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Review Paper

Rapid Detection Methods for Analysis of Fungi and Mycotoxins in Agriculture Products

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Abstract

Many commodities and products can become contaminated with mycotoxigenic fungi and mycotoxins during harvest, storage and handling, and processing. Mycotoxins are structurally diverse, deriving from a number of biosynthetic pathways and their effect upon consumers is equally diverse ranging from acutely toxic to immunosuppressive or carcinogenic. Rapid detection and quantification of fungi and mycotoxins in agriculture products and processed food is necessary for assuring safety and quality of food. This paper provides a brief review of novel approaches and technologies for mycotoxin analysis including immunological detection, nucleic acid hybridization, polymerase chain reaction (PCR), mycotoxin biosynthetic gene, and combination assays for detection of fungi and mycotoxins. Some methods have already been commercialized while others innovative tests have been reported in the literature and have the potential for providing real-time test for fungi and mycotoxin testing. The review focuses on methods for detection of fungi and mycotoxins in agriculture products.

Keywords: Rapid detection, Mycotoxins, immunological detection, nucleic acid hybridization, PCR.

Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi that have deleterious effects on human and animal consumers. Mycotoxins are structurally diverse, deriving from a number of biosynthetic pathways and their effect upon consumers is equally diverse ranging from acutely toxic to immunosuppressive or carcinogenic. The production of a particular mycotoxin is restricted to a limited number of fungal species and, in some instances, may be limited to particular strains within a species. Although over 300 mycotoxins have been described, relatively few are of major concern with respect to human and animal health[1]. Fungi from five genera (*Aspergillus, Fusarium, Penicillium, Alternaria* and *Calviceps*) are responsible for the production of the great majority of the mycotoxins that are of agricultural relevance[2]. The majorities of these fungi infect plants prior to harvest and can be regarded as phytopathogens while others become active following harvest where storage conditions are permissive of fungal growth. Not all infections can be regarded as pathogenic, however, as infection may not result in symptoms of disease, e.g. *Aspergillus flavus* infection of maize.

Aflatoxins are probably the most significant mycotoxins worldwide, with an estimated 20000 deaths linked to aflatoxin-induced liver cancer per annum in Indonesia[3]. Aflatoxins are polyketide compounds produced by several members of the *Aspergillus* section Flavi[4]. Some strains of *A. flavus* and most strains of *A. parasiticus* and *A. nomius* produce aflatoxins with *A. parasiticus* and *A. flavus* being the two most agriculturally important species, as shown in table 1. Infection of maize, peanuts, pistachio nuts, figs, cotton seed meal and other commodities by these species may result in contamination with aflatoxins. Infection may occur in the field, particularly where the plant experiences water, temperature or nutrient stress and accumulation of mycotoxin may increase if harvested material is inappropriately stored[5]. *Aspergillus flavus* produces aflatoxins B1 (AFB1) and B2 (AFB2) while *A. parasiticus* also produces aflatoxins G1 (AFG1) and G2 (AFG2), as shown in table 1. Aflatoxin B1 is considered to be the most potent naturally occurring carcinogen known. Aflatoxin M1 is a derivative of AFB1 that is formed and excreted in the milk of humans and animals following consumption of foodstuffs contaminated with AFB1. AFB1 is derived from sterigmatocystin (ST), which is itself carcinogenic[6].

Trichothecenes are sesquiterpenoid compounds produced by fungi from several genera (e.g. *fusarium, myrothecium, stachybotris, trichoderma, trichothecium, cephalosporium, cylindrocarpon* and *vertimonosporium*)[7]. Trichothecenes are structurally diverse and over 180 naturally occurring trichothecenes have been described to date. The *fusarium* species are of significance with respect to food safety because many are important pathogens and colonizers of plants, particularly cereals[8]. Four classes of trichothecenes are recognized with types A and B being produced by *fusarium* species. Type A trichothecenes include T-2 toxin, HT-2, diacetoxyscirpenol (DAS) and neosolaniol (NEO) while type B

trichothecenes include deoxynivalenol (DON) (also known as vomitoxin), 3-acetyl and 15-acetyl derivatives of DON (3-ADON and 15-ADON), Nivalenol (NIV) and 4-acetyl NIV (also known as fusarenon-X)[8], as shown in table 1. The type of trichothecene produced is characteristic of a particular species. The chief producers of type A trichothecenes are *F. sporotrichioides*, *F. poae* and *F. equiseti* while *F. graminearum*, *F culmorum* and *F. cerealis* are producers of the type B trichothecenes that are most often associated with infected plant products. Trichothecenes are potent inhibitors of eukaryotic protein synthesis and have been associated with diseases such as alimentary toxic aleukia (ATA) that led to the deaths of thousands of Russians in the 1940s as a result of consuming grain that had become contaminated with *fusarium* species while over-wintered in the field[9].

