DOI: 10.21276/IJIPSR.2018.06.03.264

**International Journal of Innovative Pharmaceutical Sciences and Research**
**www.ijipsr.com**

# MICROBIAL BIOTECHNOLOGY REVIEW IN MYCOTOXINS IDENTIFICATION METHODS AND RELATIONSHIP BETWEEN THESE SECONDARY METABOLISM AND FUNGAL DEVELOPMENT

**Adeba Tilahun*, Daniel Yimar**

*Ethiopian institute of agricultural research, National agricultural biotechnology research center, **ETHIOPIA***

**Abstract**

Filamentous fungi are unique organisms rivaled only by actinomycetes and plants in producing a wide range of natural products called secondary metabolites. These compounds are very diverse in structure and perform functions that are not always known. However, most secondary metabolites are produced after the fungus has completed its initial growth phase and is beginning a stage of development represented by the formation of spores. These review, describe secondary metabolites produced by fungi that act as sporogenic factors to influence fungal development, environmental and genetic factors that can influence the production of secondary metabolites and the techniques used for detections of mycotoxins.

**Keywords:** Secondary metabolites, Detections, Mycotoxins, Fungal developments.

**Corresponding Author:**

***Adeba Tilahun***

*Ethiopian institute of agricultural research,*

*National agricultural biotechnology research center,*

***ETHIOPIA***

***E-mail:*** *adebayortil@gmail.com*

***Phone:*** *+251966993926*

DOI: 10.21276/IJIPSR.2018.06.03.264

# INTRODUCTION

Fungi are remarkable organisms that readily produce a wide range of natural products often called secondary metabolites (Bagnara, A.; Mayer, Z.; Geisen). In many cases, the benefits of these compounds are un known. However, interest in these compounds is considerable, as many natural products are of medical, industrial and/or agricultural importance. Some natural products are deleterious (e.g., mycotoxins), while others are beneficial (e.g., antibiotics) to humankind. Although it has long been noted that biosynthesis of natural products is usually associated with cell differentiation or development, and in fact most secondary metabolites are produced by organisms that exhibit filamentous growth and have a relatively complex morphology, until recently the mechanism of this connection was not clear. A critical advance in this regard was the establishment of a G-protein-mediated growth pathway in Aspergillus nidulans that regulates both asexual sporulation and natural product biosynthesis. So Secondary metabolism is commonly associated with sporulation processes in fungi and other microorganisms. Secondary metabolites associated with sporulation can be divided into three broad categories.(i), pigments required for sporulation structures for example melanins required for the formation or integrity of both sexual and asexual spores,(ii) metabolites that activate sporulation for example, the linoleic acid-derived compounds produced by A. nidulans,(iii) toxic metabolites secreted by growing colonies at the approximate time of sporulation (for example the biosynthesis of some deleterious natural products, such as mycotoxins. Secondary metabolite production usually commences late in the growth of the microbe, often upon entering the stationary or resting phase. In early observations it was noted that the environmental conditions required for sporulation and secondary metabolism were often similar and were more stringent than those for pure vegetative growth. But in some fungal species, eventhough natural products were essential for sporulation, there are many examples of fungal strains that still sporulate but are deficient in secondary metabolite production, for example, Penicillium urticae patulin mutants and A. nidulans sterigmatocystin mutants. Some secondary metabolites have easily observable effects on morphological differentiation in fungi. Compounds excreted by mycelium can induce asexual and sexual sporulation in other fungi; this phenomenon operates across species and genera. In most cases, these compounds have not been identified but are presumed to be natural products produced as the mycelia ages. Other natural products affecting fungal development are better characterized. Fusarium graminearum produces an estrogenic mycotoxin called zearalenone that enhances perithecial production in F. graminearum. The addition of dichloros, an inhibitor of zearalenone synthesis, inhibits the sexual development of this fungus. The most deleterious

DOI: 10.21276/IJIPSR.2018.06.03.264

of natural products, in terms of health effects, are the mycotoxins. A relationship between mycotoxin production and sporulation has been documented in several mycotoxigenic genera. For example, in Aspergillus parasiticus certain chemicals that inhibit sporulation have also been shown to inhibit the production of aflatoxin. Mycotoxins production is caused by the production of sporulation and fungul developmental growth stages. These review details with the developmental process of Aspergillus with focusing on the relationship between sporulation and mycotoxin biosynthesis, and identification methods of mycotoxins. Most Aspergillus spp. propagate solely by asexual spores called conidia (e.g., Aspergillus flavus and A. parasiticus), while other species produce both conidia and sexual spores called ascospores (e.g., A. nidulans, teleomorph: Emericella nidulans). A. nidulans conidia are formed on specialized complex structures called conidiophores, while ascospores are produced inside spherical sexual fruiting bodies called cleistothecia. Most asexual species, including A. flavus and A. parasiticus, form resistant structures called sclerotia (Wolf, J. C., and C. J. Mirocha. 1973). The formation of conidiophores begins with a stalk that extends from a thick-walled foot cell. The tip of the stalk begins to swell, forming a vesicle. From the vesicle, cells called sterigmata are formed, and chains of conidia originate from the sterigmata (Sim, S. C. 2001).. Genetic and molecular studies of conidial reproduction in A. nidulans identified a gene, brlA, which encodes a transcriptional regulator proposed to govern the activation of developmental genes at the time of vesicle formation. Genes required for the synthesis of aflatoxin and sterigmatocystin are well conserved between aspergilli and are located in large gene clusters (Bennett, J. W. 1981). The relative order and transcriptional direction of some of the homologous gene pairs though are not conserved. Thus far, most of the genes in the respective clusters have been shown to encode enzymes required for toxin biosynthesis (Shimizu, K., and N. P. Keller. 2001). Several studies of aflR have demonstrated it encodes a sterigmatocystin/aflatoxin pathway-specific transcription factor. aflR deletion mutants in all three Aspergillus spp. do not express biosynthetic genes in the sterigmatocystin-aflatoxin cluster nor do they produce sterigmatocystin or aflatoxin. There are different factors which can affect mycotoxin production(Zaika, L. L., and R. L. Buchanan. 1987), conidiation, and cleistothecial and sclerotial production concurrently. Physical parameters affecting mycotoxin and/or spore production in Aspergillus spp. include temperature, availability of an air-surface interface, and pH. Nutritional factors such as carbon source and nitrogen source can also affect both mycotoxin production and morphological differentiation (Van der Voorn, L., and H. L. Ploegh. 1992). Genetic materials can affect the development and secondary metabolites of fungi.For instance The A. nidulans CCAAT binding protein complex PENR1 has been

