SYNOPSIS OF P.G. RESEARCH PROBLEM

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8. Introduction

The term mycotoxins coined by Forgacs (1962) in the aftermath of an uncommon poultry catastrophe in England, during which approximately 100,000 turkey pouls died. Thereafter, this mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites (aflatoxins) from Aspergillus flavus. Entirely mycotoxins are low-molecular-weight natural products formed as secondary metabolites by filamentous fungi. For these metabolites to be produced, fungi first gain entry into crops, synthesize the toxins which were transmitted to the final food products. The term aflatoxin is a short form derived from first letter of Aspergillus (a-) and first three letters of flavus (-fla) which makes afla- and toxin means mycotoxins (Jane et al., 2014).

Aflatoxins are polyketide secondary metabolites that are produced by fungus particularly Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius (Ellis et al., 1991; Pitt et al., 2000 and Dorner, 2004). Aflatoxins are among the most potent mutagenic, teratogenic and carcinogenic known substances. Among the most commonly found forms of aflatoxin (AFB1, AFB2, AFG1, and AFG2), AFB1 is the best-studied and the most dangerous one. The A. flavus produced AFB1 and AFB2 while A. parasiticus was responsible for AFB1, AFB2, AFG1 and AFG2 (Soso et al., 2014). The B and G nomenclature is based on blue and green colours produced under UV light on thin layer chromatography plates.
Aflatoxin contamination of crops is a serious food and feed safety issue worldwide and causes significant economic losses yearly. Because aflatoxins are potential liver toxins and carcinogens, the quantity of aflatoxins in food and feed is closely monitored and regulated in more than 100 countries. In most countries, the maximum tolerated levels for aflatoxin B1 in food range from 0 to 20 µg kg\(^{-1}\) (Egmond and Jonker, 2004).

The heavy application of insecticides, however, has been increasingly criticized from the point of environmental contamination concerns, as well as for being costly. Efficacies of agronomic practices can be negatively affected by many abiotic factors and environmental conditions (Cleveland et al., 2003).

In 1991, Cotty demonstrated 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 storage, handling, or even transportation. Breeding of resistant plant cultivars against fungal infection, the use of Bt corn and various agronomic practices, have been adopted to reduce aflatoxin contamination (Abbas et al., 2006). 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 but it is not 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 level in crops. Several organisms like; bacteria, yeasts and atoxigenic Aspergillus fungi, have been established for their capability in the control of aflatoxin contamination. However, greatest achievements to date in biological control of aflatoxin contamination in both pre- and post-harvest crops have been attained through the use of competitive atoxigenic strains of Aspergillus fungi (Dorner, 2004 and Jane et al., 2014).

When the highly competitive atoxigenic isolates are applied into crops, they produced large number of conidia, occupy the same niches as the naturally occurring toxigenic
populations, displace the toxigenic *Aspergillus* spp. and subsequently reduce aflatoxin contamination of crops. Many field experiments have shown that application of atoxigenic isolates of *A. flavus* or *A. parasiticus* in field could reduce by 90% or more of aflatoxin in cottonseed, and in pre- or postharvest peanuts (Dorner and Cole, 2002; Dorner, 2004; Jaime-Garcia and Cotty, 2007). To date, two products of atoxigenic isolates, AF36 and Aflaguard® have been approved by EPA (US Environmental Protection Agency) to be used in cotton and peanut fields for control of aflatoxin contamination in several American states (Dorner, 2004).

Development of biological control against toxigenic *Aspergillus* required screening and differentiating atoxigenic strain from toxigenic strain. For that, there are different means like chemical, morphological and microscopic methods that consumes time. Instead, molecular data differentiated the strain based on whole genome of *Aspergillus*. The comparative evaluation of whole genome sequences of atoxigenic and toxigenic *Aspergillus* species and aligning both genomes gave differences at molecular level. Based on genome differences, a specific PCR based markers are developed which quickly distinguish both is much easy and quick (Adhikari et al., 2016).

Polymerase chain reaction (PCR)-based, inter single sequence repeats (ISSR) analysis has been used successfully in the analysis of DNA relatedness of species of fungi, bacteria, plants and animals. SSR analysis of nuclear and organelle DNA can be used as molecular markers and has wide range applications in the field of genetics including kinship and population studies, represents genome region between microsatellite loci. Sequences amplified by ISSR-PCR can be used for delimiting species. Dendograms which evaluated the likeness between different isolates has also been used (Hatti et al., 2010).

### 9. Practical utility of the research work:

Biochemical, morphological and molecular characterization of *Aspergillus* species will provide the basic information and genetic diversity among aflatoxigenic and atoxigenic isolates of *Aspergillus* species. The development of molecular markers for molecular identification of toxigenic and atoxigenic *Aspergillus* species based on genome sequences will give insight to new molecular tools to identify the aflatoxigenic and atoxigenic *Aspergillus*. The genome sequencing of most efficient local aflatoxigenic and atoxigenic *Aspergillus* species will be done and genome data will be compared to analyze deletions within the aflatoxin biosynthetic gene cluster of atoxigenic isolates, so as to establish a basis for identification of atoxigenic *Aspergillus* spp. from aflatoxigenic.
10. Objectives:

1. Isolation and characterization of aflatoxigenic and atoxigenic *Aspergillus* species from groundnut seeds and various food stuff.

2. Screening of highly virulent/aflatoxigenic and atoxigenic *Aspergillus* based on aflatoxin production during infection on groundnut seeds.

3. Study *in vitro* antagonism of atoxigenic *Aspergillus* isolates against aflatoxigenic strain.

4. Genome sequencing of best aflatoxigenic and atoxigenic *Aspergillus* and functional annotation for genes related to aflatoxin biosynthesis pathways.

5. Development of molecular markers specific to aflatoxigenic and atoxigenic strain and its validation on different *Aspergillus* isolates.

11. Review of literature:

11.1. Isolation and characterization of aflatoxigenic and atoxigenic *Aspergillus* species

Saito and Machida (1999) developed a rapid method for identifying aflatoxin producing and non-producing strains of *A. flavus* and *A. parasiticus*. These *Aspergillus* grown on PDA media petriplate for 7 days at 28°C. After growth the dish was inverted and 1 or 2 drops of concentrated ammonium hydroxide solution were placed on the inside of the lid. The reverse side of aflatoxin producing colonies quickly turned plum-red or pink. Essentially color change was not occurred on the undersides of colonies that are not producing aflatoxins.

