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**Review**

# PCR-based detection and quantification of mycotoxigenic fungi*

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Mycotoxins are secondary metabolites produced by many phytopathogenic and food spoilage fungi including *Penicillium*, *Fusarium* and *Aspergillus*. The toxicity and carcinogenicity of many of these mycotoxins, and their potential to contaminate foods and animal feedstuffs is a cause of serious concern globally, both from a food safety and food trade standpoint. Thus the rapid identification of mycotoxigenic fungi would be desirable, such that early intervention steps could be applied to help limit the amounts of contaminated materials, particularly cereals and cereal-based products, gaining access to the human food chain. With this in mind a number of PCR-based methodologies have been developed for the identification of mycotoxin biosynthetic genes in different fungal genera, together with assays developed using other genes or random amplification of polymorphic DNA (RAPD) methodologies for the identification of specific toxigenic fungi. In addition, reverse transcription (RT)-PCR, competitive PCR and Real Time quantitative PCR methodologies have also been developed for this purpose. The development of each of these techniques, their usefulness, limitations and adaptability will be discussed together with descriptions of specific examples where these techniques have been utilised in different experimental settings.

## INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi which, when ingested by higher animals, cause a toxic response, mycotoxicosis. The primary genera responsible for mycotoxins found in the human food chain are *Aspergillus*, *Fusarium* and *Penicillium* (Table 1). Some *Fusarium* species are plant pathogens which can produce mycotoxins within infected plant material. They can also produce mycotoxins in plant produce under adverse storage conditions. *Aspergillus* and *Penicillium* are predominantly storage fungi, which grow and produce mycotoxins on stored foodstuffs under adverse storage conditions. Humans can ingest mycotoxins directly from plant material contaminated with mycotoxins, or indirectly by the consumption of meat or milk from animals fed on mycotoxin-contaminated feed.

There are over 300 reported mycotoxins although only about 20 of these are thought to be relevant to public health (Geisen 1998). The mycotoxins have a diverse range of structures from single heterocyclic rings to irregular six or eight membered rings (ApSimon 1994). This diverse range of structures results in a diverse range of toxic effects, which include neurotoxic, nephrotoxic, hepatotoxic, immunosuppressive and teratogenic (Hussein & Brasel 2001, Creppy 2002).

Mycotoxins have caused major epidemics in man and domesticated animals throughout historical times. Ergotism is believed to have caused thousands of deaths and reached epidemic proportions in the Middle Ages (Bove 1970). Alimentary toxic aleukia resulted in the deaths of thousands of Russians between 1942 and 1948 as a result of the consumption of cereal grains infected by *Fusarium* spp. which had been over-wintered in the field (Joffe 1986). Aflatoxins are probably the most damaging mycotoxins to human health in the world today. It is estimated that the number of deaths in Indonesia due to aflatoxin induced liver cancer is 20K $y^{-1}$ (Lubulwa & Davis 1994).

The serious consequences of mycotoxin contamination create a demand for fast and reliable techniques to detect mycotoxins and mycotoxin-producers within foodstuffs. A number of highly sensitive techniques have been developed to detect mycotoxins. These include high performance liquid chromatography (HPLC), gas chromatography – mass spectrometry (GC-MS) and immunological assays such as ELISA. In contrast, de-

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**Table 1.** Important toxigenic species and mycotoxins found in foodstuffs.

| Species | Commodity | Mycotoxin |
|---|---|---|
| *Penicillium expansum* | Fruit juice | Patulin |
| *Aspergillus flavus*, *A. parasiticus* | Dairy products | Aflatoxin M1 |
| *P. verrucosum*, *A. ochraceus* | Porcine based products, Beer, Coffee | Ochratoxin A |
| *Fusarium graminearum*, *F. culmorum* | All cereals | Type B Trichothecenes and Zearalenone |
| *F. poae* | Small grain cereals | Type A Trichothecenes |
| *F. proliferatum* | Maize | Moniliformin |
| *F. moniliforme* | Maize | Fumonisins |

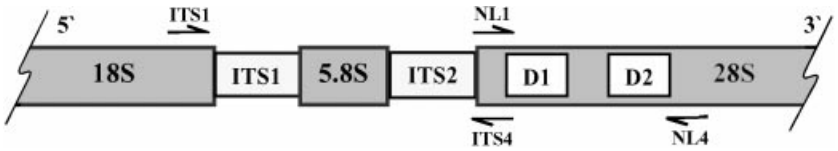


**Fig. 1.** Map of typical fungal rRNA genes showing the positions of variable regions and universal primers designed to amplify these regions. ▨, conserved; and □ variable regions of DNA. Adapted from White *et al*. (1990).

tection of mycotoxigenic fungi has relied on traditional isolation and culturing techniques. These techniques are time consuming and require taxonomical expertise. Even with expertise, identification is particularly difficult within *Fusarium* genus, which contains a large number of closely related species and is classified entirely on morphological characteristics (Gams & Nirenberg 1989). This is further confused by the numerous and often conflicting taxonomic treatments of the genus (Booth 1971, Gerlach & Nirenberg 1982, Nelson, Toussoun & Marasas 1983). It is clear from the literature that isolates of *Fusarium* are frequently misidentified (Marasas, Nelson & Toussoun 1984, O'Donnell 1997, O'Donnell, Cigelnik & Casper 1998, Niessen & Vogel 1998). The same is true for *Penicillium*, where reproductive structures are small and ephemeral (Pitt & Hocking 1997). This has important implications when dealing with mycotoxigenic species which can produce an array of different mycotoxins and secondary metabolites.

The polymerase chain reaction (PCR) is a powerful method that has revolutionised molecular biology since its development in the mid-1980s (Mullis, Ferre & Gibbs 1994). PCR allows the amplification of specific fragments of DNA from complex DNA samples. The resulting PCR product can be observed after gel-electrophoresis and staining with a DNA binding fluorescent dye such as ethidium bromide. A number of PCR-based techniques allow the taxonomic status of a fungal isolate to be determined. Amplification and direct sequencing of ribosomal DNA (rDNA) was one of the first applications of PCR within mycology (White *et al*. 1990). Ribosomal DNA genes have conserved and variable regions and are useful for the study of distantly related taxonomic groups (genera and above). The two internal transcribed spacer (ITS) regions are variable and are frequently used to distinguish at the species level. Universal primers are available which allow the ITS regions to be amplified using conserved sequences from within the rDNA gene sequences (Fig. 1). Restriction analysis of PCR amplified fragments is a rapid and inexpensive way to identify fungal species (Edel *et al*. 1997). The other common method to distinguish fungal species is by Random Amplified Polymorphic DNA (RAPD) analysis. This technique uses a single short oligonucleotide under low stringency conditions which enables the primer to anneal at a number of sites along both DNA strands allowing the amplification of a collection of variously sized DNA fragments which after gel electrophoresis provides a DNA fingerprint (Ouellet & Seifert 1993). The techniques above can be used to elucidate the identity of an unknown fungal isolate in pure culture, but they cannot be used to detect the presence of a particular fungal taxon within a crop plant or foodstuff. For this a PCR assay must be developed using oligonucleotide primers specific to the particular fungal taxon that requires detection. These are either designed from polymorphic regions of DNA from sequence alignments of target and non-target organisms (Aoki & O'Donnell 1999) or from DNA bands within a RAPD fingerprint, which are unique to the target organism (Parry & Nicholson 1996). Once unique bands have been detected they are usually used as probes to check the presence of similar DNA within related species. If the DNA does not cross-react it is sequenced and primers designed from the sequence. Primers designed by either method are tested against a number of isolates of the target taxon, preferably from a wide geographical range, and a number of closely related non-target taxon (Edel 1998).

Various PCR-based techniques have been developed to detect and quantify mycotoxigenic fungi. These fungi have been detected based on specific target DNA from mycotoxigenic genes, other genes, ribosomal DNA

or unique DNA bands from random amplified polymorphic DNA (RAPD) analysis.