DOI: 10.21276/IJIPSR.2018.06.03.264

reported to regulate both development and penicillin production. Polyamines are small aliphatic molecules important for normal cell growth and development in a wide range of organisms. The most common polyamines are spermidine, spermine, and putrescine. polyamine biosynthesis reduced mycelial growth when applied to cultures of some phytopathogenic fungi, demonstrating the importance of these molecules for fungal development. Diamino butanone, an inhibitor of ornithine decarboxylase (the enzyme required for putrescine biosynthesis), blocked the formation of aerial mycelium, asexual and sexual sporulation, and sterigmatocystin biosynthesis in A. nidulans and asexual sporulation and aflatoxin biosynthesis in A. parasiticus.

**Identifications and quantifications of mycotoxin**

Nearly every food or feed commodity can be contaminated by fungal organisms and many of the food- and feed-borne filamentous fungi are capable of producing one or more mycotoxins, which are toxic metabolites of concern to both the health of humans and animals. Mycotoxins are toxic secondary metabolitesproduced under appropriate environmental conditions by filamentous Fungi species, mainly Aspergillus, penicillium, Fusarium, Alterneria etc.The most commons are aflatoxins, citrinin, ocharatoxins, and fumonisin and putlin.The structure of mycotoxins are very complex and diverse.The majority of the being synthesized from small molecules (acetate, pyruvate etc).The most deleterious of natural products,in terms of health effects,are the mycotoxins. This diverse class of compounds can contaminate commercial foods(e.g..,wheat,maize,pea nuts,cottonseed,and coffee) and animal feedstocks.Mycotoxins can be harmful even at small concentration,creating significant food safety concerns.Concern about safety of human foods and animal feeds has led to an increased development of accurate and suitable analytical methods for mycotoxin identification and quantification. Economic losses include yield loss from mycotoxin contamination, reduced value of crops loss of animal productivity from health issues related to mycotoxin consumption and even animal death.

**Table 1: Mycotoxins and their physiological effect**

| Mycotoxins | Producing organism | Chemical structure | Effect on mammalian cells |
|---|---|---|---|
| Aflatoxins (B1, B2, G1, G2, M1, M2) | Aspergillus | Difuranocoumarin derivatives | Carcinogenic |
| Citrinin | *Penicillium* | Benzopyran derivate | Nephrotoxic |
| Fumonisin | *Fusarium, Alternaria* | Isoflavonoid compounds | Carcinogenic Hepatotoxic |
| Trichothecenes | *Fusarium, Trichoderma* | Sesquiterpenoid compound | Cytotoxic Immunosuppressive |
| Ochratoxin | *Aspergillus,Penicillium* | Dihydroisocoumarin derivatives linked to phenylalanine | Carcinogenic<br>Nephrotoxic<br>Hepatotoxic<br>Teratogeni |
| Patulin | *Penicillium, Aspergillus* | Unsaturated heterocyclic lactones | Carcinogenic<br>Nephrotoxic<br>Hepatotoxic<br>Teratogeni |

DOI: 10.21276/IJIPSR.2018.06.03.264

While some mycotoxins are produced only by a limited number of species others may be produced by a relatively large range of species from several genera. It is estimated that 25 to 50% of the crops harvested worldwide are contaminated with mycotoxins.

**Detection Methods**

**Chromatographic Techniques**

Thin layer chromatography (TLC) is a method still broadly used for quantitative and semi-quantitative measurements of mycotoxins with detection by fluorodensitometry or visual procedures (0.01 ppm detection limit). TLC based on silica gel, F254 fluorescent silica gel or silica gel impregnated with organic acid has been reported to be applied for detection of common mycotoxins (aflatoxins, citrinin, fumonisin. Although it costs less, is simple and suitable for rapid screening, the lack of automation has led to TLC being replaced by other techniques.(Lin L,Zhang J whang p).