Apart from morphological differentiation of *A. flavus* from other fungus, PCR based real-time quantitative (RTq- PCR) assay was developed by Cruz and Buttner (2008). The forward and reverse primers Asp1S/ AflRS2 (Asp1S- ATG-CCT-GTC-CGA-GCG-T and AflRS2- TTA-AGT-TCA-GCG-GGT-ATR-CC, where R= A+G) were derived from the 5.8S rRNA and 28S rRNA sequences respectively. A dual labeled probe Aslp (6-FAM-CGC-TTG-CCG-AAC-GCA-AAT-CAA-TCT-T-TAMRA) with 6- FAM at the 5’-end and TAMRA at the 3’-end was used as the reporter molecule. The size of the resulting amplicon was not given. All nine *A. flavus* isolates were PCR positive. The primers did not amplify DNA extracted from 39 other fungal species derived from 15 genera. PCR identification of *A. flavus* isolates did not necessarily guarantee aflatoxin production.

Zrari (2013) studied detection of aflatoxin from some *Aspergillus* sp. isolated from Wheat seeds. For screening aflatoxin production on the basis of Ultraviolet fluorescence *Aspergillus* isolates were grown on CMA (Coconut media agar) as single colony in the centre of plate and incubated in the dark at 28 °C for 7 days. Ultraviolet light used as a rapid method
for the identification of aflatoxin-producing isolates. The aflatoxin-producing isolates appeared as florescence colonies in the UV light, whereas nonproducing isolates appeared as white colonies.

Hina et al. (2013) isolated Aspergillus from district Larkana Sindh Pakistan on two media, Czapek Solution Agar (CZA) and Malt Extract Agar (MEA) and studied macroscopic characteristics such as colony growth, conidial color, conidiophore, vesicle, matulae, phialides and conidia. All the eight Aspergillus species viz., Aspergillus ficcum, Aspergillus flavus, Aspergillus flavus var. columnaris, Aspergillus terreus var. aureus, Aspergillus fumigatus, Emericella nidulans, Emericella rugulosa and Apergillus terricola var. americana were reported for the first time from Larkana whereas, Aspergillus terricola var. americana appeared to be a new records from Pakistan.

Navya et al. (2014) used four culture media, Czapek’s-Dox agar (CZA), Potato dextrose agar (PDA), Yeast extract sucrose agar (YESA) and Aspergillus flavus and parasiticus agar (AFPA) to know the efficacy for the growth of A. flavus. Total 38 strains of aflatoxigenic and atoxigenic A. flavus were analyzed for their growth response on PDA at different range of pH and temperatures. The results indicated that pH 5.8 and temperature 30°C were found to be optimum for the growth. Further, extracellular enzyme activities were carried out for amylase, protease and lipase. They were observed in both aflatoxigenic and non-aflatoxigenic isolates of A. flavus and did not show significant variation in extracellular enzyme activities.

Moubasher et al. (2016) isolated eleven isolates of A. flavus and A. parasiticus from Egyptian soil and air. Three types of media were used in this study; Czapek's Dox (CD), potato dextrose agar (PDA) and glucose peptone yeast (GPY). Spore suspension (100 μl) of each isolate containing 10^7 spores/ml, prepared in 0.1% (vol/vol) Tween 20, was added to 100 ml of each tested medium in 250-ml Erlenmeyer flasks. After inoculation, the flasks were incubated on a rotary shaker at 26°C for 7 days for further study.

Al-Saad et al. (2016) isolated the strain of A. flavus (AFL14) from soil in Iraq and purified using Malt extract agar (MEA) incubated for 7 days at 25 °C. The fungus was morphologically identified according to Klich (2002) and morphological and microscopic traits were used to confirm the strain identification. This strain was maintained by regular sub-culturing on MEA at 25 °C for 7 days and then stored at 4 °C until required and as spore suspension in 10% glycerol at −80 °C.
11.2. Screening of highly virulent/aflatoxigenic and atoxigenic *Aspergillus*.

Davis *et al.* (1966) demonstrated production of aflatoxins B1 and G1 by *Aspergillus flavus* in a semisynthetic medium. Isolates of *Aspergillus flavus* produced 0.2 to 63 mg of aflatoxins B1 and G1 per 100 ml in a nutrient solution consisting of 20% sucrose and 2% yeast extract. Various factors influencing the fermentation were studied. The maximal amount of toxin was produced by ATCC culture 15548 in 1-liter flasks containing 100 ml of medium incubated as stationary cultures for 6 days at 25°C.

Harry and Hugo (1967) examined production or accumulation of aflatoxin in vitro by four isolates on three substrates (acid-delinted cotton seed, shelled Spanish peanut, and rough rice) in relation to temperature in the range of 10 °C to 40 °C. Within the first 10 days after inoculation, the optimal temperature range for aflatoxin production was between 20 °C and 35 °C. Only small amount of the toxins were produced at 10 °C and 40 °C. Within the optimal temperature range, the time required for toxin production and for significant accumulation decreased as the temperature increased. More aflatoxin G was produced or accumulated in relation to aflatoxin B at lower temperatures (within the optimal range), and the aflatoxins G were metabolized more rapidly at the higher temperatures.

Schindler *et al.* (1967) examined two aflatoxin-producing isolates of *Aspergillus flavus* by growing for 5 days on Wort media at 2, 7, 13, 18, 24, 29, 35, 41, 46, and 52°C. Maximal production of aflatoxins occurred at 24 °C. Maximal growth of *A. flavus* isolates occurred at 29° and 35°C. The ratio of the production of aflatoxin B1 to aflatoxin G1 varied with temperature. Aflatoxin production was not related to growth rate of *A. flavus*; one isolate at 41°C, at almost maximal growth of *A. flavus*, produced no aflatoxins. At 5 days, no aflatoxins were produced at temperatures lower than 18°C or higher than 35°C. Color of CHCl₃ extracts appeared to be directly correlated with aflatoxin concentrations. *A. flavus* isolates grown at 2, 7, and 41°C for 12 weeks produced no aflatoxins. At 13°C, both isolates produced aflatoxins in 3 weeks, and one isolate produced increasing amounts with time. The second isolate produced increasing amounts through 6 weeks, but at 12 weeks smaller amount of aflatoxins were recovered than at 6 weeks.

Cappiello *et al.* (1995) developed a new method for the analysis of aflatoxins in food extracts, based on liquid chromatography/mass spectrometry. The chromatographic separation was performed with a reversed phase packed capillary column coupled with a modified particle beam interface capable of handling microliter per minute flow rates. This system allows higher overall sensitivity and easier operation procedures. The method had proved to be particularly suitable for the analysis of the toxins in very complex matrices. The
specificity of electron impact ionization allowed positive identification of the aflatoxins with an excellent response linearity for accurate quantization.