## DETECTION OF MYCOTOXIGENS

### *Aflatoxins*

Aflatoxins are potent mutagenic, carcinogenic and teratogenic metabolites produced mainly by *Aspergillus flavus*, *A. parasiticus* and *A. nominus*. They are difuranocoumarin derivatives (Fig. 2). The four main naturally produced aflatoxins are $B_1$, $B_2$, $G_1$ and $G_2$; the B and G nomenclature derive from the blue and green fluorescent colours produced under UV light on thin layer chromatography plates. Foods and animal feeds, particularly in warmer climates are susceptible to invasion by aflatoxigenic *Aspergillus* species with aflatoxins typically being produced before harvesting, during processing, or transportation, or in storage. Aflatoxin $B_1$ ($AFB_1$) is the aflatoxin found at the highest concentration in contaminated food and feed with *A. flavus* being reported to produce aflatoxins in commodities such as peanuts, maize, wheat and cottonseeds. The presence of aflatoxins in pistachio nuts, groundnuts and figs is

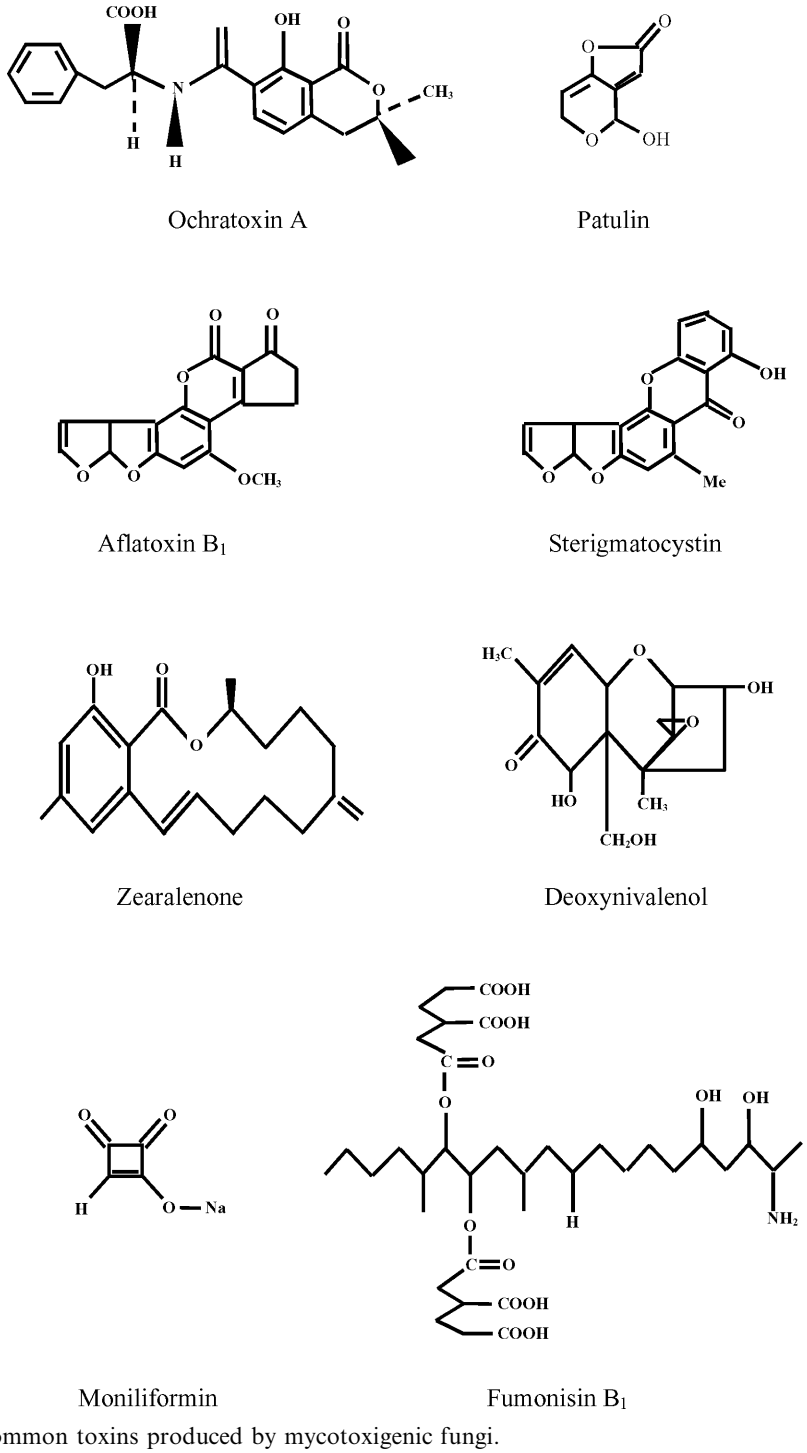


**Fig. 2.** Structure of common toxins produced by mycotoxigenic fungi.

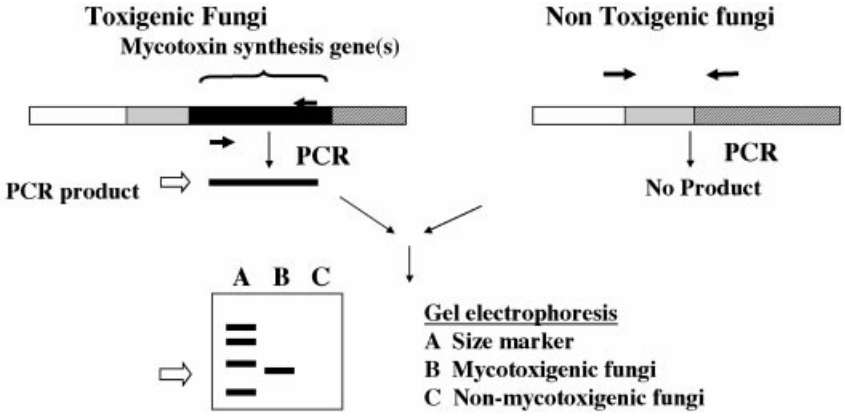


**Fig. 3.** Use of the polymerase chain reaction (PCR) for detection of mycotoxigenic fungi.

currently causing serious concerns globally, both from a food safety and food trade standpoint. $AFB_1$ is regarded as the most potent liver carcinogen known for a wide variety of animal species, including humans. Dietary exposure to $AFB_1$ has been associated with an increased incidence of hepatocellular carcinoma, particularly in populations in which exposure to hepatitis B virus is a common occurrence (Smela *et al*. 2001). As a result, maximum tolerance levels of between 5 and 20 $\mu g\ g^{-1}$ (5–20 ppb) are common in most countries, with Japan one of the world's largest importers of agricultural products having a legal limit of $AFB_1$ in foodstuffs of zero. Aflatoxin $M_1$ is a mono-hydroxylated derivative of $AFB_1$ that is formed and excreted in the milk of lactating animals including humans that have consumed $AFB_1$ contaminated material. Consumption of aflatoxin M contaminated infant milk and milk products is of concern and very low limits have been set (0.01–0.05 $\mu g\ kg^{-1}$) for infant foods; given their relatively high consumption rate of these products and the possible higher susceptibility of younger children to aflatoxins (Aksit *et al*. 1997).

For a PCR based detection method to be useful in the detection of aflatoxigenic fungi then unique genes need to be targeted which will be exclusively present in the fungus to be detected. Thus by designing primers which specifically amplify mycotoxin biosynthetic genes, these will result in their obtaining a positive PCR from DNA from the toxigenic fungus, while DNA from the non-toxigenic fungus will give no product (Fig. 3). The aflatoxin biosynthetic genes are useful targets in this regard as they will be present in aflatoxin producing fungi. Following the work of a number of research groups over recent years the aflatoxin biosynthetic pathway is now well understood (Bennett, Chang & Bhatnagar 1997) (Fig. 4). Synthesis begins with acetate and malonyl CoA, which are converted to a hexanoyl starter unit by a fatty acid synthase, which is then extended by a polyketide synthase to the first stable precursor of the pathway, norsolorinic acid. This polyketide then undergoes approx. 12–17 enzymatic conversions, through a number of pathway intermediates to eventually form aflatoxin B1, B2, G1 and G2. The aflatoxin gene cluster has now been fully elucidated, with many of the genes having been cloned and characterised (Fig. 4); for reviews see Woloshuk & Prieto (1998) and Sweeney & Dobson (1999). This has allowed the generation of specific PCR primers, which have been employed by a number of research groups in the detection of aflatoxigenic fungi. Using a multiplex PCR approach Geisen (1996), by targeting three aflatoxin biosynthetic genes namely norsolorinic acid reductase (*nor*-1), versicolorin A dehydrogenase (*ver*-1) and sterigmatocystin O-methyltransferase (*omt*-A), was able to observe a triplet banding pattern in aflatoxin producing strains of *Aspergillus flavus*, *A. parasiticus* and sterigmatocystin producing strains of *A. versicolor*. In a multiplex PCR system specificity is increased because a number of genes can be detected in one reaction. However, there were problems with the specificity of this particular system, because, while it did appear to distinguish between aflatoxigenic *Aspergillus* species and other non-aflatoxigenic food related species such as *Penicillium*, *Fusarium*, *Byssochlamys* and *Geotrichum*, it could not distinguish between aflatoxigenic and non-aflatoxigenic *A. flavus* strains. Another specificity problem centred on false positives obtained with *Penicillium roqueforti* which appeared to have cross-reacting PCR positive *nor*-1 and *ver*-1 homologues. Shapiro *et al*. (1996) outlined a similar diagnostic PCR method, again for the detection of aflatoxigenic fungi. In this case the target genes were the *omt*-1, the *ver*-1 gene and the regulatory gene *apa*-2 (since renamed *afl*R) which regulates expression of the aflatoxin biosynthetic gene cluster (Chang *et al*. 1993). Specific PCR products were obtained only with DNA from *A. parasiticus* with all three primer pairs, with the *afl*R primers failing to give a positive signal. This probably reflects the subtle sequence differences between the *afl*R gene in *A. parasiticus* (for which the primers were designed) and the *afl*R gene in *A. flavus* (Chang *et al*. 1995). Despite this no amplification products were obtained from *Fusarium*, *Penicillium* and non-aflatoxigenic *Aspergillus* species. Thus this procedure can be applied to selectively distinguish aflatoxigenic fungi from other fungi commonly found on grains. In addition the authors de-