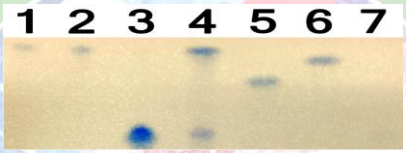



**Fig. 1: TLC analysis of DON by-products and controls. Lane 1: Mixed Culture 1; Lane 2: Mixed Culture 2; Lane 3: DON; Lane 4: 3-keto-DON and DON mixture; Lane 5: 15-A-DON; Lane 6: 3-A-DON: and Lane 7: De-epoxy-DON.**

Gas chromatography (GC) is a technique applicable to the compounds that are volatile and thermostable. Detection is achieved by linking the system to mass-spectometery (MS), flame ionization or fourier transform infrared spectroscopy. Most mycotoxins are not volatile and therefore need to be derivatised by chemical reactions such as silylation or polyfluoroacyclation in order to be quantified.

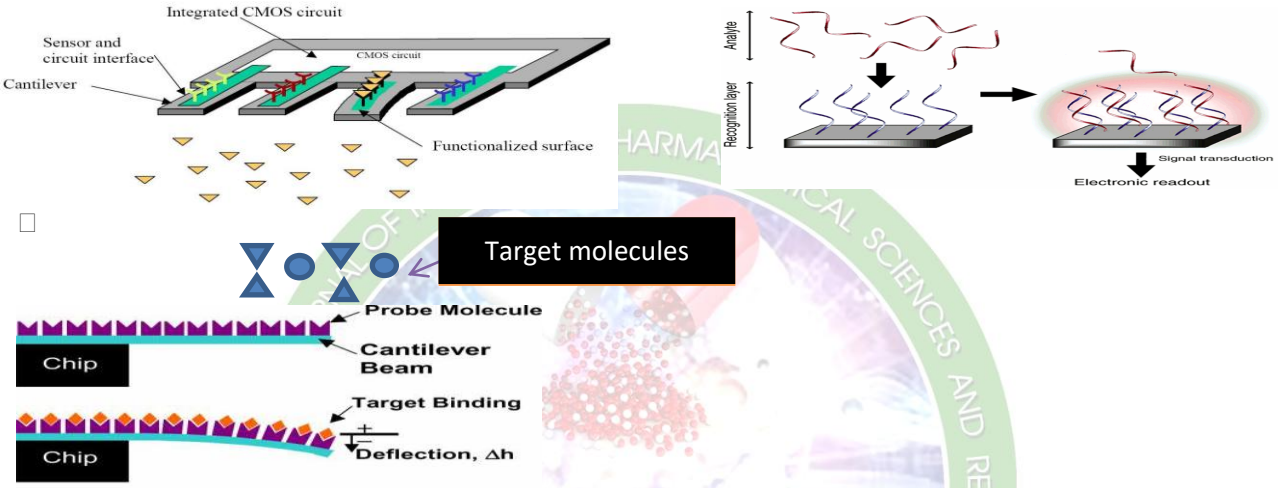
High performance liquid chromatography (HPLC) is applied in conjugation with UV, fluorescence ,amplometric or spectrofluorimetric detection. Both normal and reverse –phase HPLC are used for separation and purification(Harnandez M.A et al)

**Biological Methods**

Biosensors have emerged as rapid, sensitive, practical and convenient methods for mycotoxins analysis. They consist of a recognition element of biological origin that produces quantifiable response in a signal transduction element when contact with the target analyte. Most signal transduction mechanisms are optical (colorimetric, enhanced chemiluminescence) electrochemical or surface Plasmon resonance (Elasco-Garcia&Mattran,2002).Tissue biosensors ,optical immunosensors, enzyme ,sensors electrochemical sensors, quartz crystal, array and Plasmon resonance biosensors have been applied to detect ochratoxin, aflatoxins,

DOI: 10.21276/IJIPSR.2018.06.03.264

fumosins and deoxynivalenol in different commodities. For example, zearalenone and its derivatives were detected in milk products with a yeast whole –cell bioluminescent sensor (*genetically modified saccharomyces cerevisiae),*allowing detection at nanomolar concentration. Compared to other traditional analytical techniques, biosensors offer the possibility to monitor a large number of samples thus being a very convenient tool that can also be automated, for screening toxins in routine analysis. There are different types of biosensors. Antibody based biosensor, chemical sensor (electronic noses'"), Enzyme based biosensor, fatty acid based biosensor, and cell based biosensor and DNA biosensors.




Aflatoxins have inhibitory effect on acetylcholinesterase (AchE) and their detection is coupled with the decrease in the activity of AchE which is measured using a choline oxidase amperometric biosensor (Nayak et al., 2009).