Scherm et al. (2005) determined aflatoxin production based on fluorescence, all Aspergillus strains were cultivated in PDA at 25 °C for 7 days. A mycelium plug of each strain was placed at the centre of a Petri dish containing coconut agar medium (CAM): 100 g of shredded coconut was homogenized for 5 min with 300 ml of hot distilled water. The homogenate was filtered through four layers of cheesecloth, and the pH of the filtrate was adjusted to pH 7.0 using 2 N NaOH. Agar (20 g/l) was added and the mixture was autoclaved for 20 min at 121 °C. The plates were incubated at 25 °C for 5 days in the dark. The presence or absence of fluorescence in the agar surrounding the growing Aspergillus colonies was determined by exposing the Petri dishes to ultraviolet light (365 nm) and expressed as positive or negative (aflatoxigenic or atoxigenic).

Senyuva and Gilbert (2007) used LC/TOF-MS for rapid analysis of crude fungal extracts for secondary metabolites. He used well-characterized isolates of A. paraciticus (NRRL 2999), A. flavus, A. ochraceus, A. oryzae, A. niger, A. fumigates and P. citrinum. Fungi were inoculated onto malt extract agar (MEA), potato dextrose agar (PDA) and yeast extract sucrose agar (YES) in Petri dishes. A novel approach to study the production of secondary metabolites by fungi using LC/TOF-MS has been developed. Fungi grown on culture media for 7 to 14 days at 25 °C were solvent-extracted and directly analyzed by LC/TOF-MS. Searching against a database of 465 secondary metabolites, mycotoxins and other compounds of interest can be readily identified. They identified many secondary metabolites like Kojic acid, Cinnamic acid, 5-Methoxysterigmatocystin, Methylsterigmatocystin, Benzophenone, Aflatoxin B1, B2, G1, G2 etc.

Probst et al. (2011) indentified aflatoxigenic Aspergillus flavus isolates to reduce aflatoxin contamination of Maize in Kenya. They used A. flavus strains that were isolated during previous studies conducted by him when outbreak of an acute aflatoxicosis in Kenya in 2004 (Probst et al., 2007). Aflatoxin-producing ability of each of the A. flavus L strain isolates was determined using autoclaved maize kernels (10 g per 250-ml Erlenmeyer flask) and autoclaved at 121°C for 60 min and moisture content adjusted at 25 %. Each autoclaved maize sample was then inoculated with 1 ml of spore suspension (10^6 conidia/ml water) of the appropriate isolate and incubated for 7 days at 31°C in the dark. The methanolic extract (50 mL of 80% methanol per sample) from colonized maize samples were spotted directly onto thin-layer chromatography (TLC) plates adjacent to aflatoxin standards and the aflatoxins were visualized under 365 nm UV light as well as quantified directly on TLC.
plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc., Wilmington, NC). Isolates that produced levels of aflatoxins below the limit of detection (aflatoxin B1 at 0.5 ng/g) were considered atoxigenic and evaluated further as potential biocontrol agents.

Ghadeer and Khalaf (2012) isolated, characterized and purified aflatoxigenic Aspergillus spp. from local food items in Amman, Jordan to study the aflatoxins (AFs) production in synthetic and natural media in flasks and clay pots. The antifungal activity of garlic (Allium sativum) and black cumin (Nigella sativa) water extracts were also studied. Seven A. flavus and four A. parasiticus isolates were characterized as aflatoxigenic which were isolated from peanuts, wheat, wheat flour and corn samples. One isolate designated as P12 of Aspergillus flavus found to produce the highest aflatoxin B1 (AFB1) (450 µg.kg⁻¹) in potato dextrose agar (PDA). Among the 3 synthetic media used Potato dextrose agar (PDA), Sabauroud dextrose agar (SDA) and yeast extract sucrose agar (YES), the later was found to be most favorable for AFB1 production (625 µg.kg⁻¹). Among the 4 different natural media (crushed rice, wheat, peanut and corn) in glass flasks and in clay pots, AFB1 production was significantly higher in glass flasks (760, 710, 670 and 505 µg.kg⁻¹) compared to clay pots (645, 367.5, 440 and 287.5 µg.kg⁻¹), respectively. The minimum inhibitory concentration (MIC) of the growth of Aspergillus flavus (P12) by the aqueous extract of garlic and black cumin were 30 mg/ml and 70 mg/ml, respectively. The most favorable condition for AFs production were the natural media of crushed rice and synthetic media of YES in flask incubated at 28 ºC for 10 days.

Varga et al. (2014) postulated that comprehensive data on the occurrence of mycotoxins apart from the regulated compounds (Aflatoxins) is limited, especially in food matrices other than raw cereals. This is one reason why, in recent years, single mycotoxin methods have been increasingly replaced by LC/MS-based multitarget methods (Varga et al., 2013 and Varga et al., 2014). The harmonization of methods for different commodities, the identification of mycotoxins in unlikely matrices, and the increase in knowledge of emerging mycotoxins from Aspergillus, Penicillium, Fusarium, or Alternaria species are just a few reasons for this trend. These developments were aided by the increase in performance of modern LC/MS instruments over the last few years, and the development of software tools that enhance productivity. Modern high-resolution, accurate-mass LC/Q-TOF instruments can analyze a virtually unlimited number of contaminants (Kempe et al., 2013). They also allowed retrospective data analysis to find contaminants that were not considered at the time of the measurement (Wust et al., 2013).
Jane et al. (2014) proposed that aflatoxin production is determined by several factors. Normally, these factors have been classified into; physical and biological factors. Studies clearly showed that, aflatoxins were produced between 25 °C – 28 °C temperature under acidic pH. Relative humidity between 83% - 88% has been found to be appropriate and suitable level of CO₂ & O₂ has also been reported to influence the mold growth and aflatoxin production (Bankole and Adebanjo, 2003). Research showed that 20% CO₂ and 10% O₂ in air reduce the aflatoxin production. Presence and absence 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 and Anklam (2002), a mixture of cadmium and iron lowers the mold growth and therefore aflatoxin production.