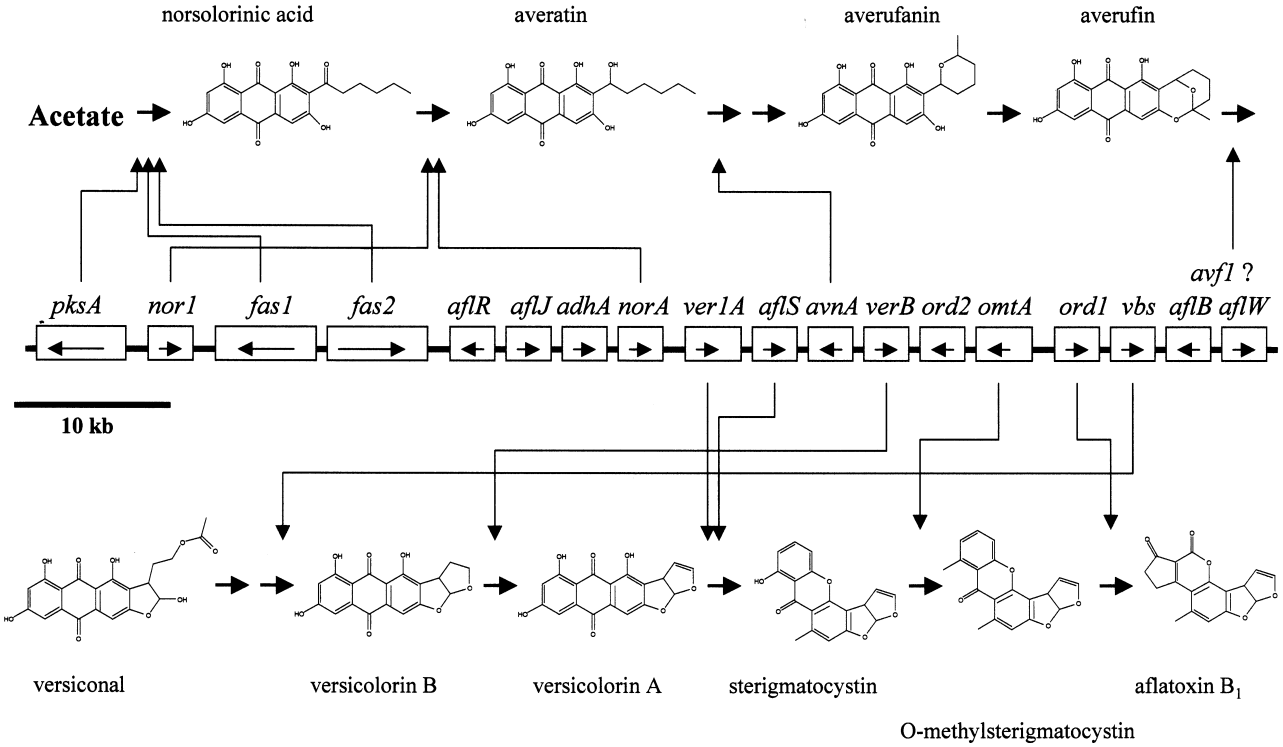


**Fig. 4.** The biosynthetic pathway for sterigmatocystin and aflatoxin $B_1$ biosynthesis by *Aspergillus flavus*. Biosynthetic pathway is outlined on the perimeter, with the genes encoding the enzymes involved depicted in boxes, with arrows indicating the position of each enzyme in the pathway. The direction of transcription of each gene is depicted by an arrow within the box. *Pks* A, polyketide synthase; *nor* 1, *nor* A, norsolorininc acid reductase; *fas*1, *fas*2, fatty acid synthases; *afl*R, transcription factor; *afl* J, unknown; *adh* A, alcohol dehydrogenase; *ver* 1A, *afl*S, *O*-methyltransferase; *avn* A, versiconal hemiacetyl acetate reductase; *ver* B, desaturase; *ord* 1, *ord* 2, *omt* A, *O*-methyltransferases; *vbs*, oxidase/dehydrogenase; *avf* 1 (*afl* B *afl* W), esterases. Adapted from Woloshuk & Prieto (1998).

scribed an enrichment procedure which involved suspending the corn samples in a potato dextrose agar for times ranging from 24 to 48 hours which increased the sensitivity of the PCR method allowing $1 \times 10^2$ *A. parasiticus* spores to be detected. Farber, Geisen & Holzapfel (1997) described a PCR-based method, which allowed the detection of aflatoxigenic fungi in contaminated figs. The *ver*-1, *nor*-1 and *omt*-A genes were targeted and the expected amplicons obtained from DNA isolated from infected figs, with uninfected figs giving no signal. There was some interference however when fig DNA was present in the reaction, with the sensitivity being reduced ten fold when compared with pure fungal DNA. Interference by inhibitory substances such as fats, proteins and carbohydrates with the sensitivity of a diagnostic PCR reaction is common. However, recent methods developed for the isolation of yeast DNA from various food matrices, such as repeated washes with phosphate buffered saline may help in this regard (Kosse *et al.* 1997).

### *Ochratoxin*

Ochratoxin A (OTA), produced by *Aspergillus ochraceus* and related species in tropical climates, is a derivative of isocoumarin linked to L-phenylalanine (Fig. 2). It is also widely produced by *Penicillium* spp., and particularly by *P. verrucosum*, in temperate regions. It has been detected in food products such as coffee (Mantle & Chow 2000) as well as in wine, beer and grape juice. OTA has been shown to be a nephrotoxic, hepatotoxic and teratogenic and has been classified as a LB cancer compound, being possibly carcinogenic for humans (Petzinger & Ziegler 2000, Lindsey 2002), leading to intake limits of below 5 ng $kg^{-1}$ body weight $day^{-1}$ being recommended. The degree to which the average European consumer is exposed to OTA is demonstrated by the regular detection of OTA in human milk (Skaug *et al.* 2001) and blood (Peraica *et al.* 1999). Unlike the aflatoxin biosynthetic pathway however, the biosynthetic steps involved in OTA production are not as yet well established. It is believed to be formed from a number of combined pathways. The isocoumarin group is a pentaketide skeleton formed from acetate and malonate via a polyketide pathway. A chlorine atom is incorporated most probably through the action of a chloroperoxidase to form the isocoumarin portion of OTA. A C1 unit is also added and oxidized to a carboxyl group at C-8. L-Phenylalanine derived from the shikimic acid pathway is linked through the additional carboxyl group (Moss 1998) (Fig. 5). To develop a PCR based molecular detection system for ochratoxin A the polyketide synthase gene in *Aspergillus ochraceus* has been targeted. Two pairs of primers (one degenerate pair) based on the ketosynthase

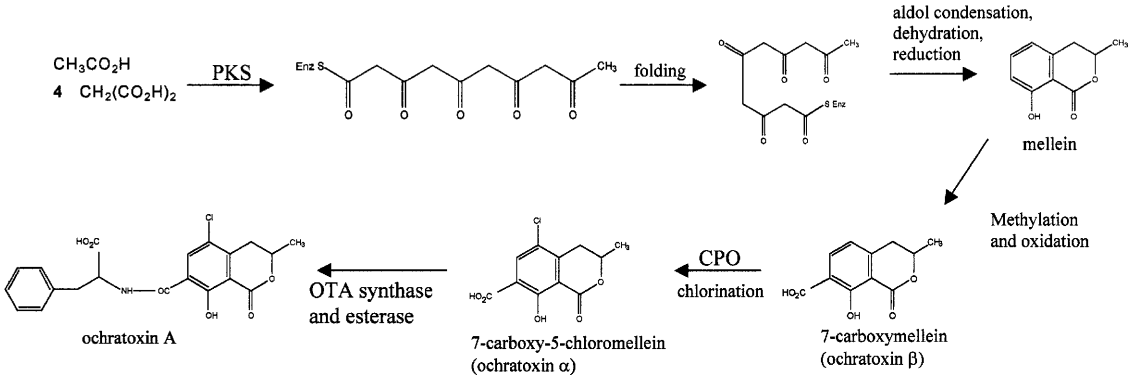


**Fig. 5.** Proposed biosynthetic pathway for ochratoxin A. PKS, polyketide synthase; CPO, chloroperoxidase. Adapted from Moss (1998).

