives a carryover advantage in storage. Now that, there are fewer toxigenic strains during storage and also the atoxigenic strains dwell in the crop and continue to offer protection until they are used.

The atoxigenic strains are selected from the environment through a powerful process like; microbiological procedures, DNA, and field-based methodologies to ensure that they are environmentally friendly and improved to provide effective, long-lasting, and area-wide reductions in aflatoxins (Mehl et al., 2012).

4. Advantages of Biocontrol of Aflatoxins Using Atoxigenic *A. flavus*

Generally, biocontrol methods have been characterized as; effective, environmentally friendly, cost- effective and innovative means of reducing aflatoxins levels in crops. In addition to that, modifications to fungal communities caused by application of biocontrol strains carry over through the value chain, preventing contamination in storage and transport even when conditions favor fungal growth. Biocontrol is a simple invasion in the field that by itself noticeably reduces aflatoxin contamination in crops from harvest until use. Moreover, since fungi can spread, as the safety of fungal communities within treated fields improves, hence the safety of fungal communities in areas neighboring treated fields (Bandyopadhyay et al., 2005)

Positive influences of atoxigenic strain applications carry over between crops provide benefits to plants for several years. That is, a single use of atoxigenic strains may benefit not only the treated crop but also rotation crops and second season crops that miss a treatment (Bandyopadhyay et al., 2005).

5. Criteria for Selection of Atoxigenic *A. flavus* for Aflatoxin Biocontrol

Studies show that *Aspergillus spp.* can be isolated from air-borne dust particles, insects, plants and soil. Both toxigenic and non-toxigenic strains co-exist in all the above mentioned environments. The ability of atoxigenic Aspergillus strains to compete successfully for the same ecological niche offers the heart for biological control.

Various approaches have been devised to characterize different *Aspergillus spp.* strains in the exploration for non-aflatoxigenic strains appropriate for use in biocontrol. Some methods are founded on phylogenetics, while others are based on phenotypes for example sclerotium size. According to Horn (2003), those strains with small sclerotia (<400 µm in diameter) are linked with toxin production. Whereas those producing large sclerotia (>400 µm) may or may not be atoxigenic. Molecular approaches established on DNA sequences have greatly thrived and can expose phylogenetics relationships between isolates. PCR – based and pyro sequencing approaches have so far employed (Das et al., 2008). Further, cultural traits revealing non- production of aflatoxins and also, the presences or absences of toxin biosynthetic genes have been employed search for biocontrol agents. Abbas (2004) revealed the isolation of several atoxigenic strains of *A. flavus.* These strains comprised of; K49 (= NRRL 30797, isolated from corn), F3W4 (= NRRL 30796, isolated from soil), in addition to other four (NRRL 58976, NRRL 58988, NRRL 58975 and NRRL 58974) *A. flavus* strains from various environmental. All strains were characterized with respect to toxin production in culture and various cultural characteristics such as growth rate, fluorescence and pigmentation.

6. Efficacy of *A. flavus* as a biocontrol agent of aflatoxins production

Wu et al. (2013) reports that,over 100 countries have imposed regulations for levels of aflatoxin both in feeds and foods. The levels are so low such that they affect the intended grains for export. Both laboratory and field experiments have been carried out in testing the effectiveness of atoxigenic *A. flavus* as a biocontrol agent of aflatoxins. The principal aim of developing biocontrol strategies is to lessen mycotoxins contamination in crops, precisely corn. Globally, the use of atoxigenic *Aspergillus spp*. has been investigated. Most studies carried out in the US, have demonstrated the abilities of atoxigenic Aspergillus flavus strains (NRRL 21882 (Afla-Guard®), AF36, and K49) to decrease aflatoxin contamination in commercial corn production (Dorner et al., 2000). A current report by Abbas et al. (2011) demonstrated a comparison of the capabilities of NRRL 21882, AF36, and K49 to reduce aflatoxins in corn tested with equal numbers of conidia of toxigenic *A. flavus* strains (F3W4 and K54). , aflatoxins were finally reduced by 83 and 98% by K49 and NRRL 21882, respectively, while AF36 was able to reduced aflatoxins by 20%.

In West Africa, precisely Nigeria, *A. flavus* strain La3279 was found to be the most effective atoxigenic isolate in reducing aflatoxin contamination both in laboratory tests and during the two-year field study with an average aflatoxin reduction of>99.3% (Atehnkeng et al.,2008). In an earlier study by Brown et al. (1991), an atoxigenic isolate reduced aflatoxin by 80–95% in co-inoculated ears compared with ears inoculated with an aflatoxin producer alone. In a recent similar study, Abbas et al. (2006) reported reductions of 65–95%.Drought and high temperatures after silking generally enhance the potential for aflatoxin contamination in maize (Payne, 1992). Most of the examined atoxigenic isolates from West Africa achieved over 90% reductions in contamination.

7. Factors Affecting the Efficacy of this Aflatoxins Biocontrol Agent

7.1 Inoculation Method

Soil application of non-toxigenic *A. flavus* in maize field has been widely used. Lyn et al. (2009) reported that, soil application of non-toxigenic *A. flavus* K49 strain reduced levels of aflatoxin by 65%. However, direct application to corn ears was more effective. A sprayable system called “clay-based water-dispersible granules” has been developed to spray *A. flavus* directly on corn silks. Spray treatment reduced aflatoxin contamination by 97%.