Lamboni et al. (2016) studied diversity in secondary metabolites including mycotoxins from strains of Aspergillus section Nigri isolated from raw Cashew nuts from Benin, West Africa using UHPLC-QTOF-MS. To investigate the diversity of secondary metabolites, including mycotoxins, 150 strains were isolated from cashew samples and assayed for their production of secondary metabolites using liquid chromatography high resolution mass spectrometry (LC-HRMS). Seven species of black Aspergilli were isolated based on morphological and chemical identification: A. tubingensis (44%), A. niger (32%), A. brasiliensis (10%), A. carbonarius (8.7%), A. luchuensis (2.7%), A. aculeatus (2%) and A. aculeatinus (0.7%). The secondary metabolites identified were aurasperone and pyranonigrin-A, fumonisins (B2 and B4), ochratoxin A, secalonic acids etc. All of these metabolites were species specific. Thus, chemical profile clustering showed 5 groups confirming the species specific metabolites production.

Moubasher et al. (2016) extracted aflatoxin from A. flavus and A. parasiticus mycelial mat harvested by filtering through Whatman no. 1 filter paper. The filtrate was extracted twice with 100 ml chloroform. Pre-coated silica gel TLC plates (20x20) (Sigma) were used for the aflatoxin detection. Fifty microliters of each sample was spotted onto the TLC sheets. TLC was developed in toluene: ethyl acetate: acetic acid (50: 30: 4) solvent system. Authentic samples of aflatoxins (Sigma) were used as standard. Aflatoxins were visualized under a UV lamp at 365 nm and their presence was confirmed by spraying 50% sulphuric acid on the developed plates which reacts with the blue and green fluorescent aflatoxins to give yellow fluorescent derivatives.
11.3 Biological control of aflatoxin production by atoxigenic *Aspergillus* spp.

11.3.1 Advantages of biocontrol of aflatoxins using atoxigenic *Aspergillus*

Dorner and Cole, (2002) treated peanut field plots with atoxigenic strains of *A. flavus* (NRRL 21882) and *A. parasiticus* (NRRL 21369) at 67 days 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 atoxigenic 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 indicated that field application of the atoxigenic strains had a carry-over effect and reduced aflatoxin contamination that occurred in storage. This leads to reduced crop aflatoxin contamination. This has been termed as shift of strain profile from toxigenic to atoxigenic.

Moreover, according to Bandyopadhyay *et al*. (2005), 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. 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.

Jane *et al*. (2014) characterized biocontrol methods as effective, environmental friendly, cost-effective and innovative means of reducing aflatoxins level 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.

11.3.2 Atoxigenic *Aspergillus* for aflatoxin biocontrol

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 based on DNA sequences have greatly thrived and can expose phylogenetics relationships between isolates. PCR – based and pyrosequencing 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.

Jane *et al*. (2014) concluded from study of other researchers 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.

11.3.3 Efficacy of *Aspergillus* as a biocontrol agent for aflatoxins production

In an earlier study by Brown *et al.* (1991) showed that atoxigenic isolate reduced aflatoxin by 80–95% in co-inoculated ears compared with ears inoculated with an aflatoxin producer alone. In a similar study, Abbas *et al.* (2006) reported reductions of 65–95%. Most of the examined atoxigenic isolates from West Africa achieved over 90% reductions in contamination.

Study governed by Atehnkeng *et al.* (2008) in West Africa, precisely Nigeria, reported that *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%.

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) and found aflatoxins were finally reduced by 83 and 98% by K49 and NRRL 21882, respectively, while AF36 was able to reduced aflatoxins by 20%.

Wu *et al.* (2013) reported 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, peanut, cottonseed etc. 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).

11.3.4 Current world scenario of *Aspergillus* as a biocontrol

Since applications of atoxigenic *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, atoxigenic 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).
Recently, a commercial biopesticide product (called Afla-guard®) has been developed based on the *A. flavus* strain NRRL 21882. This strain is the active ingredient in an EPA-registered biopesticide Afla-guard® currently sold in United States market by Syngenta Crop Protection, USA. Additionally, the atoxigenic *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).

In China, Yin *et al.* (2008) screened one highly competitive strain of *Aspergillus flavus* AF051 from more than 30 atoxigenic strains of *A. flavus*. Field tests showed that this strain reduced natural *Aspergillus* populations by up to 99% in the soil of peanut fields. Pitt and Hocking (2006) used biocontrol in the field of peanut in Australia, that resulted in 95% reduction of aflatoxin formation in peanuts. These results indicated that atoxigenic strains could be used in different agro-ecozones for the control of aflatoxin contamination.

In India, very little work done in the area of developing indigenous biological control as compared to other countries. It is not an easy task to isolate any atoxigenic strain and postulate it as a biocontrol. Instead, it needs many experiments to be conducted in order to check its feasibility in field, pre- and post- harvest effect in retarding or replacing toxigenic *Aspergillus*. To isolate a potential biocontrol requires tremendous efforts, huge manpower, time and money. So identifying biocontrol by studying its genetics at molecular level may reduce the efforts, need of huge manpower, time and money. But main reason behind unavailability of accurate molecular markers without sufficient data related to whole genome of *Aspergillus* spp. for the identification of atoxigenic *Aspergillus*. In India the aflatoxin contamination in food and feed is greater as compared to other developed courtiers of world. So there is a need to work potentially in this regards.

**11.4. Molecular aspects of aflatoxigenic and atoxigenic *Aspergillus***

**11.4.1 Genetic diversity analysis among *Aspergillus* using molecular markers.**

Doohan *et al.* (1999) had first time designed an alternative approach other than mere PCR, which is based on the use of reverse transcription–polymerase chain reaction (RT-PCR) to study the expression of the *Tri5* gene, involved in trichothece biosynthesis in *Fusarium culmorum*, *Fusarium graminearum*, and other species. The same method was later applied for monitoring aflatoxin production of *A. parasiticus* by Sweeney *et al.* (2000).

Sweeney *et al.* (2000) extracted total RNAs of the *A. parasiticus* strain 439 grown in inducing and non-inducing media to be amplified by RT-PCR with specific primers matching two genes of the aflatoxin biosynthetic pathway (*aflR* and *aflQ*). They demonstrated that
aflatoxin production monitored by thin layer chromatography was correlated with transcription of aflR and aflQ in this particular strain.