Fumonisins are another group of mycotoxins produced by *fusarium* species, chiefly those associated with pink ear rot of maize. Consumption of fumonisin contaminated maize has been linked to high rates of esophageal cancer[10]. Fumonisins consist of a linear carbon backbone substituted at various positions with hydroxyl, methyl and tricarboxylic acid groups. The forms generally present in naturally contaminated maize are members of the B series, FB1, FB2, FB3 and FB4[11], as shown in table 1. Three species are most commonly associated with pink ear rot, *F. proliferatum*, *F. verticillioides* and *F. subglutinans*. Only *F. verticillioides* and *F. proliferatum* are able to produce fumonisins while *F. subglutinans* and *F. proliferatum* can produce moniliformin and beauvericin.

Ochratoxins are cyclic polyketides, the most potent of which is ochratoxin A (OTA)[12], as shown in table 1. OTA has been associated with human endemic neuropathy occurring in the Balkan region[12]. This mycotoxin was first detected in *Penicillium ochraceus*, but a large number of other *Penicillium* and *Aspergillus* species have since been reported to be OTA producers. While *Penicillium* species are the predominant OTA producers in temperate regions, *Aspergillus* species are more significant in warmer regions.

Patulin is a polyketide lactone that is produced by a number of *Aspergillus* and *Penicillium* species as well as fungi from other genera[13], as shown in table 1. *Penicillium expansum* is probably the species of greatest agricultural significance. Patulin can occur in mouldy fruits; major sources of human contamination are apples and apple juice. The ability of phylogenetically dissimilar species to produce patulin or OTA indicates that the genes for the biosynthesis of these mycotoxins have been gained (or lost) several times during evolution.

Ergot alkaloids are also produced by a range of fungi, including species within the genera *Acremonium, Balansia, Aspergillus* and *Penicillium*[14], as shown in table 1. *Calviceps purpurea* is perhaps the best-known species having been associated with epidemics of St Anthony's fire, a severe disease of humans, in the middle Ages. Ergot alkaloids have a tetracyclic ergovaline ring that has homology to neurotransmitters. Peptide alkaloids have strong affinity for adrenaline receptors while clavines and D-lyergic acid amides have strong affinity for serotonin receptors[14].

However, there is no standard method of analysis due to the chemical diversity of mycotoxins and matrices they are in. A need to obtain representative samples for quantitative analysis is essential. Also, reliable analytical methods are unavailable due in part by a deficiency in surveillance data for less known mycotoxins[15].

Detection Methods of Mycotoxigenic Fungi

Using immunological assays to detect mycotoxigenic fungi: Although polyclonal antibodies (PAbs) derived from immunizing animals have largely been superseded by the use of monoclonal antibodies (MAbs), this procedure can still provide the basis for useful assays. While the cost and time required developing MAb-based immunoassays is considerable, this is offset by the potential to produce, indefinitely, unlimited quantities of a specific MAb. A broad range of immunoassay formats have been developed, although the Enzyme-linked immunosorbent assay (ELISA) is the most widely used for the detection of plant pathogens[16]. Immunoassays have also been adapted for on-site field work in dip-stick or dot-blot formats providing a user friendly system for rapid pathogen detection and disease diagnosis[17]. More recently, lateral flow devices have been produced that simplify detection of the target molecule in a one-step procedure. In addition to detection, such immunoassays can be used for quantification of the target species.