(A)

| Partial ketosynthase domain alignment | Species | (mycotoxin) |
|---|---|---|
| SPSGRSRMWDAGADGYARGEAVCSVVLKTLSQA | *Aspergillus parasiticus* nonaketide synthase | (lovastatin) |
| SSDGRCHSFDDTASGYGRGEGAAVVILKNMAEA | *Aspergillus parasiticus* pksl2 | (unknown function) |
| SSDGISYSFDSRADGYGRGEGVAAIVLKTLPDA | *Aspergillus terreus* diketide synthase | (lovastatin) |
| SPDGRCYAFDERANGYARGEGVAVLLLKRLEDA | *Cochliobolus heterostrophus* pks1 | (T-toxin) |
| SPDASCKTFDANANGYARGEAINAIFLKPLNNA | *Giberella fujikoroi* Fum 5 | (Fumonisin) |
| STEGRCLSFDEDAKGYGRGEGAAVVILKRLSTA | *Aspergillus terreus* MSAS | (Patulin) |
| STEGRCLSFDEDAKGYGRGEGAAVVILKALSTA | *Aspergillus terreus* pksM | (unknown function) |
| SADGKCRSFDDSANGYGAGEGAGVVILKALEKA | *Penicillium griseofulvin* pks2 | (unknown function) |
| SPHGRSRMWDAGADGYARGEGYAAVALKRLSDA | ***Aspergillus ochraceus* pks Aopks 7** | |

(B)

| Partial ketosynthase domain alignment | Species | (mycotoxin) |
|---|---|---|
| GTAYCGIPSRISYLLDLMGPSVALDAACASSLV | *Penicillium griseofulvin* PKS | (unknown function) |
| GTAYCGVPNRISYHLNLMGPSTAVDAACASSLV | *Penicillium patulum* PKS | (patulin) |
| GTAYCGVPNRISYHLNLMGPSTAVDAACASSVV | *Penicillium. freii* PKS | (unknown function) |
| GTAYCGVPNRISYHLNLMGPSTAVDAACASSLV | *Aspergillus. terreus* PKS | (unknown function |
| GTAYCGVANRISYHLNLMGPSTAVDAACASSLV | *Aspergillus parasiticus* pksL2 | (unknown function) |
| GTAYCGVPNRISYHLNLMGPSTAVDAACASSLV | *Byssochlamys nivea* 6-MSAS | (unknown function) |
| GTAYCGVANRISYHLNLMGPSTAVDAACASSLV | ***Aspergillus ochraceus* AOPKS-J1** | |

**Fig. 6.** Partial alignment of the ketosynthase domains of fungal PKS genes with the cloned PKS PCR products from *Aspergillus ochraceus*. (A) AOPKS-7, (B) AOPKS-J1. Areas of dark background indicate common amino acids. The amino acid sequences were either experimentally determined or deduced from nucleotide sequences.

domain of previously characterised fungal polyketide synthases (PKSs): Primer pair A: (Forward GGGTGTTAACTCAGACGACTA and Reverse GCGATTTCGCCCACGGAGTGTC) and Primer pair B: (Forward GAGCAGATGGATCCCARCAGCG and Reverse GGAGATRGCYCCCCSGCTTTRTC) (where R = A+G, Y = C+T and S = G+C) were designed and employed using genomic DNA from *A. ochraceus* ATCC 22947. Both sets of primers produced products which, on the basis of sequence homologies appear to be from different genes. The PCR product amplified with the ketosynthase primer pair B (AOPKS-J1) was almost exactly homologous to the *A. parasiticus pks*L1 gene at the nucleotide level. The translated product displayed a high degree of homology to other fungal PKS protein sequences, including sequences from *Penicillium patulum*, *P. griseofulvin*, *P. freii*, *A. terreus* and *Byssochlamys nivea* (Fig. 6A). The PCR product amplified by primer pair A (AOPKS7) (designed to a different part of the ketosynthase domain sequence), was not homologous to other fungal PKS genes at the nucleotide level. The degree of homology was higher at the amino acid level but in general, appeared to be less well conserved than the AOPKS-J1 homologues (Fig. 6B).

Reverse transcription (RT)-PCR analysis with primers designed from the AOPKS-7 gene was performed on RNA harvested from *A. ochraceus* grown under both OTA permissive (Yeast Extract Sucrose) and non-permissive conditions (Yeast Extract Peptone). Results

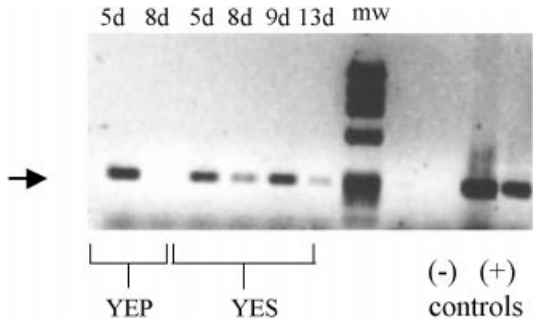


**Fig. 7.** Reverse transcription (RT)-PCR analysis of AOPKS-7 gene transcription under permissive (YEP) and non-permissive (YES) growth conditions. The 290 bp transcript is arrowed. In the (−) control no RNA was added to the RT-PCR reaction. (+) control contained DNA from a *aopks*-7 genomic template.

obtained indicated the presence of AOPKS-7 mRNA transcripts under both sets of growth conditions tested and revealed higher levels of transcription in *A. ochraceus* ATCC 22947 grown under non-permissive conditions for OTA production (Fig. 6). Preliminary Northern Blot analysis performed with AOPKS-J1 indicates similar findings; suggesting that while these *pks* genes are expressed in *A. ochraceus* it is likely that they are not involved in OTA biosynthesis, given that upregulation of expression of these *pks* genes would be likely under OTA permissive growth conditions. Therefore further work in this area focusing on the cloning of the OTA biosynthetic genes not only in *A. ochraceus* but in *P. verrucosum* will be required before a molecular-based detection system can be developed for OTA production.

### Fusarium *mycotoxins*

*Fusarium* is important within agriculture as it includes a number of important plant pathogens and they produce a wide assortment of mycotoxins. In their review of *Fusarium* metabolites, Vesonder & Goliński (1989) listed 48 trichothecenes, 12 zearalenone derivatives and 10 other mycotoxins. This list did not include the then, recently discovered fusarochromanone (Pathre *et al*. 1986) and fumonisins (Gelderblom *et al*. 1988), or the since discovered mycotoxins beauvericin (Gupta *et al*. 1991, Logrieco *et al*. 1998), sambutoxin (Kim & Lee 1994) and fusaproliferin (Santini *et al*. 1996). The *Fusarium* mycotoxins are primarily found in cereal grains, where the predominant mycotoxins are trichothecenes, moniliformin, fumonisins and zearalenone (D'Mello, Pacinta & Macdonald 1999). Many toxigenic *Fusarium* species have been associated with infected grains but the predominant pathogens found worldwide are *F. graminearum* and *F. culmorum*. These fungi cause fusarium head blight (scab) in small grains and gibberella ear rot in maize. These diseases are associated with the temperate grain growing regions. *F. graminearum* tends to be more dominant in warmer regions (North America and China) and *F. culmorum* is more dominant in cooler regions (northern Europe) (Miller 1994, Parry, Jenkinson & McLeod 1995). *Microdochium nivale* (syn. *F. nivale*) and *F. poae* are also common head blight pathogens in northern Europe (Parry *et al*. 1995). *M. nivale* produces no known mycotoxin (Logrieco *et al*. 1991).

The trichothecenes are a closely related group of sequiterpenes. They are classified based on substituent functional groups into A or B types dependent on the presence or absence of a keto group at the C-8 position. The structure of deoxynivalenol (DON), a type B trichothecene is shown in Fig. 2. Their primary mechanism of toxicity is inhibition of protein synthesis, although their biological effects are diverse. It is important to note that the toxicity of trichothecenes to experimental animals varies widely. For example, DON, which is the predominant trichothecene detected, is 14 times less toxic than nivalenol when administered intraperitoneally to mice (Ueno 1983). In humans, trichothecenes are thought to be the most important *Fusarium* mycotoxins. Large-scale human toxicosis has been reported from China, Russia, Japan and India (Yoshizawa 1983, Joffe 1986, Luo 1988, Bhat *et al*. 1989). Symptoms include anorexia, nausea, vomiting, abdominal pain, diarrhoea, and convulsions (Yoshizawa 1983). Pigs are the most sensitive farm animals to trichothecenes, resulting in feed refusal and vomiting (Williams, Blaney & Magee 1989). The US Food and Drug Administration has established advisory levels for DON at 1 $\mu g\ g^{-1}$ for finished wheat products for human consumption, 10 $\mu g\ g^{-1}$ for grain and grain by-products destined for cattle older than 4 months and chickens and 5 $\mu g\ g^{-1}$ for grain and grain by-products destined for pigs and all other animals (Trucksess *et al*. 1995).

Moniliformin, zearalenone and fumonisins are polyketide-derived secondary metabolites, although their biosynthesis is unclear. The recent sequencing of the fumonisin gene cluster [EMBL Accession No. AF1557-73] should lead to the elucidation of the fumonisin pathway. Moniliformin exists either as a sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (Fig. 2). Moniliformin was first isolated from a culture of *F. proliferatum*, which had been misidentified as *F. moniliforme* (Cole *et al*. 1973). Since then, many *Fusarium* spp. have been identified as producers of moniliformin. Of these, many are important pathogens of cereals, *F. proliferatum* and *F. subglutinans* being of particular importance on maize (Marasas *et al*. 1986, Schütt, Nirenberg & Deml 1998). Moniliformin is a potent cardiotoxic mycotoxin causing cardiac failure in broiler chickens injected with 1 $\mu g\ g^{-1}$ body weight (Nagaraj *et al*. 1995). The acute and long-term effects of moniliformin on human health are not known although it has been implicated as the aetiological agent of an endemic heart disease (Keshan disease) in regions of China (Zang & Li 1989).