Criseo et al. (2001) developed a quadruplex-PCR for detection of aflatoxin producing A. flavus isolates targeting the aflR, nor-1, ver-1 and omtA genes of the aflatoxin biosynthetic pathway. The primer pairs nor1/nor2 (nor1- ACC-GCT-ACG-CCG-GCA-CTC-TCG-GCA-C and nor2- ACC-GCT-ACG-CCG-GCA-CTC-TCG-GCA-C), ver1/ver2 (ver1- GCC-GCA-GGC-CGC-GGA-GAA-AGT-GGT and ver2- GGG-GAT-ATA-CTC-CGG-CGA-CA-CAGC-C), omt1/omt2 (omt1- GTG-GAC-GGA-CCT-AGT-CCG-ACA-TCA-C and omt2- GTC-GGC-GCC-ACG-CAC-TGG-GTT-GGG-G) and APA-450/APA-1482 (APA-450-TAT-CTC-CCC-CCG-GGC-ATC-TCG-CGG and APA-1482- CCG-TCA-GAC-AGC-CAC-TGG-ACA-CGG) producing 400 bp, 537 bp, 797 bp and 1032 bp fragment in PCR respectively, comprised the quadruplex PCR. Three different culture media and TLC were used to determine the production of aflatoxins. Among a total of 12 strains of A. flavus, nine were non-aflatoxin producers and only three were aflatoxin producers. All three aflatoxigenic strains yielded the expected four amplicon bands. In contrast, non-aflatoxigenic strains resulted in varying band patterns comprised of 1, 3 or 4 bands. The presence of all 4 bands in some non-aflatoxigenic strains indicates that the absence of aflatoxin production may be due to base-pair substitution mutations that resulted in the formation of non-functional gene products.

Manonmani et al. (2005) targeted the aflR gene in PCR assays for detection of both A. flavus and A. parasiticus since the sequence of this gene is considered essentially identical in both species. Primers aflR1-F/aflRS1-R (F- AAC-CGC-ATC-CAC-AAT-CTC-AT and R- AGT-GCA-GTT-CGC-TCA-GAA-CA) were utilized. A total of 28 different fungal isolates were tested and included 7 strains of A. flavus, one strain of A. parasiticus, four additional Aspergillus species, in addition to isolates representing three additional fungal genera. All 7 strains of A. flavus and the single strain of A. parasiticus yielded the expected 798-bp amplicon. Multiple bands were observed in Fusarium isolates indicating the formation of nonspecific products. No amplicons were produced from DNA derived from the remaining 19 fungal isolates. A wet mycelial mass of 0.05 g and 100 spores were found to be the minimum required for positive PCR results.

Criseo et al. (2008) assayed 85 of 134 natural atoxigenic A. flavus isolates using quadruplex polymerase chain reaction (PCR)-based assay. The result showed that 36.5% of the strains revealed DNA fragments that correspond to the complete set of genes as found in toxigenic A. flavus. The results indicate that some atoxigenic A. flavus strains may have the
complete aflatoxin gene cluster but might have various deletions in the cluster. Thus, analysis of deletion within aflatoxin gene cluster is an effective method for rapid identification of true atoxigenic isolates for the development of biocontrol agents.

Rahimi et al. (2008) screened 230 fungal isolates from pistachio nuts for identifying aflatoxin production by amplifying DNA sequences of aflIR, aflI and omtB genes to correlate the results with TLC. Among 150 isolates of A. flavus tested for aflatoxin production by TLC, 46 were found to produce aflatoxins B1 and B2, while a few produced low amounts of G1. All of the A. parasiticus isolates yielded all aflatoxins detected in TLC. The aflIR primer pair aflR-F/aflR-R (F- CGC-GCT-CCC-AGT-CCC-CTT-GAT-T and R- CTT-GTT-CCC-CGA-GAT-GAC-CA) yielded the expected 630-bp amplicon with all isolates of A. parasiticus and with 33 of 46 TLC confirmed aflatoxin producing isolates of A. flavus. The aflI primer pair aflj-F/aflj-R (Forward- AGT-CAA-AGG-TTG-AAT-ACC and Reverse- GCT-CAG-CCA-TGA-CCT-TGA-CTG) produced the expected 840-bp amplicon with 39 of the 46 TLC aflatoxin positive isolates of A. flavus. The OmtBII primers OmtBII-F/OmtBII-R (F- ATG-TGC-TTG-GCI-TGC-TGT-GG and R- GGA-TGT-GGT-CTA-TGC-GAT-TGA-G), Inosine (I) was used in the forward primer because it binds to all nucleotides except guanine, yielded the expected 611-bp amplicon with all 46 TLC positive aflatoxin producing isolates of A. flavus and with all isolates of A. parasiticus. The authors concluded that since the omtBII 611-bp amplicon resulted from all aflatoxigenic isolates of A. flavus and A. parasiticus examined and its absence in non-aflatoxin producing Aspergilli, the omtBII primers are specific for aflatoxin producing fungi. The omtB gene was targeted because it had been postulated to be capable of discriminating between sterigmatocystin-producing fungi and isolates of A. flavus and A. parasiticus.

Yin et al. (2009) performed molecular characterization of toxigenic and atoxigenic Aspergillus flavus isolates, collected from peanut fields in China. Presence of aflatoxin genes in all 59 tested isolates was analyzed by PCR amplification with 13 pairs of PCR primers. the norB-cypA-F/norB-cypA-R primer pair was used to amplify unique deletion regions found in A. flavus. Except for primers pksA-F/ pksA-R and omtA-F/omtA-R, 21 primers were designed based on the sequences of aflatoxin biosynthetic pathway genes in A. flavus (GenBank accession no. AY510451.1). The PCR primer pair norB-cypA-F / norB-cypA-R amplified a 1.8 kb fragment from each A. parasiticus isolate. This primer pair generated 0.8 and 0.3 kb fragments in 26 and 27 A. flavus isolates, respectively. In other words, there was a 1.0 kb deletion (type II) or a 1.5 kb deletion (type I) in these 26 and 27 isolates, respectively. No fragments were amplified from remaining three A. flavus isolates by this primer pair. The
PCR amplifications with the other 12 primer pairs showed that 3 A. parasiticus and 45 A. flavus isolates had 12 tested aflatoxin genes. The 11 remaining A. flavus isolates had 5 deletion patterns in the aflatoxin genes. Thus analysis of deletions within the aflatoxin biosynthetic gene cluster might be an effective marker for identification of atoxigenic isolates. In this study, they found that 11 of 35 A. flavus isolates, which contained no detectable aflatoxins, had various deletions in aflatoxin genes.

Gautam and Bhadauria (2012) characterized Aspergillus species using PCR based markers associated with commercially stored triphala powder. For molecular characterization, internal transcribed spacer (ITS)-4, a universal fungal primer was utilized. Difference in banding patterns and number of bands obtained after polymerase chain reaction (PCR) amplification clearly differentiates between the Aspergillus species. Results also revealed that only A. flavus showed amplification with all the three aflatoxinogenic primers apa-2, ver-1 and omt-1, which means that only A. flavus was identified as aflatoxinogenic and other Aspergillus species as non-toxigenic according to PCR analysis. Hence, morphological, microscopic and molecular methods are important for the complete identification of important Aspergillus species.