Many antigens appear to be common to fungi of different species or genera such that polyclonal antibodies that are raised against a particular fungus may cross-react with another that is taxonomically distinct[17]. The specificity of the antibodies may reflect that of the antigens against which they were raised. For example, the immunodominant extracellular polysaccharide (EPS) antigens are more highly conserved than soluble macromolecules (exoantigens) obtained from surface washings of mycelium[18]. Such conservation may be exploited where fungi from more than one genus produce a particular mycotoxin. For example, the EPS of *Aspergillus* and *Penicillium* species both contain galactofuranoside residues so that antibodies raised against these targets may react to both. Indeed PAbs raised against culture filtrate of *A. parasiticus* cross-reacted with *Aspergillus* and *Penicillium* species but not with *Fusarium* species and a monoclonal antibody raised against *A. flavus* EPS also cross reacted with high specificity to *Aspergillus* and *Penicillium* species[18]. Polyclonal antibodies were raised to products of two genes, ver-1 and apa-2 involved in aflatoxin biosynthesis following their expression in *Escherichia coli*. Polyclonal antibodies to these chimeric proteins were highly specific towards *A. flavus* and *A. parasiticus* and did not cross-react with the other species tested.

Table-1
Fungal genera and species of major significance and their associated mycotoxins

Fungi	Mycotoxins
Aspergillus species (e.g. *A. flavus, A. parasiticus* and *A. nomius*)	Aflatoxins, predominantly B1 and B2 *A. parasiticus* and *A. nomius*) (*A. flavus*) and, in addition, G1 and G2 (*A. parasiticus*). B1 is metabolized to M1 which is excreted in milk of humans and animals
Aspergillus species (e.g. *A. flavus, A. parasiticus*, *A nidulans*)	Sterigmatocystin
Fusarium species (e.g. *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. sporotrichioides*, *F. poae* and *F. cerealis*)	Trichothecenes: Type A (e.g. T-2, HT-2, diacetoxyscirpenol (DAS), neosolaniol) produced by *F. sporotrichioides*, *F. poae* and/or *F. equiseti* Type B (e.g. nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives) produced by *F. graminearum*, *F. culmorum* and/or *F. cerealis*
Fusarium proliferatum and *F. verticillioides*	Fumonisins
Fusarium species (e.g. *F. proliferatum* and *F. avenaceum*)	Enniatins and beauvericin
Penicillium and Aspergillus species (e.g. *P. verrucosum*, *P. ochraceus* and *A. carbonarius*)	Ochratoxin A
Penicillium and *Aspergillus* species (e.g. *P. expansum*)	Patulin
Penicillium species	Citrinin and roquefortine
Claviceps purpurea, *Aspergillus*, *Penicillium* and *Acremonium* species	Ergot alkaloids
Alternaria species	Alternariol, altertoxin, tenuazonic acid

Polyclonal antibodies raised to soluble protein fractions of *F. culmorum*, detected *F. culmorum*, *F. graminearum* and *F. poae* when used in an ELISA but did not cross-react with *Microdochium nivale* or Tapesia species[19]. However, the assay could not differentiate between the three *Fusarium* species. In a separate report, PAbs raised against *F. graminearum* and *F. sporotrichioides* were found to cross-react with other *Fusarium* species while those to *F. poae* were species specific[20]. Samples of grain of hard and soft wheat suffering from FHB were assayed with the *F. sporotrichioides* antiserum and the results found to correlate well with the levels of ergosterol and DON. Monoclonal antibodies have been raised against a number of *Fusarium* species including *F. culmorum* and *F. avenaceum* and, more recently, *F. graminearum*[20].

However, they have been used only in preliminary studies for the detection of these fungi within plant tissues. While the MAb-based assays for detection of these *Fusarium* species are still under development, it is anticipated that ELISA based formats utilizing the monoclonal antibodies to the *Fusarium* species described above will be available in the near future for the detection of these pathogens in plant tissues. In addition to disease diagnosis, antibodies have considerable potential in epidemiological studies. For example, fluorescently labeled antibodies to *Fusarium vasinfectum* have been used to detect the pathogen in host tissues and soil.

A third method, phage display libraries, has been developed for the production of antibodies[21]. The gene segments encoding the light and heavy variable antibody domains are rearranged in vitro which, when expressed in E. coli, results in the production of a single polypeptide chain that forms a functional antibody fragment (scFv). The whole sequence can be expressed, fused to the minor coat protein pIII, on the surface of a filamentous phage. Populations (libraries) of phage have been produced containing in excess of 108 different scFv clones[22]. There are very few reports of the use of this approach to produce assays to detect mycotoxigenic fungi but a scFv has been used to detect melanin within *Alternaria alternata*[22, 23].