Zearalenone is a non-steroidal oestrogen (Fig. 2). Its major metabolites, α- and β-zearalenol bind to oestrogen receptors causing hyperoestrogenism resulting in

various reproductive disorders. Pigs are particularly susceptible to zearalenone because their oestrogen receptors have a high affinity for α-zearalenol (Fitzpatrick *et al*. 1989). The actual toxicity of zearalenone is very low (Hidy *et al*. 1997), and consequently it has been suggested that zearalenone should be referred to as a mycoestrogen rather than a mycotoxin (Hagler *et al*. 2001). Zearalenone is produced primarily by *F. graminearum*, *F. culmorum*, *F. equiseti* and *F. crookwellense* and can be found in all cereal products (Placinta, D'Mello & Macdonald 1999).

Fumonisins consist of a 20-carbon aliphatic chain with two ester linked hydrophilic side chains (Fig. 2). This structure closely resembles sphingosine, an essential phospholipid within cell membranes. The toxic activity is a result of the competition with sphingosine in sphingolipid metabolism (Riley *et al*. 1996). The fumonisins were the most recent group of mycotoxins to be identified after they were found to be the cause of the fatal syndrome, equine leucoencephalomalacia (Kellerman *et al*. 1990). They have since been shown to have numerous toxic effects in animals (Shephard 2001). A high level of fumonisin within the human diet has been linked to high incidence of oesophageal cancer in South Africa (Rheeder *et al*. 1992) and China (Chu & Li 1994). The major producers of fumonisins are *F. moniliforme* and closely related species. Maize is the only significant food source contaminated by these mycotoxins (Placinta *et al*. 1999). Guidance levels for fumonisins were recently set by the US Food and Drug Administration (Trucksess 2001). Levels for total fumonisins ($B_1 + B_2 + B_3$) range between 2 and 4 $\mu g\ g^{-1}$ for human foods depending on the product and between 5 and 100 $\mu g\ g^{-1}$ for animal feeds, depending on the animal.

Various PCR-based techniques have been developed to detect and distinguish between *Fusarium* spp. These fungi have been differentiated by either mycotoxigenic genes, other genes, ribosomal DNA or unique DNA bands from random amplified polymorphic DNA (RAPD) analysis.

Restriction analysis of PCR amplified DNA (PCR-RFLP) has been used to distinguish a large number of *Fusarium* species. Bateman, Kwaśna & Ward (1996) distinguished 18 *Fusarium* haplotypes using eight restriction enzymes to cut a PCR product of approximately 600 bp consisting of ITS1, 5.8S and ITS2 ribosomal DNA. Edel *et al*. (1997) amplified a further 600 bp into the 5′ end of the 28S rDNA gene, which includes the variable domains D1 and D2 (Fig. 1), and were able to distinguish a further five *Fusarium* haplotypes. Neither of the methods could distinguish between *F. crookwellense*, *F. culmorum* and *F. graminearum*, indicating they are closely related. Schilling, Möller & Geiger (1996) sequenced the ITS regions and showed that the ITS1 regions of *F. graminearum* and *F. culmorum* were identical and species-specific primers could not be designed, based on the minor differences found in the ITS2 region.

Sequencing of the variable domains at the 5′ end of the 28S rDNA determined that trichothecene-producing *Fusarium* species could be resolved into two monophyletic groups (Mulè *et al*. 1997). These clades consisted of type A and type B trichothecene-producers and both clades could be readily distinguished from a third group of trichothecene non-producers. Such a system could be used to rapidly identify the trichothecene status of unidentified *Fusarium* isolates. The phylogenetic distinction between Type A and Type B trichothecene-producers was clarified further by a study aimed at reclassifying the identity of the Quorn mycoprotein fungus. Phylogenetic analysis of 28S, ITS and β-tubulin DNA sequences of the Quorn mycoprotein isolate were all shown to be identical to *F. venenatum* sequences and occurred within a Type A trichothecene cluster and it was therefore not an isolate of *F. graminearum*, as previously identified (O'Donnell *et al*. 1998). RAPD analysis published the same year (Yoder & Christianson 1998) also concluded that the Quorn mycoprotein fungus was a *F. venenatum* isolate. A number of mycotoxigenic Japanese *Fusarium* isolates were identified by sequence analysis to represent a third cluster, more closely related the Type A trichothecene cluster than the Type B cluster, even though they produce nivalenol (O'Donnell 1997).

Grimm & Geisen (1998) developed a PCR-ELISA assay specific to fumonisin-producing *Fusarium* spp., based on the ribosomal ITS1 sequence. After amplification of a product by PCR a third internal oligonucleotide probe was required to achieve the required specificity.

RAPD analysis has been used for many years to distinguish between pure cultures of *Fusarium* spp. (Oeullet & Seifert 1993, Hering & Nirenberg 1995, Voight, Schleier & Bruckner 1995, Amoah *et al*. 1996, Yli-Mattila 1996, Altomare *et al*. 1997) and isolates (Yli-Mattila 1996, Kerenyi *et al*. 1997, Satyaprasad, Bateman & Ward 2000, Walker *et al*. 2001). RAPD analysis of *F. moniliforme* isolates was able to distinguish between low and high fumonisin producers (Jiménez *et al*. 2000). It is not known if these groups correlate to the *Gibberella fujikuroi* mating populations F and A respectively, which are reported to have different abilities to produce fumonisins (Leslie *et al*. 1992, Leslie *et al*. 1996). Other DNA-based techniques have successfully been used to distinguish *Fusarium* spp. in pure culture, including Restriction Fragment Length Polymorphism (RFLP) (Benyon, Burgess & Sharp 2000), Amplified Fragment Length Polymorphism (AFLP) (Leissner, Niessen & Vogel 1997) and Single Strand Conformational Polymorphism (SSCP) (Rogers, Clapp & Jeffries 2000).

Species-specific primers have been designed for a number of toxigenic *Fusarium* species based on RAPD bands unique to a particular species. Yoder & Christianson (1998) designed species-specific primers for *F. crookwellense*, *F. sambucinum*, *F. torulosum* and *F. venenatum* from unique RAPD bands. One primer pair