Levin (2012) concluded by studying the research work done by many investigators that no single set of PCR primers can reliably be used to detect aflatoxin producing molds since aflatoxin precursor genes are also involved in the synthesis of other fungal toxins such as sterigmatocystin by atoxigenic molds. In addition, the successful amplification of a targeted gene sequence cannot be taken as proof of aflatoxin production since the gene may be cryptic and not expressed due to an undetected mutation external to the amplicon sequence. The use of conventional multiplex PCR, utilizing primers targeting the aflR, nor-1, ver-1 and omt-A genes appears to offer some promise in detecting aflatoxin producing molds, particularly with respect to the ability to distinguish characteristic DNA banding patterns derived from amplicons of appropriate size. Further work in this area in conjunction with multiplex PCR assays appears to be necessary.

Moubasher et al. (2013) performed molecular differentiation experiment between aflatoxigenic and non-aflatoxigenic strains of A. flavus and A. parasiticus using multiplex PCR procedure. The PCR primers used were the set of four primers of aflR, nor-1, ver-1 and omt-A genes of the aflatoxin biosynthetic pathway. Multiplex PCR showed that the four aflatoxigenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the aflatoxin biosynthetic pathway which encode for the products. Non-aflatoxigenic strains gave varying results with two, three, or four banding patterns. A banding
pattern in seven non-aflatoxigenic strains resulted in non-differentiation between these and aflatoxigenic strains proving that more accurate molecular technique need to be developed.

Mohamed et al. (2014) studied ten fungi belonging to five genera isolated from barley (*Hordeum vulgare* L.) grain collected from three main regions of Saudi Arabia. Eight (61.6%) were found aflatoxigenic out of 13 *Aspergillus flavus* isolates screened by HPLC for aflatoxins (AFs) production. Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR) analysis revealed a high level of genetic diversity in the *A. flavus* population, which was useful for molecular characterization but could not discriminate between aflatoxigenic and non-aflatoxigenic isolates, but the ISSR primers were somewhat better.

Cesar et al. (2014) studied the genetic variability of *A. flavus* isolates from different seasons, inoculum sources, and years, from a no-till corn field in the Mississippi Delta. Out of 175 *A. flavus* isolates examined, 74 and 97 had the typical norB-cypA type I (1.5 kb) and type II (1.0 kb) deletion patterns, respectively. Variability in the sequence of the *omtA* gene of the majority of the field isolates (*n* = 118) was compared to strain K49 (atoxigenic *Aspergillus flavus* strain), used as a biological control agent in corn fields. High levels of haplotypic diversity (24 *omtA* haplotypes; Hd = 0.61} 0.04) were found. Among the 24 haplotypes, two were predominant, H1 (*n* = 71), which consists of mostly toxigenic isolates, and H49 (*n* = 18), which consists of mostly atoxigenic isolates including K49. Toxigenic isolates were prevalent (60%) in this natural population. This study provides valuable information on the diversity of *A. flavus*.

Davari et al. (2015) evaluated the contamination of feedstuffs with *Aspergillus* spp. and detected genes involved in the aflatoxin biosynthesis pathway of *A. flavus* and *A. parasiticus* isolates. A total of 110 cow feed samples were examined using cultural and PCR methods. Sixty eight (61.82%) *Aspergilli* were isolated from 110 samples of feedstuff. Using four sets of primers, a quadruplex PCR was developed to detect genes (*nor1, ver1, omtA* and *aflR*) at different loci coding enzymes in the aflatoxin biosynthesis pathway of *A. flavus* and *A. parasiticus* strains. Out of 28 strains of *A. flavus* and *A. parasiticus*, 10 isolates (35.71%) showed a quadruplet pattern indicating the important genes involved in the aflatoxin biosynthesis pathway encoded for functional products. These isolates were confirmed to be aflatoxigenic by Thin layer chromatography. Eighteen isolates (64.29%) had three, two and single molecular banding patterns. They concluded that using multiplex PCR primers targeting the *aflR, nor-1, ver-1* and *omt-A* genes appears to offer some promise in detecting aflatoxigenic molds.
Adeela et al. (2015) developed a reliable and quick method for the detection of aflatoxin producing strains in groundnut by using molecular approaches. Total 80 samples of infected groundnut were collected from four different cities of Punjab and checked for their aflatoxin contamination. For aflatoxin detection, three target genes nor1, ver1 and aflIR were selected which was involved in the aflatoxin biosynthesis. In all examined cases, 24 out of 80 (30%) samples successfully amplified all three genes indicating aflatoxigenic activity. Discrimination between aflatoxigenic and non-aflatoxigenic strains were also determined on the basis of amplification of these three target DNA fragments. In this study, it was also demonstrated that only specific strains were able to produce the aflatoxin contamination in groundnut.

Mylroie et al. (2016) identified and quantified toxigenic and non-toxigenic Aspergillus flavus strain in contaminated maize using quantitative real-time PCR. Using polymorphisms found in the fungal rRNA intergenic spacer region (IGS) between a toxigenic strain of A. flavus (NRRL 3357) and the non-toxigenic strain used in the biological control agent Afla-Guard® (NRRL 21882), they developed a set of primers (Af2-F ATCATTACCGAGTGTAGGGTTCCT/ Af2-R GCCGAAGCAACTAAGGTACAGTAAA –amplicon size for Af2 is 73 bp, 3357-F2 GGAGCGGGATCTCAGACC / 3357-R8 GTAGGAGGTAGGGTGTACAGACC and 21882-F2 GAGCGGGATCTCAGAGA / 3357-R8 GTAGGAGGTAGGGTGTACAGAGC amplicon size for both 3357 and 21882 is 51 bp) that allowed the identification and quantification of the two strains using quantitative PCR. It must be noted that these primers were developed only for these two particular A. flavus strains and thus their ability to differentiate among other toxigenic and non-toxigenic strains is currently unknown. A further study is needed for determining the usefulness of these primers with other toxigenic and non-toxigenic strains of A. flavus as well as with other maize genotypes.