Nucleic acid hybridization: While relatively few assays have been developed for the immunological detection of mycotoxigenic fungi, a large number of assays have been produced to detect these fungi on the basis of nucleic acid sequences that are specific to the target organism. Nucleic acid hybridization assays involve the selection, cloning and chemical labeling (e.g. biotin, digoxigenin, 32P) of sequences specific to the target organism. These are then used as probes to detect RNA or DNA of the pathogen in extracts or tissue squashes of plant material. The assay may involve immobilization and detection of nucleic acid on a membrane or, in some instances, utilize a microplate format similar to that used in immunoassays[24]. The development and use of nucleic acid hybridization assays to detect and identify plant pathogenic fungi has been limited, although species-specific DNA probes have been developed towards several *Fusarium* species, including *F. culmorum*, *F. graminearum* and *F. avenaceum*[25]. Hybridization is relatively insensitive and there are few instances where this method has been used to detect fungi directly in extracts from plant tissues. Hybridization assays are sufficiently sensitive for the identification of fungi cultured from plant tissue (bio-amplification) but this, of course, incurs

the problems associated with selection during isolation and greatly increases the time required to complete the analysis.

Polymerase chain reaction (PCR)-based assays for detecting mycotoxigenic fungi: In contrast to hybridization, the PCR has found widespread use throughout plant pathology, and a significant number of PCR-based assays have been developed for use with species associated with the production of mycotoxins[26]. PCR can also be used in a one- or two-step reverse-transcriptase (RT) PCR protocol to detect genes that are being expressed rather than to detect the presence of the DNA that encodes a particular gene. Reverse transcriptase is used to generate a DNA copy (cDNA) from RNA (mRNA or total RNA) present in a sample. The cDNA is then subjected to PCR using primers designed for the target sequence. When designed to genes characteristic of a particular species or to genes involved in mycotoxin biosynthesis and assayed in a real-time or competitive PCR format the mRNA level of target genes can be estimated in a manner similar to that for DNA[27].

Detection of mycotoxigenic species by PCR: A number of approaches have been taken to develop assays with the desired level of specificity, whether to detect a single species or to detect strains from different species with the potential to produce a common mycotoxin. Two broad approaches have been adapted to developing assays to detect individual fungal species. The first has been by using 'universal' primers to isolate specific DNA regions followed by comparison of the sequence of the target species with that of other species within databases. The coding portions of many fungal 18S, 5.8S and 28S rDNA genes are highly conserved and primers to these regions have been generated[28]. These allow the isolation of the internal transcribed spacer sequences (ITS-1 and ITS-2), which lie between the coding regions, from a wide range of fungi. The ITS region is amplified from the target fungus and sequenced to identify regions of DNA unique to the fungus of interest. Polymorphism within the ITS region is generally at the level of species, rather than between isolates of the same species, making it an ideal target for the development of species-specific PCR assays. For example the ITS-1 and -2 of *F. avenaceum* was found to differ markedly from that of *F. graminearum* and *F. culmorum* and primers to this species were obtained which did not cross-react with DNA from a range of other fungal or plant species[29]. Where such species are not associated with a particular plant or plant product, such reactions may not pose a practical problem, but they may limit the use of the assay on other crops or food products. More highly conserved regions within the ITS is suitable for designing primers to differentiate one genus from another such as was done for *Fusarium*[30].

In instances where the ITS region is of limited use, other characterized regions of the genome may be used. For example, galactose oxidase is produced by only a few fungal species and used this to produce PCR primers specific to *F. graminearum* based upon the sequence of the galactose oxidase (gaoA) gene[31]. As the amount of sequence information from fungi deposited in databases increases, the number of potential targets for the design of PCR assays based upon characterized genes will increase. An alternative approach is to develop assays based upon polymorphisms detected within anonymous DNA regions. Two methods are commonly employed to detect such polymorphisms between fungal species. Random amplified Polymorphic DNA (RAPD) analysis has been used by a large number of workers to study variability within and between species, including many *Fusarium* species (*F. graminearum*, *F. cerealis* (*F. crookwellense*), *F. venenatum*, *F. torulosum*, *F. sambucinum* and *F. proliferatum*) [31].