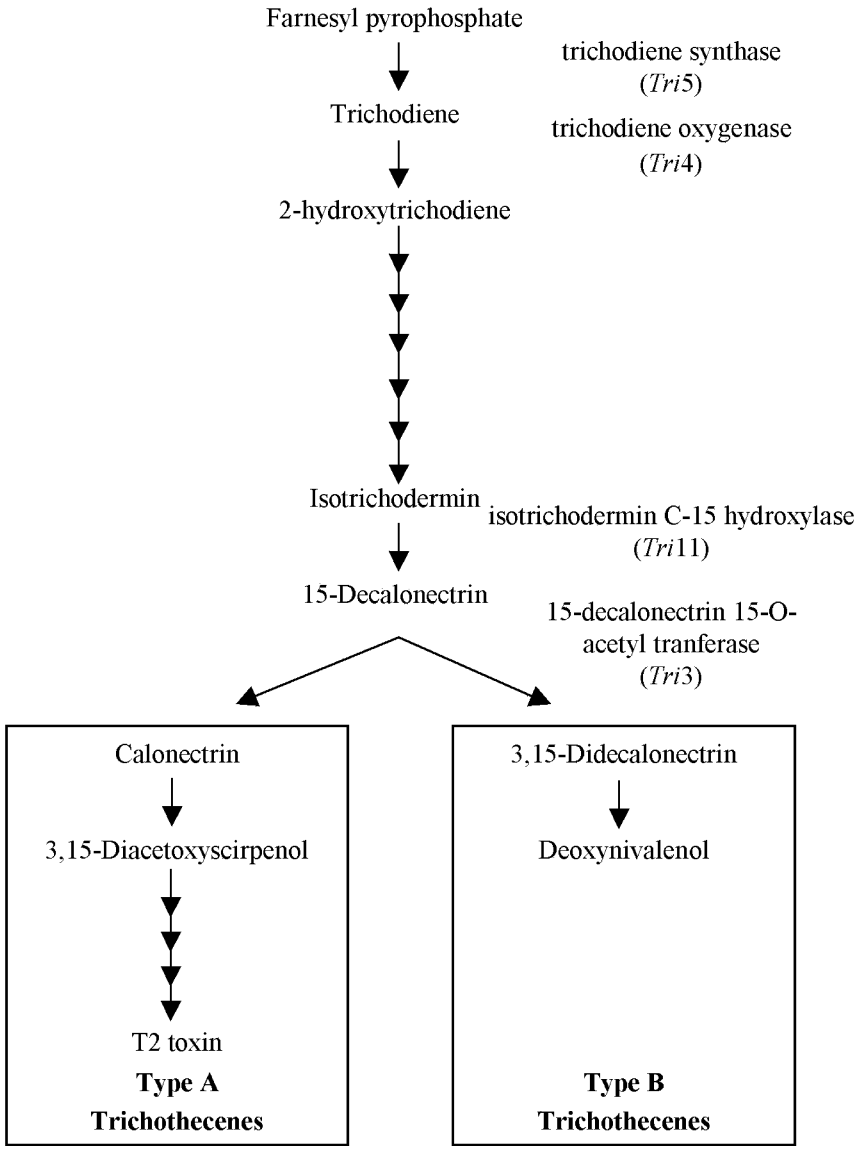


**Fig. 8.** Trichothecene biosynthetic pathway.

designed from a *F. crookwellense* unique band also amplified *F. culmorum* and one primer pair designed from a *F. culmorum* unique band also amplified *F. graminearum*, again indicating the close relationship between these three species. This technique has been used by other researchers to develop primers for *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. moniliforme*, *F. poae*, *F. pseudograminearum* and *F. subglutinans* (Ouellet & Seifert 1993, Parry & Nicholson 1996, Schilling *et al.* 1996, Doohan *et al.* 1998, Nicholson *et al.* 1998, Turner *et al.* 1998, Möller, Chelkowski & Geiger 1999, Williams *et al.* 2001). A number of these primer pairs were designed with matching melting temperatures so they could be combined in a multiplex PCR to detect more than one species in a single PCR reaction (Nicholson *et al.* 1998). There are some cases where species-specific primers have not been adequately tested against closely related species. Schilling *et al.* (1996) used ITS sequence information and Lees (1995) used a unique RAPD band to design primers specific for *F. avenaceum* that were later shown to cross-react with *F. tricinctum* (Turner *et al.* 1998). Murillo, Cavallarin & Segundo (1998) developed primers, which were supposed to be specific to *F. moniliforme* from a genomic library of the species. Unfortunately, these primers were not tested against the closely related species of the *G. fujikuroi* complex, with which they were later shown to cross-react (Möller *et al.* 1999).

PCR assays have been developed for *F. graminearum* and *F. pseudograminearum* based on DNA sequence information for galactose oxidase and the translation elongation factor genes respectively (Niessen & Vogel 1997, Aoki & O'Donnell 1999). An alternative method to the identification of specific species is the use of toxigenic genes to identify *Fusarium* spp. as functional groups. The trichothecene pathway has been elucidated (Desjardins, Hohn & McCormick 1993) and several trichothecene biosynthetic genes have been characterised (Fig. 8). Several researchers have developed primers towards the *Tri5* gene (Niessen & Vogel 1998, Doohan

*et al*. 1999b, Edwards *et al*. 2001). This gene encodes for trichodiene synthase, the enzyme that catalyses the isomerization and cyclization of farnyl phosphate to trichodiene, the first step in the biosynthetic pathway of trichothecenes (Hohn & Beremand 1989). These PCR assays allow the detection of trichothecene-producing *Fusarium* species in pure culture and within cereal samples. Such tests could indicate the presence or absence of all *Fusarium* trichothecenes in a single test. Where samples gave a positive result the individual trichothecene mycotoxins could then be screened for. One problem with this assay is that the predominant trichothecene-producing *Fusarium* species found on cereals are *F. culmorum* and *F. graminearum*. Isolates of both these pathogens exist as two chemotypes, which predominantly produce either DON or nivalenol (Chelkowski, Bateman & Mirocha 1999). Which one they produce is important as nivalenol is more toxic than DON. A genetic basis for DON and nivalenol chemotypes has been determined for Korean isolates of *F. graminearum* (Lee *et al*. 2002). However, it has recently been shown that seven phylogeographically distinct lineages of *F. graminearum* exist across the world, with evidence that they all contain both chemotypes (O'Donnell *et al*. 2000). Any genotype assay developed to distinguish DON and nivalenol-producing isolates of *F. graminearum* would have to be tested against examples of both chemotypes from each lineage.

Sequencing of the fumonisin biosynthesis gene cluster (Proctor *et al*. 1999) will allow the development of an assay based on mycotoxin genes for fumonisin-producing *Fusarium* spp. At present there is no sequence information for the genes involved in the biosynthesis of beauvericin, moniliformin or zearalenone.

## Penicillium *toxins*

### *PR toxin*

*Penicillium roqueforti* used in the manufacture of mould ripened cheeses produces a range of mycotoxins including patulin, penicillic acid, citrinin, roquefortine C, isofumigaclavines, PR toxin and mycophenolic acid. Of these, PR toxin is the most acutely toxic metabolite produced by *P. roqueforti*, and it regarded as a potential carcinogen (Smith *et al*. 1994). PR toxin is a sesquiterpenoid secondary metabolite that is derived from acetyl-CoA. *P. roqueforti* has recently been reclassified into three species named *P. carneum*, *P. paneum* and *P. roquefortii* based on molecular and biochemical profiling (Boysen *et al*. 1996). Each of the three species are capable of producing secondary metabolites with *P. carneum* producing roqueforine C, mycophenolic acid, patulin, and penicillinic acid and *P. paneum* producing patulin, botryodiploidin as well as other secondary metabolites. *P. roqueforti* produces PR toxin, marcfortines, roquefortine, isofumigaclavine A and mycophenolic acid. Even though many *P. roqueforti* strains isolated from blue cheese have been shown to produce PR toxin (Geisen 1998) and roquefortine C (Finola *et al*. 2001) and these metabolites have been shown to be present in cheese; they are not deemed to pose a health risk to consumers as they are known not to be stable in cheese. Nevertheless, *P. roqueforti* strains that do not produce secondary metabolites or PR toxin would be preferable as starter cultures from a food safety standpoint. With this in mind Geisen and co-workers have been trying to develop a molecular-based detection system to distinguish between *P. roqueforti* strains which are producers of secondary metabolites and those that are not (Geisen 1998). Initially they targeted a key enzyme involved in the biosynthesis of PR toxin, namely aristolochen synthase (*ari*1), a sesquiterpene cyclase (Geisen 1998). By using sequence data available from the previously cloned gene (Proctor & Hohn 1993), a gene specific PCR targeting the *ari*1 gene was developed and *P. roqueforti* strains were screened for the presence of aristolochen synthase. Using the *ari*1 specific PCR primers a product of the expected length was shown to be present in many of the 21 strains tested. However some of the strains which were negative in the PCR were able to produce toxin. These were subsequently shown to be positive following dot-blot hybridisation using an *ari*1 specific gene probe (Geisen 1998), thus indicating the presence of the *ari*1 gene in some species with altered PCR primer binding sites. Another potential problem with the use of this method was also identified where *ari*1 gene homologues were observed in fungal species, which are known not to produce PR toxin. Specifically both *P. camemberti* and *Byssochlamys nivea*, were PCR positive with the *ari*1 primers while with the dot-blot analysis, even more species gave positive results, indicating the presence of *ari*1-like gene sequences in a wide variety of species that are unable to produce the toxin, such as *P. italicum*, *P. nalgiovense* and even *Aspergillus nidulans*. A possible reason for these results, as postulated by the author, could be that homologues of enzymes involved in fungal secondary metabolism such as *ari*1 may be present in strains which do not produce these secondary metabolites, but may encode for related enzymes which act upon other substrates participating in the production of different and as yet unidentified related end-products by the fungus. These results taken together with the specificity problems previously discussed for the aflatoxigenic fungi indicate that care should be taken when using a monomeric PCR reaction which targets only one mycotoxin biosynthetic gene, in a diagnostic setting; as the primers used may be insufficiently specific for the detection of the specific mycotoxin-producing fungus. More recent work by Geisen's group, in which they performed a RAPD analysis of a large number of *P. roqueforti* strains has proven more successful. By using three primers *ari*1 (CTGCTTGGCACAGTTGGCTTC), *nor*1 (ACCGCTACGCCGGCACTCTCGGCAC) and *omt*1 (GTGGACGGACCTAGTCCGACATCA) they were able to observe slight genotypic differences in seventy six strains of *P.*