7.2 Inoculum rate

Inoculum rate has been documented as one of the most vital factors affecting the efficacy of biocontrol agents. Studies that have been done clear elucidate that there exist a robust association between inoculum rate and effectiveness of biocontrol agent in decreasing the levels of aflatoxin. Several try-outs have been conducted to demonstrate the effects of inoculum rate of biocontrol agents on aflatoxin reduction in pre- and post-harvest maize. In an earlier research carried out in USA, nontoxigenic *A. flavus* strain NRRL21368 was applied at varying rates in a maize field in 1994, and the concentrations of aflatoxins in total kernels were shown to be; 337.6, 73.7, 34.8 and 33.3 µg/kg for the 0, 2, 10 and 50 g/m row treatments, correspondingly. In the following year (1995), the same try out was repeated and aflatoxin concentrations in total kernels averaged 718.3, 184.4, 35.9 and 0.4 µg/kg, representing, 74.3%, 95.0% and 99.9% aflatoxin reduction. (Dorner et al., 2000). It is worth mentioning that, when plots or fields are retreated with biocontrol agents in subsequent years, a higher degree of control might be attained. The same experiment done in Australia, Pitt and Hocking (2006) obtained similar results.

7.3 Optional time for application of nontoxigenic strain

Research shows that the prime time for the biocontrol agent application depends on the prevailing environmental conditions. Soil temperature has been reported as a major factor, affecting the growth and sporulation the nontoxigenic fungus. Pitt and Hocking (2006) indicated that *A.* flavus grows best at temperatures below 10 °C under laboratory conditions on the other hand, field experiments showed no growth when soil temperature was below 20 °C. Therefore,

application of nontoxigenic strains to soil should be delayed till when the conditions are favourable.

7.4 Ways that incapacitate aflatoxin production in nontoxigenic strains

A. flavus and *A. parasiticus* produce aflatoxins since they have complex pathways for biosynthesis these toxins. Ehrlich et al.(2005) clear indicates that the enzymes and regulatory proteins responsible for aflatoxin synthesis are represented by more than 25 clustered genes in a 70-kb region. Among these genes, *hexA*, *hexB* and have been studied widely and they are said to be larger than 5kb in size., *hexA* encodes for fatty acid synthase (FAS) alpha, *hexB* codes for FAS beta while *pksA* codes for polyketide synthase.

Intensive investigations on the molecular mechanisms accountable for the loss of aflatoxin production in *Aspergillus* spp. have been done. In a study done by Cotty (2006) analysis of aflatoxin synthesis gene cluster through DNA sequencing technique displayed point mutation or deletion in the aflatoxin gene cluster in several nontoxigenic strains. On the other hand, Chang et al. (2005), NRRL21882 strain of *A. flavus* had a deletion of the entire *hexA* gene. The study further demonstrated a common deletion pattern in aflatoxin gene cluster for 38 atoxigenic strains of *A. flavus*. However, Yin et al., (2008) discovered two novel deletion patterns in the atoxigenic strains of A. flavus found in China. Therefore, deletion patterns in aflatoxin gene cluster is said to be diverse amongst atoxigenic strains of *A. flavus.*

In a controversial polymerase chain reaction experiment done by Criseo et al. (2008), 36.5% of 134 atoxigenic strains of *A. flavus* exhibited DNA fragments that resemble the complete set of genes as those for toxigenic *A. flavus*, meaning that, some atoxigenic *A. flavus* could be having the complete aflatoxin gene cluster. Consequently, the atoxigenicity is suggested to be due defects at other molecular levels for example, post transcriptional level, however exact mechanisms are not known so far.

7.5 Global warming has a challenge in the utilization of atoxigenic *A. flavus* as aflatoxin biocontrol agent.

Global warming has an effect in biocontrol of aflatoxins. Maize-growing regions globally are experiencing increase in temperatures. Climate change has resulted to unpredictable weather difficulties like high heat and drought in areas practicing agriculture, yet aflatoxin contamination are more predominant under these environmental conditions (Reverberi et al., 2013)

Several Agricultural areas facing drought frequently suffer contamination (Samuel et al., 2013). Since 1901 the average global surface temperature has increased by 0.8 ^{o}C, with most of that upsurge happening for the last three decades. Therefore, it is predicted that by the end of the 21st century the conducing climate for aflatoxin contamination may include more of the maize-growing regions worldwide hence, outbreaks will become more common. Also, climate change can lead to increased plant damage. Alongside, changes in the soil environment and its microbiome due to increase in temperature may well also subject the crop to increased damage.

8. Conclusion

Globally, aflatoxins pose risks to corn, both in the field and in store. Biocontrol of aflatoxins using atoxigenic *A. flavus* has been the most used method worldwide. Its efficacy is dependent on; the method used to inoculate it, inoculum rate and finally the prime time for application of the agent. Up to 99.9% reduction of aflatoxin using atoxigenic *A. flavus* has been recorded. It is noted that, this biocontrol agent lack the ability to produce aflatoxins due to a mutation in the gene responsible for the toxin production. Competitive exclusion of toxigenic strains by the atoxigenic strains is reported to be the main principle for aflatoxin biocontrol. With increase in climate change cases, the efficacy of biocontrol of aflatoxins using atoxigenic *A. flavus* is reduced.

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