**Physico Chemical Methods**

Capillary electrophoresis(CE) is an electrophoretic methods leading to a fast separation of components based on charge and mass dependent migration in electrical fields.In combination with fluorescence detection CE allows the detection of mycotoxins at trace levels(Narang,U et al )

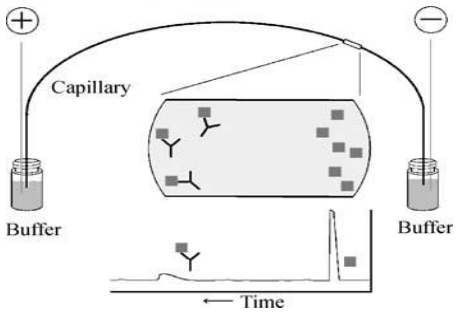



**Fig.2: Schematic of one form of capillary electrophoretic immunoassay. In this format antibody (Y) is combined with sample and a fluorescently labeled toxin (tracer). Bound and unbound tracer are separated in an electric field. In the presence of free toxin the amount of unbound tracer increases and the amount of bound tracer decreases, changing the relative sizes of the two peaks.**

DOI: 10.21276/IJIPSR.2018.06.03.264

**Immunological Methods**

These are binding asseys based on monocolonal or polycolonal antibodies raised againist toxins (antigens),which can be performed as immunoaffinity column-based analysis(IAC) or enzyme-linked immunosorbent assay(ELISA).The principle of the ELISA test is the atigen-antibody reaction (Bacigalupo.M.A 1994).The compititive assay format in which the toxin compete with enzyme conjugate to the toxins for specific immobilized antibodies ,is often used in commercial avoilable kits(Redascrine ,Aflatoxincup,etc).The technology is based on the ability specific antibody to distinguish the three dimensional structure of specific myco toxin(Kolosova A.Y et al 2006).Currently most of the commercialyavoilable ELISA test kits for mycotoxins are working in the kinetics phase of antibody-antigen binding,which reduces the incubation time to minutes from which it was 1-2h by convensinal methods .

Although reduction of incubation time may lead to some loss of assay sensitivity, the kit can provide accurate and reproducible results.

**Fluorescence**

All the aflatoxins have a maximum absorption around 360 nm (Akbas and Ozdemir, 2006).Letters 'B' and 'G' of the aflatoxins refer to its blue (425nm) and green-blue (450nm) fluorescence colours produced by these compounds under Ultra Violet (UV) light. AFB1 is the most common aflatoxin; it is followed by the AFB2. AFG is fairly rare. The fluorescence emission of the G toxin is more than 10 times greater than that for the B toxin (AlcaideMolina et al., 2009).

**Black light test**

The black light test is a method which correctly identifies negative AFs samples with minimum expenditure of time and money. It consists on the illumination of the sample witha UVlamp. Fluorescence may be bright or dim, depending on the amount of fluorescing agent present. It is highly recommended to use safety goggles when working with the black light test. These goggles eliminate blue haze resulting from eye fluorescence caused by reflected long wave UV radiation. The black light test is commonly applied on animal feed. However, it is only a preliminary confirmatory test; it does not give a quantitative indication. Thus confirmatory and quantitative measurements are needed to be applied to those samples that reacted positively to the black light test. Non-fluorescing samples need not be subjected to this. A quantitative screening test which commonly follows the black light test is small chromatographic column (mini-column) (B-100 Series Ultraviolet Lamps, UVP). After the quantitative test a judgment can be made as to whether or not accept a lot.

DOI: 10.21276/IJIPSR.2018.06.03.264

**Laser-Induced Fluorescence (LIF) screening method**

LIF detection technique was pioneered by Yeung (Novotny & Ishii, 1985). This screening method consists on a mobile phase which contains an eluted sample of aflatoxins. Such mobile phase passes through a detection window in the LIF detector. Thus, the whole fluorescence induced by the laser is collected by the detector (Alcaide-Molina et al., 2009). In LIF detection, the number of molecules that are photo-degraded is inversely proportional to the velocity of the fluorophore in front of the laser beam (Simeon et al., 2001). It has been said that AFB1 is the most toxic and one of the less fluorescent of the aflatoxins. However, the poorest sensitivity of the method may correspond to some other AF.Sensitivity tests should be applied for different AFs to select the one with the lowest sensitivity. The system should be calibrated with the curve of such aflatoxin; thereby, a signal provided by other AF is going to be translated into a higher concentration of this AF, leading to a confirmatory analysis on the screening method. This strategy, then, eliminates false negatives (Alcaide-Molina et al., 2009). Thus, LIF detection shows as an appropriate detection technique with applications on very low concentrations of sample with native fluorescence or that fluoresce after derivatization (Simeon et al., 2001). However, LIF detection is a technique restricted to a limited number of laboratories because the high cost of the lasers, and because most of the analyte molecules have to be labeled with dyes that match the laser wavelength. Moreover, when the labeling reactions are not well understood, they can lead to contradictory results (Lalljie & Sandra, 1995).

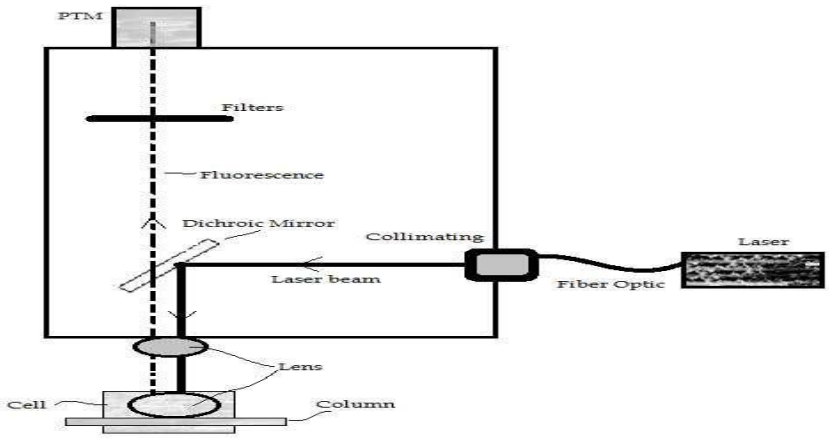



**Fig.3: Scheme of a LIF detector (adapted from Simeon et al., 2001)**

**Molecular methods**

For the detection of mycotoxin-producing fungi unique DNA sequences of the respective organisms have to be chosen as primer binding sites. It is concluded that genes involved in the mycotoxin biosynthetic pathway may form a perfect basis for an accurate, sensitive, and

DOI: 10.21276/IJIPSR.2018.06.03.264

specific detection system for mycotoxigenic strains in agricultural commodities, foods and animal feeds.