Moubasher et al. (2016) used expression profile of aflatoxin genes Nor-1, Omt-B, Omt-A and Ord-A to differentiate between aflatoxigenic and non-aflatoxigenic strains of Aspergillus flavus and Aspergillus parasiticus. Eleven strains of Aspergillus flavus and Aspergillus parasiticus, were isolated from Egyptian soil and air. The isolated strains have been screened for their ability to produce aflatoxins (B1, B2, G1 and G2) in three types of media (Czapek's Dox, potato dextrose and glucose peptone yeast). Differentiation between aflatoxigenic and non-aflatoxigenic strains was carried out using TLC to detect B1, B2, G1 and G2. Types of produced toxins were differed according to the constituent of media and fungal species, as well as its strains. The expression of four aflatoxin biosynthetic pathway
genes nor-1 (Afl-D), omt-B (afl-O), omt-A (afl-P) and ord-A (afl-Q) was evaluated in the eleven strains using RT-PCR. Mostly the results of the conventional method were concomitant with that of RT-PCR. The omt-B, omt-A and ord-A are important in differentiation between aflatoxigenic and nonaflatoxigenic strains.

11.4.2. Genome sequencing and functional annotations of aflatoxigenic and atoxigenic genes.

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

Yu et al. (2004) and Ehrlich et al. (2005) reported complex pathways for biosynthesis of aflatoxins in *A. flavus* and *A. parasiticus*. Enzymes and regulatory proteins for aflatoxin synthesis in these two fungi are encoded by more than 25 clustered genes in a 70-kb region. 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*).

Ehrlich et al. (2005) studied aflatoxin biosynthesis gene clusters and flanking regions in *Aspergillus* species. Clones from fosmid libraries (Agencourt Bioscience Corporation, Beverly, MA, USA) were selected by hybridization to PCR amplicons from the ends and middle of the *A. parasiticus* AF biosynthesis pathway gene cluster (GenBank no. AY371490). Sequencing was performed with at least six fold redundancy to obtain a probable sequencing error rate <1 bp per 1000 bp. Sequencing of genes related to aflatoxin biosynthesis pathway was carried out on fosmid clones selected by homology to *Aspergillus parasiticus* sequence. Alignments revealed that gene order is conserved among AF gene clusters of *Aspergillus nomius*, *A. parasiticus*, two sclerotial morphotypes of *Aspergillus flavus* and an unnamed *Aspergillus* spp. Although gene order within the AF cluster is highly
conserved among AF producers in section Flavi, the gene order differs markedly from that of the ST cluster of *A. nidulans*. In addition, the AF cluster contains three pairs of sister genes (*norB* and *norA*, *hypB1* and *hypB2* and *omtA* and *omtB*) that occur only singly in the ST cluster. Differences between AF and ST clusters in both gene order and homologous gene pairs were resulted from gene duplication and/or adaptive translocation events after divergence of the AF and ST clusters.

Chang *et al.* (2005) reported 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. Analysis of DNA sequence of aflatoxin gene cluster for 38 atoxigenic strains of *A. flavus* showed that deletions in the aflatoxin gene cluster among *A. flavus* strains are very common. Thirty eight atoxigenic strains had 8 different deletion patterns in the gene cluster.

Bhatnagar *et al.* (2006) opined that 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. 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).

Moubasher *et al.* (2016) postulated that PCR-based methods, including monomeric, quadruplex and multiplex PCR of different aflatoxin biosynthesis genes have been used by various researcher viz. Manonmani *et al.*, 2005, Somashekar *et al.*, 2004, Criseo *et al.*, 2001, Shapira *et al.*, 1996 etc. However, PCR detection of aflatoxin biosynthesis genes is not always successful in distinguishing aflatoxigenic from non-aflatoxigenic strains due to inter-and intra-specific genetic mutations within the primers’ targeted binding site (Levin, 2012). Criseo *et al.* (2001) have developed a multiplex PCR protocol to differentiate aflatoxin-producing and non-producing strains within the *A. flavus* group; Mayer *et al.* (2003a) have used real-time PCR to monitor the expression of an aflatoxin biosynthetic gene of *A. flavus* in wheat, and to compare the copy numbers of the *nor-1* gene to conventional CFU data (Mayer *et al.*, 2003b).

Mylroie *et al.* (2016) used toxigenic *A. flavus* NRRL 3357 and atoxigenic *A. flavus* NRRL 21882 strains to study polymorphism and identification at molecular level. Previous studies have used genes in the aflatoxin pathway to identify and quantify toxigenic isolates of *A. flavus* as well as other *Aspergillus* species and other mycotoxin producing fungi. The IGS region was chosen for sequencing as it is a region of higher variability than ITS region.
Sequencing of the IGS region using the primers LR12R and INVSR1R revealed multiple polymorphisms. A 2-base pair indel between NRRL 3357 and 21882 was used to design primer pairs which amplified an approximately 51 bp fragment. Multiple indels and SNPs were found between NRRL 3357 and NRRL 21882 and one 2 bp indel was chosen to design primers to differentiate between the two A. flavus strains. PCR amplification and visualization showed that the two primer pairs were successful at indentifying the target fungal strain while not producing a product in the non-target strain.

Adhikari et al. (2016) studied degeneration of aflatoxin gene clusters in Aspergillus flavus from Africa and North America. To study degeneration of genes, whole genome sequencing was performed at Arizona Genomics Institute (AGI) located at the University of Arizona’s BIO5 institute using Illumina HiSeq 2000. Libraries were sequenced with 100-bp paired-end reads and an insert size of 250 bp. Whole genome sequence of the non-aflatoxigenic genotypes analyzed in this study revealed aflatoxin gene clusters from 35 genotypes. Deletions found in the aflR, aflJ, avfA, norB–cypA, pksA, verA, verb and avfA genes region present in all A. flavus. The current study revealed both large (>1 kb) and small (< 1 kb) deletions through alignment of sequences of 35 non-aflatoxigenic genotypes. Inability of some genotypes to produce aflatoxin resulted from deletion of biosynthesis genes. In other genotypes, non-aflatoxigenicity originated from SNP formation. The process of degeneration differed across the gene cluster; genes involved in early biosynthesis stages were more likely to be deleted while genes involved in later stages displayed high frequencies of SNPs. They concluded SNP polymorphism analysis might help in differentiating atoxigenic strain from toxigenic.

12. Materials and methods

i. Location: Department of Biotechnology, College of Agriculture, Junagadh Agricultural University, Junagadh.

ii. Year of experiment: 2016-18

iii. Treatments details:
   - Aspergillus isolates – 21
   - Screening for Aflatoxigenic and atoxigenic Aspergillus (Zrari, 2013)
   - Molecular characterization of aflatoxigenic and atoxigenic Aspergillus (Mylroie et al., 2016)
   - Genome sequencing and functional annotation of aflatoxigenic and atoxigenic genes (Adhikari et al., 2016).
vi. Experimental details and observations to be recorded

Exp. 1. Isolation and characterization of toxigenic and atoxigenic Aspergillus species.