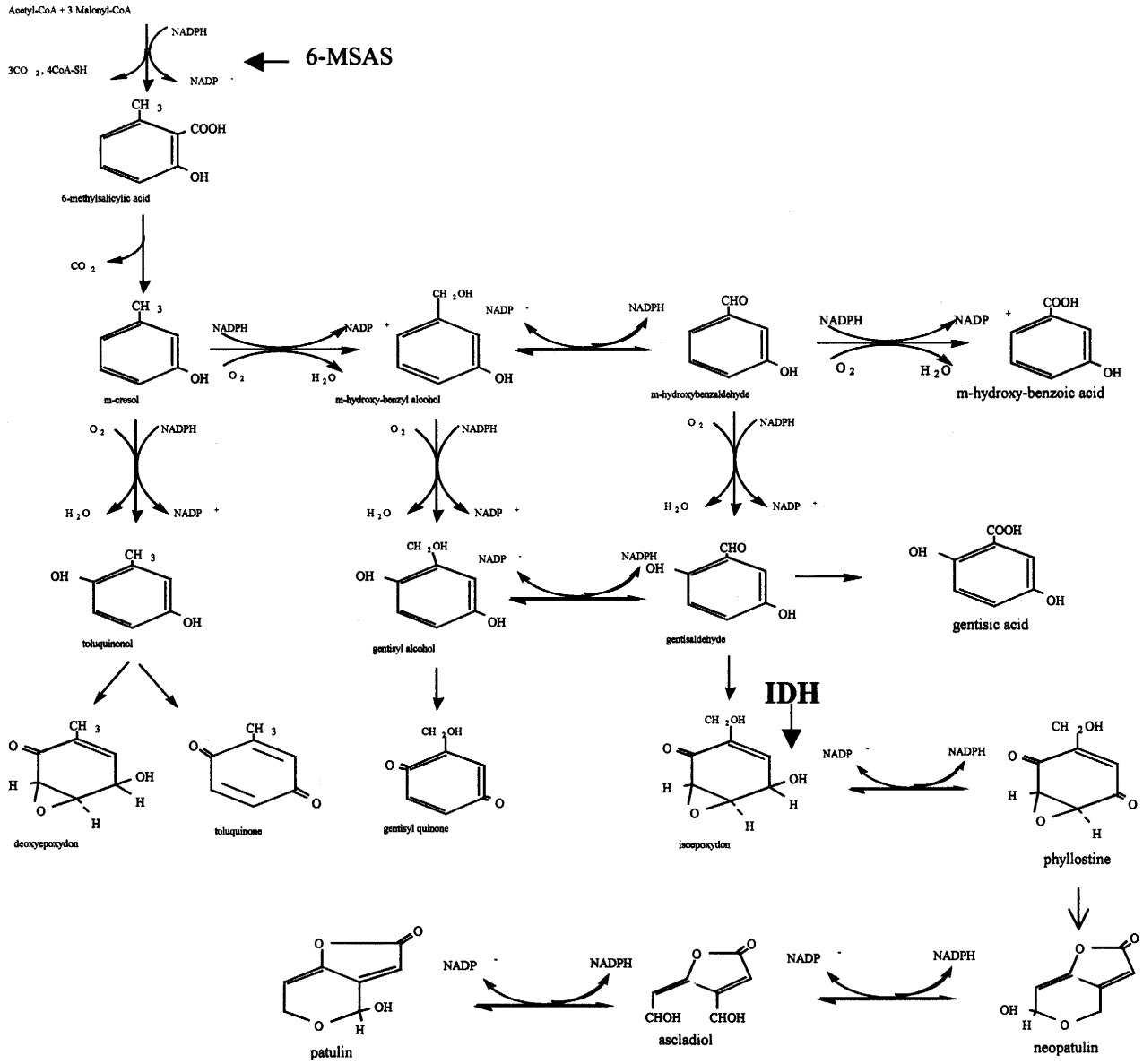


**Fig. 9.** Patulin biosynthetic pathway. 6-MSAS, 6-methylsalicylic acid synthase; IDH isoepoxy dehydrogenase. Adapted from Gaucher *et al.* (1981).

*roqueforti* used as starter cultures for production of blue cheeses, which allowed the strains to be divided into different RAPD groups (Geisen *et al.* 2001). In addition, these RAPD groupings correlated with the production of secondary metabolites. They found that by using primers with a length of 21 and 25 nucleotides, as much more reproducible RAPD patterns and micropolymorphisms were obtained. Significantly they report on one genotype, distinguishable with the *ari*1 primer, which produced fewer secondary metabolites than other genotypes, a finding that might be useful in the selection of starter strains with reduced or impaired capacity to produce undesirable secondary metabolites.

## *Patulin*

Another important penicillium mycotoxin is patulin, which is produced primarily by *Penicillium expansum*. Although patulin can occur in many mouldy fruits, grains and other foods, the major sources of contamination are apples and apple products such as juices. It has been postulated that the fungus can live as an endophyte since apple juice made from unblemished fruit can contain patulin. Also, patulin may be translocated into apples through fungus-contaminated soil (Mantle 2000). Data on the carcinogenic potential of patulin are as yet incomplete, but it has been shown to cause immunotoxic, neurotoxic and adverse gastrointestinal effects (Pfeiffer, Gross & Metzler 1998). The patulin biosynthetic pathway has been well characterised and involves at least ten steps in the conversion of acetyl- and malonyl CoA to patulin, (Gaucher *et al.* 1981) (Fig. 9). Nine of the pathway specific enzymes have been characterised. The polyketide synthase gene from *P. patulum*, a key enzyme in the pathway has been cloned (Beck *et al.* 1990). Given that polyketide synthase genes contain domains that are very highly conserved, even between species, this

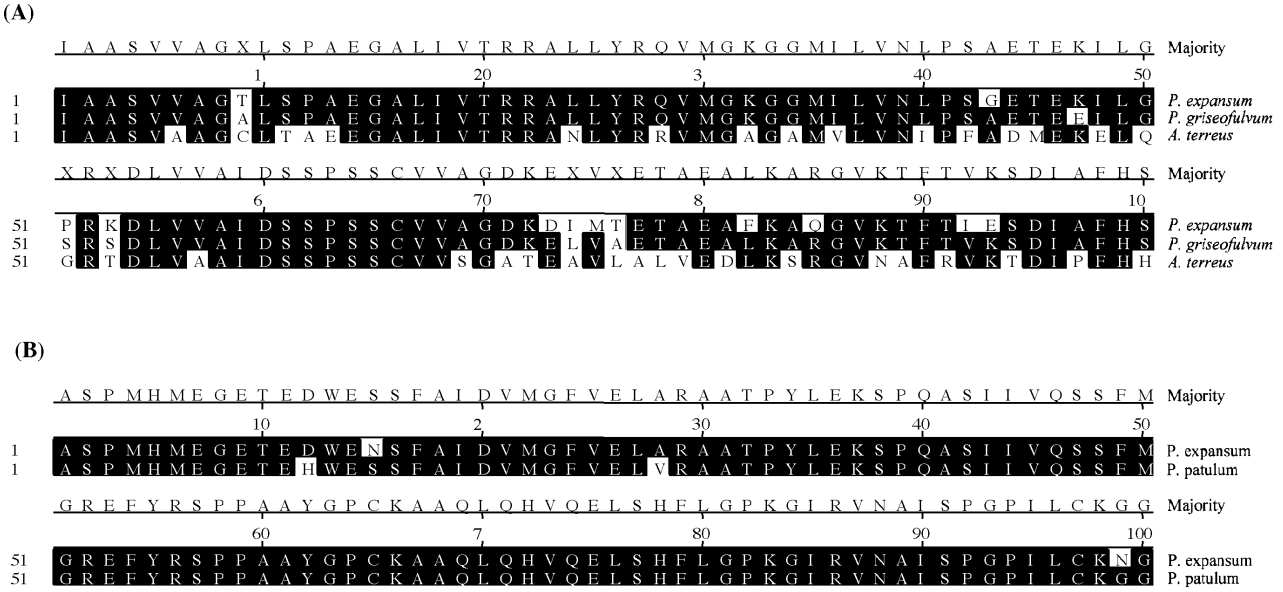


**Fig. 10.** Partial amino acid alignments of two genes involved in the biosynthesis of patulin. (A) A partial alignment of part of the 6-methylsalicyclic acid synthase amino acid sequence from *Penicillium expansum*, *P. patulum* and *Aspergillus terreus*. (B) A partial alignment of the isoepoxydon amino acid sequence in *P. expansum* and *P. patulum*. Areas of dark background indicate common amino acids. The amino acid sequences were deduced from nucleotide sequences.

gene is often unsuitable as a target for a specific PCR-based diagnostic approach (Mayorga & Timberlake 1992). However, this problem can be overcome by targeting other patulin biosynthetic genes such as the isoepoxydon dehydrogenase (IDH) gene which catalyses the conversion of isoepoxydon to phyllostine. With this in mind we have cloned part of the 6-methylsalicylic acid synthase gene (6-MSAS), the polyketide synthase involved in the first step of the patulin biosynthetic pathway and part of the IDH gene from *P. expansum* (White & Dobson unpublished results). The 6-MSAS gene shows 82% similarity to *P. patulum* 6-MSAS and the IDH gene shows 84% similarity to *P. patulum* IDH at the amino acid level (Fig. 10). Preliminary work indicates that these genes may prove useful in the detection of patulin production in *P. expansum*, given that they appear to be upregulated under conditions in which patulin is produced by the fungus. Indeed the isoepoxy dehydrogenase (IDH) gene has previously been shown to be a useful target for the detection of patulin production given that PCR primers designed to amplify a 600 base pairs of the IDH gene (CAATGTGTCGTACTGTGCCC) and (ACCTTCA-GTCGCTGTTCCTC) have been developed which are capable of detecting *P. expansum* and *P. brevicompactum* isolates from environmental samples, with all of the patulin-producing *P. expansum* strains tested being PCR positive for the IDH gene (Paterson *et al.* 2000).