These methods are the best and the most advantages than other detection methods. Some advantages of molecular methods are that it is fast (direct detection),is not influenced by environmental condition, no special expertise in phenotypic identification is needed, Phenotypically similar species can be distinguished ( eg. yeasts, black Aspergilli. A.ustusA. calidoustus, P .roquefortii P. paneum), several fungi can be identified in one run, Viable but non culturable fungi can be detected.

**DNA extraction**

Isolates of mycotoxins producing fungi were cultured on PDA for 7 days. Afterwards, mycelia were scraped into Eppendorf tubes with a sterile spatula. Mycelia were harvested by centrifugation (15 min, 4500 x g), washed once with sterile distilled water and lyophilised.Total DNA was the norsolorinic acid reductase encoding gene nor1, the versicolorin A dehydrogenase encoding gene ver1, the sterigmatocystin O-methyltransferase encoding gene omtA, and the regulatory gene aflR have been described. The trichodiene synthase encoding gene tri5, a yet non-identified protein encoding gene tri7, and the regulatory gene tri6 have been the targets for detecting trichothecene-producing Fusaria.The target sequence for the detection of patulin-producing Penicillum strains is within the isoepoxydon dehydrogenase encoding gene IDH. If there is only limited or even no information available about genes involved in a certain mycotoxin biosynthetic pathway, specific primers can be designed, for example, by random amplified polymorphic DNA (RAPD) analysis or by analyzing the fungal ribosomal region. Instead of using two primers that are designed based on pre-existing knowledge of the target sequence, RAPDs are produced from single, with respect to sequence randomly chosen, oligonucleotide primers, typically 10 bases long.extracted from mycelium of each isolate using CTAB methods

**Development of A PCRAssay**

For the detection of mycotoxin-producing fungi unique DNA sequences of the respective organisms have to be chosen as primer binding sites. It is concluded that genes involved in the mycotoxin biosynthetic pathway may form a perfect basis for an accurate, sensitive, and specific detection system for mycotoxigenic strains in agricultural commodities, foods and animal feeds, since those genes are supposed to be exclusively present in organisms potentially producing mycotoxins (Mishra P.K.; Fox, R.T.V. 2003)many genes involved in the biosynthesis of these mycotoxins have been identified and their DNA sequences have been published. In the meantime, PCR methods for the detection of aflatoxigenic Aspergilli based

DOI: 10.21276/IJIPSR.2018.06.03.264

on the norsolorinic acid reductase encoding gene nor1, the versicolorin A dehydrogenase encoding gene ver1, the sterigmatocystin O-methyltransferase encoding gene omtA, and the regulatory gene aflR have been described. The trichodiene synthase encoding gene tri5, a yet non-identified protein encoding gene tri7, and the regulatory gene tri6 have been the targets for detecting trichothecene-producing Fusaria.The target sequence for the detection of patulin-producing Penicillum strains is within the isoepoxydon dehydrogenase encoding gene IDH.

If there is only limited or even no information available about genes involved in a certain mycotoxin biosynthetic pathway, specific primers can be designed, for example, by random amplified polymorphic DNA (RAPD) analysis or by analyzing the fungal ribosomal region. Instead of using two primers that are designed based on pre-existing knowledge of the target sequence, RAPDs are produced from single, with respect to sequence randomly chosen, oligonucleotide primers, typically 10 bases long.

There are strategies to develop specific PCR to detect mycotoxigenic fungi

**1.** Primer design ——— based on
- Gene of the mycotoxin biosynthetic path way
- RAPD fragments
- *ITS* region

**2.** Primer alignments ——— with all DNA sequences entered in data base using BLAST program

**3.** PCR optimization ——— Based on
- Annealing temp.
- Number of cycles
- $Mg^{+2}$-concentration
- Hot start polymerase
- Additives etc.

**4.** Primer specificity ——— Check with purified DNA from different food related Fungi, bacteria, insects as well as agricultural Commodities, food and animal feed potentially Contaminated.

**5.** DNA extraction ——— adapt the DNA extraction procedure to the matrix of DNA Sources and establish the detection limits using artificial Contaminated samples.