Different Aspergillus spp. will be isolated from groundnut seeds and other sources like soil, fruits and foods collected from different parts of Saurashtra region of Gujarat.

The pure culture of Aspergillus spp. will be isolated from seed, soil, fruits and food samples inoculated in Potato dextrose agar (Hi-media) media plate containing 200 gm/l of Potato infusion, 20 gm/l Dextrose, Agar 15 gm/l and final pH 5.6. After grown on PDA plate, the brown/yellow/green/black colour colonies resembling to Aspergillus spp. will be picked up from plates and will be re-inoculated onto centre of another fresh PDA medium to obtain pure culture of fungus (Zrari, 2013). For isolation of Aspergillus from groundnut the seeds will be surface sterilized with 0.5 % sodium hypochloride solution. The genus level confirmation of Aspergillus will be carried out by morphological and microscopic study. Pure cultures will be maintained fresh and viable by periodic transfer on PDA medium (Schindler, 1967).

Screening for aflatoxigenic Aspergillus species

Toxin producing ability of the fungi will be carried out using the ammonia solution or ammonium hydroxide (NH₄OH) vapour test (Kumar et al., 2007). The fungal culture of each species or strain will be grown in PDA media for 5 days at 28°C. Then 2 drops of concentrated (27%) NH₄OH solution will be added to the inverted lid of the Petri dish and allowed (30 min) to react. The formation of a pink to plum-red colour on the underside/reverse of the fungal colony indicates aflatoxin production (positive test) while negative tests have no observable colour changes (Zrari, 2013).

The isolates from pure culture plate will be examined for morphological and microscopic characters as described by Salano et al., 2016, Gautam and Bhadauria, 2012.

1. Morphological observations:- Colony diameter, colony surface colour, reverse colour, colony growth etc.

2. Microscopic observations: - Diameter of conidia and Vesicles, texture of conidia, conidial shape, conidiophore branching etc. under a dissecting microscope.
Exp. 2. Screen highly virulent and toxigenic aflatoxin producing *Aspergillus* using groundnut variety GG-20.

The seeds of groundnut variety GG-20 will be collected from Main Oil Seed Research Station, Junagadh Agricultural University, Junagadh, Gujarat. Ten gram seed of GG-20 variety of peanut will be rehydrated with 1 ml of water, sterilized at 121°C for 15 min and inoculated with 1 ml of a spore suspension (approximately 6 x 10⁵ spores/ml) from different isolates of a fungus (Adye and Mateles, 1964). The aflatoxin detection from *Aspergillus* infected groundnut seeds will be detected by ELISA / LCMS Q TOF (Peter et al., 2010). The flasks will be incubated at 28°C and the seeds will be crushed in 70 % methanol after 10 days after inoculation for aflatoxin detection by LCMS Q TOF. For aflatoxin extraction 10 gram of infected groundnut seeds will be crushed in 50 ml of 70 % Methanol then Shaken at 150 rpm for 30 minutes at room temperature on rotary shaker in amber/brown colour 250 ml flasks. The extract will be filtered by Whatman filter paper and then concentrated under N₂ evaporator till it becomes to 2 ml and stored in amber colour 2 ml bottles till further use. The highest aflatoxin producing strain will be selected as a virulent or toxigenic strain as of data obtained from LC Q TOF.

**Observations:** Aflatoxin B₁, B₂, G₁, G₂ and total.

Exp. 3. Characterization and *in vitro* antagonism of atoxigenic *Aspergillus* isolates with virulent aflatoxigenic *Aspergillus* to derive best biocontrol.

The *Aspergillus* isolates derived from various sources as described earlier will be used for this study. The isolate producing highest aflatoxin detected in LC Q TOF as compared to other *Aspergilli* will be used as toxigenic isolate. Those *Aspergillus* isolates producing below MRL (Maximum residual limit) of aflatoxin will be used as atoxigenic for antagonism study. To derive best biocontrol all isolates of atoxigenic *Aspergillus* will be co-inoculated with highly virulent and aflatoxigenic *Aspergillus* strain. The antagonism experiment will be carried out in Petriplate as well as on Groundnut seeds.

i. The one end of PDA media petriplates is inoculated with most toxic strain and opposite end with atoxigenic isolates. These plates are incubated at 28°C for some days to study antagonism (Cotty, and Bhatnagar, 1994).

**Observation:** Inhibition of aflatoxigenic isolate by atoxigenic *Aspergillus*.

ii. Simultaneously to study reduction in aflatoxin production of aflatoxigenic strain by atoxigenic isolates in groundnut seeds, 10 gm of Groundnut seeds will be inoculated
with toxic strain and each atoxigenic isolates in brown flask. These co-inoculated aflatoxigenic and atoxigenic strains will be allowed to grow simultaneously on seeds for 10 days at 28 °C in dark. After 10 days the methanolic extraction of aflatoxin will be carried out as described previously. The most competent atoxigenic isolate will reduce aflatoxin production at maximum level (Probst et al., 2011).

If the atoxigenic isolate reduces aflatoxin production of aflatoxigenic strain at maximum level as well as grows well to inhibit growth of toxigenic strain in Petriplate then it will be selected as a best atoxigenic antagonist for further experiments.

**Observations:** Aflatoxin B$_1$, B$_2$, G$_1$, G$_2$ and total.

**Exp. 4. Genome sequencing of aflatoxigenic and atoxigenic potent biocontrol agent Aspergillus and functional annotation for development of gene specific molecular markers.**

- The most virulent *Aspergillus* and potent atoxigenic *Aspergillus* derived from previous experiments will be subjected to genome sequencing (Adhikari et al., 2016).

1. Isolation, purification and quantification of genomic DNA.
2. Genome sequencing
   - Library preparation
   - Ligation of adaptors, Nick repair and purification of ligated DNA
   - Size selection of DNA fragments
   - Amplification and purification of library.
   - Template preparation and genome sequencing.
4. Comparative evaluation of aflatoxigenic genes with atoxigenic genome.
5. Identification and development of molecular markers related to aflatoxin biosynthesis pathways.

**13. Statistical analysis:**

Appropriate bioinformatics and statistical tools will be used throughout the study to analyze molecular and biochemical data (Adhikari et al., 2016 and Rohlf, 1994).
14. References


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