The second method to generate anonymous markers is the amplified fragment length polymorphism (AFLP) assay[32]. This method has been used to develop a species-specific assay for *A. ochraceus* that did not cross-react with other *Aspergillus* or *Penicillium* species tested[33]. The development of truly species-specific PCR assays for mycotoxigenic fungi is entirely dependent upon a robust taxonomic foundation. Primer sets should be tested against closely related species to ensure specificity. Unfortunately, it is not always obvious from traditional, morphology-based taxonomies to determine which species are closely related. For example PCR assays to *F. avenaceum* designed from ITS and RAPD sequences were found subsequently to cross-react with *F. tricinctum*[34]. Molecular analyses are also revealing, sometimes, unsuspected distinctions within species. For example, sub-groups/lineages have been recognized within *F. graminearum*[35]. Sequence analysis has revealed that the product of the Fg16F/R primer pair is diagnostic of the lineage/groups tested to date and hence this primer pair may be used to detect *F. graminearum* and simultaneously determine lineage/group[36]. Furthermore, because particular lineages/groups appear to be associated with geographic regions and mycotoxin chemotypes, the assay could be used to aid the detection of migrants and monitor pathogen movement. Although a number of PCR assays have been developed against mycotoxigenic species there are relatively few reports of their use to detect pathogens in plant material[37]. However, PCR assays are sufficiently sensitive that *Fusarium* and other cereal pathogens can be detected, even in symptomless plants[38]. Thus species-specific PCR-based assays to mycotoxigenic species are potentially both specific and highly sensitive.

Using mycotoxin biosynthetic gene clustering for identifying mycotoxins: Many mycotoxin biosynthetic genes are present within gene clusters, and some of these appear to have undergone horizontal transfer from one species to another and are now present in several species[39]. If the sequences of genes associated with the biosynthesis of particular mycotoxins are available then a more generic approach is possible. Regions of homology within mycotoxin biosynthetic genes from the different species can be used to develop primers to detect the genes from all of the relevant mycotoxigenic species. Where mycotoxin biosynthetic genes are present in clusters, identification of one gene frequently facilitates the identification

of other genes involved in the pathway. Such an approach has been broadly successful for a number of mycotoxins.

Trichothecenes: Although our understanding of the biosynthesis of trichothecene mycotoxins is incomplete, many steps in the pathway are known, and a large number of genes that are involved in trichothecene biosynthesis have been isolated[40]. While a significant number of genes appear to be present within a 23 kb gene cluster, several genes involved in trichothecene biosynthesis lie outside this region, and it is not known whether they form a second cluster or are present within a distal portion of the same cluster[40]. The first gene in the pathway (*Tri5*), which encodes trichodiene synthase, is highly conserved among trichothecene-producing *Fusarium* species[41]. This has facilitated the development of PCR assays to detect species that produce trichothecenes[41]. Generic assays to detect trichothecene producing *Fusarium* species have also been developed to other genes within the cluster. *Tri6* encodes a transcriptional regulator of trichothecene biosynthetic genes and a PCR assay to this gene has been used to detect *F. gramine*arum in cornmeal and to differentiate it from a fumonisin producing species (*F. verticillioides*)[42]. In addition, RT-PCR assays have been developed against *Tri5* to study regulation of trichothecene biosynthesis in *F. culmorum* and *F. graminearum*. These assays have been used to study biosynthesis of trichothecenes during colonization of cereal hosts and in response to fungicides[43].

Generic assays to detect trichothecene producing *Fusarium* species have limited use with respect to risk assessment and the focusing of additional analysis. The most common trichothecene in blighted grain is DON which often occurs along with acetylated derivatives (3-ADON or 15-ADON) that are less toxic[43]. In other instances the predominant toxin produced by the isolate or species present may be Nivalenol (NIV). Analysis of 28S ribosomal DNA sequences revealed that type-A and type-B producing species clustered together in two monophyletic groups.

Recent works show that *Tri13* and *Tri7* genes responsible for the conversion of DON to NIV and the acetylation of NIV to 4-acetyl nivalenol (4-ANIV) respectively. Both genes are non-functional in DON producing isolates[44]. A PCR assay to *Tri7* based on product size polymorphism between DON and NIV chemotypes of *F. graminearum* has been described [44]. The PCR results obtained from *F. graminearum* and *F. culmorum* isolates concurred with known toxin production in all cases. Understanding the molecular genetic basis of trichothecene biosynthesis has been used to develop assays with differing specificities to detect species capable of trichothecene production or to producers of a particular class of toxin or even a specific toxin.