The complexity of eukaryotic nuclear DNA is sufficiently high, that by chance these RAPD primers bind to the DNA in both forward and reverse orientations close enough to one another for PCR amplification. With some randomly chosen RAPD primers no sequences are amplified, with others, the same length products are generated from DNA of different genera, species or subspecies and with still others, patterns of bands are different on genus, species or

DOI: 10.21276/IJIPSR.2018.06.03.264

subspecies level. RAPD fragments unique for the fungi to be detected are sequenced and based on these sequences it should be possible to design primers for the development of a specific PCR system. The organization of the ribosomal genes is conserved in fungi. Eukaryotic fungal ribosomal genes are arranged in a tandem repeat and within the ribosomal DNA repeat; the two variable non-coding internal transcribed spacer regions (ITS regions) are nested between the highly conserved 5.8S nuclear small subunit ribosomal RNA and the two large subunit ribosomal RNA genes. The ribosomal region spanning ITS1, 5.8S and ITS2 is often between 600 and 800 bp long. The fungal ribosomal genes are highly conserved at the genus level or even higher, but the internal transcribed spacers ITS1 and ITS2 and the intergenic spacer IGS have evolved faster than the ribosomal genes and may therefore be more useful for the development of specific oligonucleotide primers, aimed at differentiating at the genus, species or subspecies level. Several studies have shown that the ITS regions are highly variable among and within different fungal species. In order to identify specific primer binding sites for a PCR to detect mycotoxin-producing fungi, either ITS1 or ITS2 or even both are amplified in several mycotoxin producers and non-producers by using primers binding to the conserved regions of the structural ribosomal RNA genes. The sequences of the obtained PCR products are compared in order to identify regions which may serve as target sites in a PCR to distinguish toxigenic from non-toxigenic fungi or to detect fungi at the genus or species level.

**RNA-Based Approach**

It has to be kept in mind that the absence of a PCR band does not necessarily imply that the analyzed strain is unable to produce mycotoxins. For example, a mutation in the primer binding site not affecting the functionality of the encoded protein could be the reason for a missing PCR product. In addition, the generation of the expected PCR product does not necessarily imply that the analyzed strain is capable of producing mycotoxins, because the presence of a PCR product does not allow to draw any conclusion about expression of the respective gene. However, the presence or lack of mRNAs may permit direct differentiation between mycotoxin-producing and non-producing strains. The detection systems based on reverse transcription PCR (RT-PCR) have been developed (Sweeney, M.J.; Pàmies, P) to monitor aflatoxin production in Aspergillus parasiticus and trichothecene production in Fusarium culmorum . RT-PCR has been demonstrated to be 100-1000 times more sensitive than Northern hybridization analysis. In addition, several specific mRNAs may be detected simultaneously in a single RNA sample by multiplex RT-PCR. RT-PCR allows the detection

DOI: 10.21276/IJIPSR.2018.06.03.264

of mRNAs transcribed by specific genes by the PCR amplification of cDNA intermediates synthesized by reverse transcription.

- Total RNA was used as a template to synthesize cDNAs with random hexamer primers and reverse transcriptase. The resulting cDNA was amplified by PCR using the specific primers. Since genomic DNA sequences homologous to RNA targets present in the PCR reaction may interfere with the detection of RNA by RT-PCR, the RNA preparations were treated with DNAse I first.
- As a control, the gene transcription of a housekeeping gene, ß-tubulin, was monitored by RT-PCR. To distinguish cDNA from genomic DNA targets, the ord1 and ß-tubulin primers were designed from coding regions flanking introns, so that the decreased size of the RT-PCR product relative to the PCR product derived from genomic DNA could be discerned.
- This strategy allows the detection of false positive reactions resulting from contamination of the RNA treated with DNase I with genomic DNA from foreign cellular material or carry-over contamination from previous PCR reactions and also assessed the efficiency of the DNase I treatment of the isolated RNA. The developed RT-PCR was successfully used to distinguish aflatoxin-producing from non-producing Aspergillus parasiticus strains, but this technique has also the potential to be employed as a tool to investigate the effects of a variety of physiological factors on the transcription of the aflatoxin genes.

TAQMAN™ PCR

- The TaqMan™-technology makes use of the 5'-3' exonuclease activity of the polymerase to generate a template-specific fluorescent signal after hydrolyzing an internal probe during each step of the PCR. The internal probe is 5'-labeled with a reporter fluorescent dye and 3'-ligated to a quencher dye. As long as the reporter fluorescent dye and the quencher dye are located in close proximity on the internal probe, the quencher dye greatly reduces the fluorescence emitted by the reporter dye by Förster energy resonance transfer (FRET) through space. During PCR, the reporter dye is separated from the quencher dye, which results in an increase of the reporter dye signal. Only if the internal probe is binding to the DNA in between the two PCR primers a fluorescence signal during PCR is generated ( Schnerr, H et al).

DOI: 10.21276/IJIPSR.2018.06.03.264

**Hybridization based techniques**

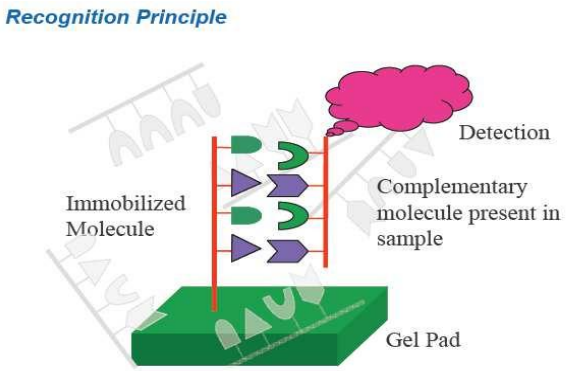



**Fig.4**

Detection of the labels are:

Colorimetry, Radioactivity,Fluorescence, Luminescence, Magnetometry, Etc

Therefore, this additional hybridization step increases the specificity of the PCR. False negative results due to PCR inhibitors could be excluded by using an internal amplification control in each PCR. The parameter measured is the threshold-cycle (CT) where each reaction trespasses a certain fluorescence level. In comparison to the Light Cycler™ PCR, the TaqMan™ PCR needs more time, but on the other hand, more samples can be run at the same time. A PCR with 45 cycles is completed within about 2 h with at maximum 96 single reactions per run. For quantification, a calibration curve with a serially diluted solution of pure fungal genomic DNA has to be generated with each set-up of the real time PCR (Meyer, R.; Chardonnens 1996). A TaqMan™ quantitative PCR system directed against the nor1 gene of the aflatoxin biosynthetic pathway as a target sequence has been developed by Mayer et al.

## CONCLUSION

It has been noticed that filamentous fungi are readily synthesize complex compounds that are putatively helpful but not necessary for survival and whose production is presumably costly to maintain. Furthermore, production is often linked to fungal development. Natural products are often produced late in fungal development, and their biosynthesis is complex. This complexity is due to a number of factors that affect secondary metabolite production such as external and internal factors, involvement of many sequential enzymatic reactions and genetic factors. Some natural products directly enhance sporulation of the producing organisms. . Most natural products play no obvious roles in sporulation or spore protection but are secreted into the environment at a time in the life cycle of the fungus that corresponds with sporulation. However, some of the compounds that are excreted into the environment could have subtle effects on the organism that are not immediately obvious.Mycotins are one of the natural

DOI: 10.21276/IJIPSR.2018.06.03.264

products secreted by flamentuss fungi. Aflatoxin and sterigmatocystin production in Aspergillus spp. has been extensively studied due to the deleterious effect of the mycotoxin on human health. Thus why these review study is needed to identify the better detection methods of the fungal secondary metabolites. While medical investigations have focused on understanding the mechanisms of aflatoxin toxicity and carcinogenicity in humans, these effects of sterigmatocystin and aflatoxin on human health are a consequence of ingesting infected crops and are not the primary function of these compounds. In recent years, great progress has been made in the discovery of signaling pathways that connect fungal development with natural product biosynthesis. However, much remains to be learned. The complexity of these regulatory networks, with multiple target sites and interconnections with other regulatory mechanisms, makes their full elucidation a challenging task. Different methods for detection and quantification of aflatoxins have been discussed along this document. It has been found that the most popular methods are: ELISA, electrochemical immunosensors, chromatography and fluorescence. The most promising breakthroughs in the detection of mycotoxins or mycotoxigenic fungi are expected to be made in the area of sensor technology. Bio-sensors can be used for the detection of very different analytes such as pathogens, pesticides and toxins. They are a subgroup of chemical sensors where the analytical devices are composed of a biological recognition element such as enzymes, antibodies, receptors, proteins, oligonucleotides, or even a whole cell coupled to a chemical or physical transducer, which measures the changes that occur when the sensor couples to its analyte. PCR may be applied to the screening of agricultural commodities for the detection of mycotoxin. The usefulness of the PCR methods developed so far to monitor quality and safety in the food and feed industry was already demonstrated. It seems to be even possible by using a quantitative approach to correlate the number of target molecules with fungal biomass or even mycotoxin content. The real time PCR systems have furthermore the potential to be adapted to quantify mRNA, which can be used for monitoring the expression of genes involved in mycotoxin biosynthesis under particular environmental conditions in different foods. Preliminary results on this field are very encouraging.

**REFERENCES**

1. Bagnara, A.; Mayer, Z.; Geisen, R. Quantification of Aspergillus flavus BFE96 by SYBR green Q-PCR in black pepper. Mycotox. Res., 16:244-247, 2000
2. Sweeney, M.J.; Pàmies, P.; Dobson, A.D.W. The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in Aspergillus parasiticus 439. Int. J. Food Microbial., 56:97-103, 2000.