Fumonisins: The first fumonisin biosynthetic gene to be isolated, *Fum5* from *F. verticillioides*, encodes a polyketide synthase[45]. As with many other mycotoxins, the genes involved in fumonisin biosynthesis appear to be clustered[45]. At least 15 genes within the fumonisin cluster are co-regulated and have patterns of expression that correlate with production of fumonisin. Some of these genes provide potential targets for the development of additional assays to detect fumonisin producing species. A PCR assay developed to sequence from this gene has been used in a multiplex format along with assays to *Tri5* and ITS to provide genus-level detection combined with determination of mycotoxin production potential (trichothecene and fumonisin).

Aflatoxins and sterigmatocystin: Twenty five transcripts were found to be co-regulated within the sterigmatocystin gene cluster of *Aspergillus nidulans*[46]. Although the arrangement of the genes within the aflatoxin biosynthetic cluster is different, many of the gene products are conserved at the amino acid level. Recently, it was shown that 24 genes were differentially expressed during aflatoxin biosynthesis by *A. flavus* and *A. parasiticus*[46]. The sequence of many of the genes in both pathways is known, and this information has been used to design a number of PCR assays to detect aflatoxin producing fungi and to monitor biosynthesis of aflatoxins. The former assay targeted *nor-1, ver-1* and *omt-A* while the latter selected *ver-1* and *omt-1* and a regulatory gene, *apa-2* (now called *aflR*) [47]. The *omt-1* (*omt-A*) gene encodes the O-methyltranferase that converts sterigmatocystin to O-methylsterigmatocystin, and its inclusion in multiplex assays can be used to differentiate between fungi that produce aflatoxins and those that can only synthesize sterigmatocystin. Both assays were broadly successful in differentiating aflatoxin-producing isolates from other fungi, but difficulties were encountered in obtaining the required level of specificity.

A real-time PCR assay has been developed towards *nor-1* to detect aflatoxigenic fungi, but in all probability this assay will also be unable to differentiate between non-aflatoxin and aflatoxin producing isolates. Furthermore, this assay was not tested against sterigmatocystin producing *Aspergillus* species. A real-time RT PCR assay has been reported for *pks-A* and *aflR* and has been used to study the effect of nutritional factors and inhibitors of aflatoxin production on these two genes. A second gene (*aflJ*) is involved in the regulation of aflatoxin biosynthesis. This gene has not been identified in the sterigmatocystin gene cluster, indicating that production of these toxins may be differentially regulated. This gene may provide a suitable target for differentiating between fungi producing the two types of mycotoxin.

Patulin: The biosynthesis of patulin involves at least ten biosynthetic steps and has been relatively well characterized at the biochemical level[48]. In contrast the organization of the genes involved in patulin biosynthesis is not known. A PCR assay was, however, designed to the iso-epoxydon dehydrogenase (*IDH*) gene from the patulin biosynthetic pathway of *Penicillium expansum*[48]. The assay also reacted to all isolates of *P. brevicompactum*, although no patulin was detected in cultures from this species. It was suggested that isolates of this species might produce patulin under different conditions and evidence

was presented to support this in later work. The primers to the *IDH* gene have also been shown to amplify from *Aspergillus* species able to produce patulin and thus may provide a generic tool to detect patulin producing species. A second gene involved in biosynthesis of patulin has also been targeted for use in PCR assays. A portion of the 6-methylsalicylic acid synthetase gene (*6-MAS*), the first step of the patulin biosynthetic pathway, has been cloned from *P. expansum*, but a PCR assay for the specific detection of this gene from patulin producing species has yet to be reported.

Ergot alkaloids: The first step in the biosynthesis of ergot alkaloids is the formation of 4-dimethylallyltryptophan (DMAT) from L-tryptophan and dimethyl-allyldiphosphate. This step is catalyzed by DMAT synthase (DMATS) encoded by the gene cpd1[49]. PCR primers designed towards this gene have been shown to amplify a product from *Penicillium* and *Claviceps* species able to produce clavine alkaloids.

Combination assays and alternatives to PCR: PCR is reliant upon the isolation of DNA of the target species from contaminating substances that may compromise the reaction. A combination of antibody and PCR assays can be used to both purify and concentrate the target in order to enhance sensitivity. Immunocapture PCR uses antibodies to purify the target organism from complex substrates[50]. The DNA is isolated from the bound organism and used in subsequent PCR assays. The antibody step aids purification, thus enhancing sensitivity while the specificity resides in the PCR component of the assay. While the majority of antibodies to mycotoxigenic fungi react to several species or genera, when combined with species/mycotoxin specific PCR assays they may be used for the sensitive, specific detection of mycotoxigenic species within complex substrates. Immunocapture-PCR has been used to detect phytopathogenic bacteria and may be useful for detecting resting structures, such as chlamydospores, in soil[50]. The ligase chain reaction (LCR) is an alternative to PCR that also employs a temperature cycling reaction[4]. In this process oligonucleotide primers anneal to adjacent points in the DNA of the target organism. The primers are then ligated using a thermostable DNA ligase. If the target DNA is present, the primers anneal and are covalently joined by the ligase. The new molecule then acts as a template for a second set of complementary primers which are included in the reaction. The ligated product is exponentially produced in a process analogous to that of PCR. LCR can exploit single base pair differences between the target organism and other species if the primers are designed such that the 3' end of one of them is complementary to the critical nucleotide. Although LCR has not been used, to date, for the detection of myotoxigenic fungal species it has been used for the detection of bacterial plant pathogens including *Erwinia* stewartii.

A number of other nucleic acid-based technologies have been developed and these may also be of use in studies involving *Fusarium* species. Nucleic acid sequence-based amplification (NASBA) or self-sustained sequence replication (SSR) employs the concerted action of three enzymes (Rnase H, AMV RT and T7 RNA polymerase) to amplify RNA targets in an isothermal reaction. This assay has been used to detect RNA of the *Fum5* gene involved in fumonisin biosynthesis and *Tri5* from trichothecene producing *Fusarium* species[51]. NASBA, however, like RT-PCR is only able to detect fungi when they are actively producing mycotoxins and is thus of limited use where the fungi are quiescent or growing under conditions not suitable for mycotoxin production.

Conclusion

Although PCR is a very sensitive assay, various limitations have hindered the widespread uptake of this technology for the control of plant disease for the detection of mycotoxigenic fungi. The plant material has to be processed to extract and purify the DNA. The DNA is then subjected to PCR and, unless using 'realtime', then PCR products are size separated prior to analysis. Sample sonication can greatly speed up the DNA extraction process. Sonication has been successfully used to prepare DNA of *Fusarium* species from wheat grain for PCR in only five minutes[52]. Immunocapture of the pathogen or DNA offers an alternative, rapid method to purify DNA for PCR analysis. Recently, portable PCR machines have been developed and are now being marketed (e.g. Smart Cyler® II TD (transportable device) system). This device comprises 16 independently programmable modules and incorporates four-colour real-time PCR detection. This allows simultaneous detection of several target organisms, even where the PCR protocols differ. The advent of DNA microarrays is poised to revolutionize many aspects of plant pathology, disease diagnosis and disease management, including the detection of mycotoxigenic fungi. Microarrays consist of DNA or oligonucleotides bound (or synthesized), at discrete locations, on a glass slide. Originally, DNA microarrays were designed to permit analysis of gene expression of a large part or the whole genome (transcriptome) in a single assay. DNA arrays on membranes require more reagents but are a step towards true microarrays. In a further advance, the detection of hybridization can be achieved electronically[53]. Electronic detection of hybridization may permit further miniaturization of equipment and increase portability of assays. An alternative form of microarray consists of biotinylated antibodies immobilized onto streptavidin-coated slides. Where antibodies are available to both the mycotoxin producing species and to the mycotoxin itself, it is possible to detect the organism(s) and the metabolites in a single assay. Flow-through devices currently permit the use of 88 capture antibodies and provide analysis within minutes rather than hours. While improved, rapid DNA extraction procedures, 'real-time' PCR and the development of portable PCR machines enhance our ability to detect and quantify mycotoxigenic fungi the future lies with the microarray technology. The physical capacity of current DNA microarrays would permit all mycotoxigenic fungi to be detected in a massive parallel analysis of any plant tissue or product[54]. The

robustness of such assays could be increased by inclusion of anonymous species-specific DNA fragments and phylogenetically informative sequence alongside sequence for genes involved in mycotoxin biosynthesis. Whatever the assay, it relies upon robust phylogenetic information and a detailed understanding of the molecular genetic basis of mycotoxin biosynthesis.

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