DOI: 10.21276/IJIPSR.2018.06.03.264

3. Schnerr, H.; Niessen, L.; Vogel, R.F. Real time detection of the tri5 gene in Fusarium species by LightCycler™-PCR using SYBR® Green I for continuous fluorescence monitoring. Int. J. Microbial., 71:53-61, 2001.
4. Mishra P.K.; Fox, R.T.V.; Culham, A. Development of a PCR-based assay for rapid and reliable identification of pathogenic Fusaria. FEMS Microbiol. Lett., 218:329-332, 2003.
5. Meyer, R.; Candrian, U. PCR-based DNA analysis for the identification and characterization of food components. Lebensm. Wiss. u. Technol., 29:102-111, 1996.
6. Abdollahi, A., and Buchanan R. L.1981. Regulation of aflatoxin biosynthesis: characterization of glucose as an apparent inducer of aflatoxin production. J. Food Sci. 46:143-146. CrossRefGoogle Scholar.
7. Adams, T. H., and J.-H. Yu. 1998. Coordinate control of secondary metabolite production and asexual sporulation in Aspergillus nidulans. Curr. Opin. Microbiol. 1:674-677. CrossRefMedlineGoogle Scholar
8. Asakawa, H., K. Kitamura, and C. Shimoda. 2001. A novel Cdc20-related WD-repeat protein, Fzr1, is required for spore formation in Schizosaccharomyces pombe. Mol. Genet. Genom. 265:424-435. CrossRefMedlineGoogle Scholar
9. Bennett, J. W. 1981. Loss of norsolorinic acid and aflatoxin production by a mutant of Aspergillus parasiticus. J. Gen. Microbiol. 124:429-432. Google Scholar
10. Bennett, J. W., Leong P. M., S. Kruger, and D. Keyes. 1986. Sclerotial and low aflatoxigenic morphological variants from haploid and diploid Aspergillus parasiticus. Experientia 42:841-851. Google Scholar
11. Boylan, M. T., P. M. Mirabito, C. E. Willett, C. R. Zimmerman, and W. E. Timberlake. 1987. Isolation and physical characterization of three essential conidiation genes from Aspergillus nidulans. Mol. Cell. Biol. 7:3113-3118
12. Burow, G. B., Nesbitt T. C., J. Dunlap, and Kelle N. P.. 1997. Seed lipoxygenase products modulate Aspergillus mycotoxin biosynthesis. Mol. Plant-Microbe Interact. 10:380-387. Cross Ref Google Scholar
13. Calvo, A. M., Gardner H. W., and N. P. Keller. 2001. Genetic connection between fatty acid metabolism and sporulation in Aspergillus nidulans. J. Biol. Chem. 276:20766-20774.
14. Dorn, G. L. 1970. Genetic and morphological properties of undifferentiated and invasive variants of Aspergillus nidulans. Genetics 66:267-279.

DOI: 10.21276/IJIPSR.2018.06.03.264

**15.** Akbas and Ozdemir, 2006, AlcaideMolina et al., 2009,Lalljie& Sandra, 1995, Novotny & Ishii, 1985

**16.** Maragos, C. M. (1998). Analysis of mycotoxins with capillary electrophoresis. Sem. Food Anal. 3:353–373

**17.** Mullett, W., Lai, E. P. C., Yeung, J. M. (1998). Immunoassay of fumonisins by a surface plasmon resonance biosensor. Anal. Biochem. 258:161–167.

**18.** Nasir, M. S., Jolley, M. E. (2003). Fluorescence polarization (FP) assays for the determination of grain mycotoxins (fumonisins, DON vomitoxin and aflatoxins). Comb. Chem. High Throughput Screen. 6:267–273.

**19.** Olsson, J., Borjesson, T.Lundstedt, T., Schnu¨rer, J. (2002). Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. Int. J. Food Microbiol. 72:203–214.

**20.** Narang, U., Gauger, P. R., Kusterbeck, A. W., Ligler, F. S. (1998). Multianalyte detection using a capillary-based flow immunosensor. Anal. Biochem. 255:13–19.

**21.** Bacigalupo, M. A., Ius, A. Meroni, G., Dovis, M., Petruzzelli, E. (1994). Determination of aflatoxin B in agricultural commodities by timeresolved fluoroimmunoassay and immunoenzymometric assay. Analyst 119:2813–2815.

**22.** Sim, S. C. 2001. Characterization of genes in the sterigmatocystin gene cluster and their role in fitness of Aspergillus nidulans. Master's thesis. Office of Graduate Studies of Texas A & M University, College Station. Google Scholar

**23.** Folestad et al., 1985; Diebold et al., 1979, Elasco-Garcia &Mottram,2002, Nayak et al., 2009.

**24.** Thrane, U.,2002. XXXXX International Journal of Food Microbiology (current issue).

**25.** Yli-Mattila, Mach, R. L.; Alekhina, I. A., Bulat, S. A.; Kullnin-Gradinger, C. M. Cubicek, C. P., Klemsdal., S. S., 2002. Phylogenetic relationship of Fusarium langsethiaeto Fusarium poae

**26.** Geisen, R. PCR methods for the detection of mycotoxin-producing fungi. In: Bridge, P.D.; Arora, D.K.; Reddy, C.A.; Elander, R.P. (eds). Application of PCR in mycology. CAB International, New York, 1998,p.243-266.

**27.** Grimm, C.; Geisen, R. A PCR-ELISA for the detection of potential fumonisinproducing Fusarium species. Lett. Appl. Microbiol., 25:456-462, 1998.

**28.** Liewen, M.B.; Bullerman, L.B. Toxigenic fungi and fungal toxins. In: Vanderzant, C.; Splittstoesser, D.F. (Eds). Compendium of methods for the microbiological

DOI: 10.21276/IJIPSR.2018.06.03.264

examination of foods. 3rd ed., American Public Health Association, Washington DC, 1999, p.811-819.

**29.** Meyer, R.; Chardonnens, F.; Hübner, P.; Lüthy, J. Polymerase Chain Reaction (PCR) in the quality and safety assurance of food: Detection of soya in processed meat products. Z. Lebensm. Unters.Forsch., 203:339-344, 1996.

**30.** Paterson, R.R.M.; Archer, S.; Kozakiewicz, Z.; Lea, A.; Locke, T.; O´Grady, E. A gene probe for the patulin metabolic pathway with potential for use in patulin and novel disease control. Biocontrol Sci. Technol., 10:509-512, 2000.

**31.** Yong, R.K.; Cousin, M.A. Detection of moulds producing aflatoxins in maize and peanuts by an immunoassay. Int. J. Food Microbiol., 65:27-38, 2001