## REVERSE TRANSCRIPTION PCR

PCR-based diagnostic methods have been shown to be useful in the identification of mycotoxigenic fungi and in many cases in distinguishing them from non-toxin producing strains. However, one of the problems with such an approach is that even if the fungus is present as would be evident from a PCR positive reaction, this is still not definitive of mycotoxin-production. For example it is well established that water activity ($a_w$) has an affect on toxin production. The minimum $a_w$ for growth in many mycotoxigenic fungi is less than the minimum $a_w$ required for toxin production. A clear example of this can be seen with *Aspergillus ochraceus* where the minimum $a_w$ for growth is 0.77 but is 0.85 for ochratoxin production (Kozakiewicz & Smith 1994). A clearer indication of mycotoxin production in this instance would be the detection of messenger RNA (mRNA) transcripts for genes involved in ochratoxin production, given that if these biosynthetic genes are being transcribed then the mycotoxin is being produced. Reverse transcription PCR (RT-PCR) is a technique that allows the detection of such mRNA species. It allows the detection of mRNAs transcribed by specific genes by the PCR amplification of complementary DNA (cDNA) intermediates synthesised by reverse transcription. The technique has been widely used in the area of fungal genetics to monitor the expression of specific genes under different physiological conditions, such as extracellular ligninolytic enzyme production in the white-rot fungus *Phanerochaete chrysosporium* (Broda *et al.* 1995) and genes involved in fungal-plant interactions such as mitogen-activated protein kinase gene expression in the phytopathogen *Pyrenophora teres* (Ruiz-Roldan, Maier & Schafer 2001). It has also been used to monitor the expression of mycotoxin genes involved in trichothecene production (Kimura *et al.* 1998). RT-PCR is more sensitive than other methods used for RNA analysis, such as S1-nuclease analysis,

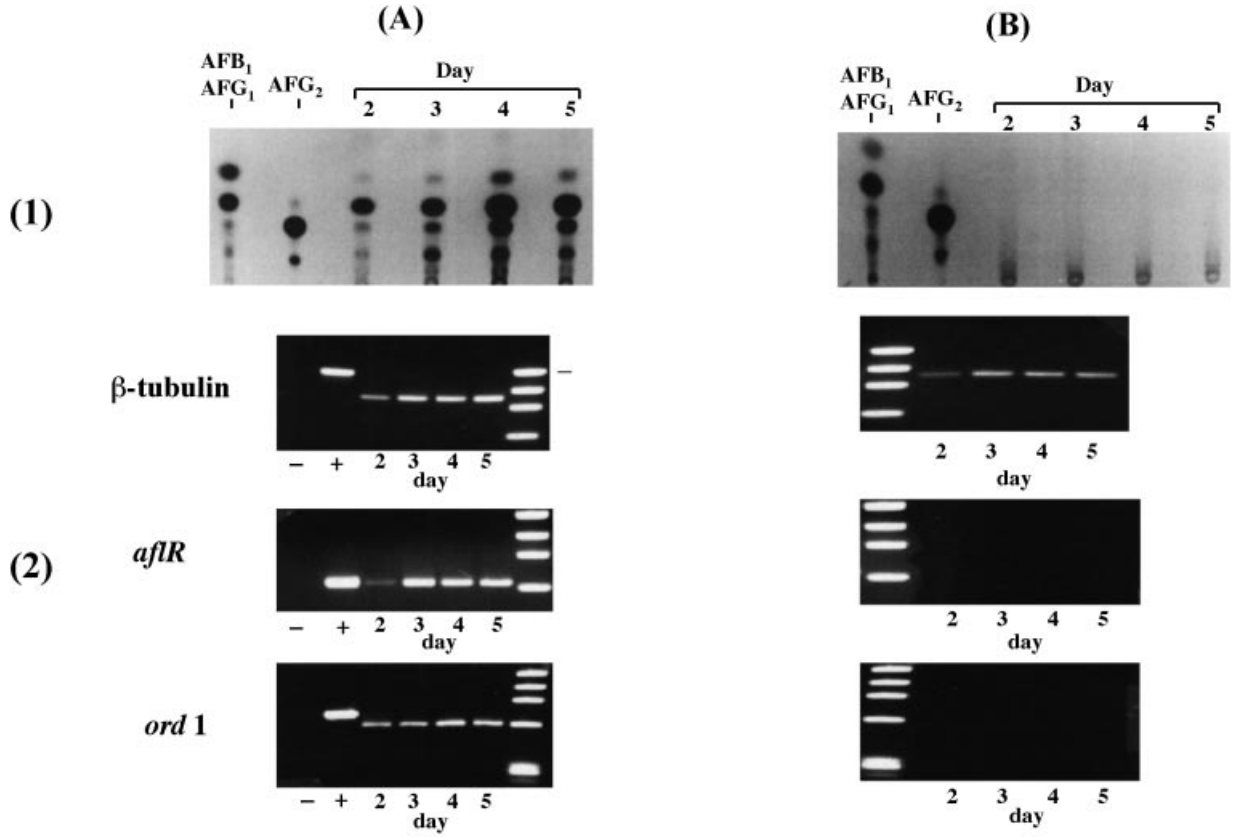


**Fig. 11.** RT-PCR detection of aflatoxin production by *Aspergillus parasiticus*. (A) Cultures grown in YES medium that allows aflatoxin production. (B) Cultures grown in YEP medium that does not allow aflatoxin production. (1) TLC analysis of aflatoxin production. $AFB_1$ $AFG_1$ and $AFG_2$ indicate aflatoxin $B_1$, $G_1$ and $G_2$ standards (500 ng each), Days 2–5 indicate 10 μl extracts from cultures harvested at days 2–5. (2) RT-PCR analysis of *afl*R, *ord*1 and β-tubulin transcription taken on days 2–5. (−) negative control PCR reaction (genomic DNA template absent). (+) positive control PCR product (20 ng genomic DNA template). Adapted from Sweeney, Pamies & Dobson (2000).

RNase A protection assay, *in situ* hybridisation or Northern hybridisation. The first step is the synthesis of cDNA from RNA using reverse transcriptase. First strand cDNA is typically synthesised from total RNA using random hexamers (Collins & Dobson 1997). The newly synthesized first strand cDNA is then used as a template for PCR, using specific primers that will amplify the target gene. Contaminating genomic DNA which can also serve as a template for amplification is a common problem encountered in RT-PCR reactions, which can be overcome by the use of RNase-free Dnase1 digestion to remove contaminating genomic DNA before synthesis of the cDNA (Ausubel *et al*. 1995). PCR products from cDNA can be distinguished from those of genomic DNA by their smaller size if the primers amplify across an intron (Doohan *et al*. 1999b). This is a useful check for the presence of genomic DNA.

A RT-PCR based detection system has been employed to monitor aflatoxin production and aflatoxin gene expression in *A. parasiticus* (Sweeney, Pamies & Dobson 2000). Two pairs of oligonucleotide primers were designed to specifically amplify the coding regions of the structural gene *ord*1, which encodes a cytochrome P450 monooxygenase involved in the conversion of the penultimate aflatoxin pathway intermediate *O*-methylsterigmatocystin to $AFB_1$, and the regulatory gene *afl*R the positive transcriptional regulator of the aflatoxin biosynthetic genes (Fig. 4) (Flaherty & Payne 1997, Prieto & Woloshuk 1997). Total RNAs from the aflatoxigenic *A. parasiticus* 439 grown in aflatoxin permissive and non-permissive media was used as a template to synthesise cDNAs with random primers and M-MLV reverse transcriptase. The resulting cDNA was amplified with the specific primers. General gene transcription under both growth conditions was also assessed by monitoring transcription of β-tubulin. The technique enabled the detection of the three specific gene transcripts (Fig. 11), when *A. parasiticus* was cultured under both permissive and non-permissive conditions. The production of aflatoxin in *A. parasiticus* correlated with the detection of transcripts for *ord*1 and *afl*R by RT-PCR. Significantly no *ord*1 or *afl*R transcripts were produced under non-permissive conditions, correlating with lack of aflatoxin production under these conditions.

Thus this work demonstrates the use of RT-PCR as a method to monitor mycotoxin production in mycotoxigenic fungi. It is a rapid, sensitive, highly specific and non-isotopic alternative to conventional methods

such as Northern hybridisation analysis. In fact RT-PCR may be superior to Northern analysis when very low levels of transcript detection is required. For example, Prieto & Woloshuk (1997) could not detect *ord*1 transcripts from total RNA and detection required the isolation of poly $(A)^+$ RNA; while *ord*1 transcripts can be detected from 1 μg total RNA using RT-PCR. The use of nested RT-PCR can also further increase detection sensitivity limitations if they arise (Green *et al*. 1998). The technique has an added specificity in that very similar mRNAs can be distinguished from each other by either designing very specific primers or by increasing the stringency of primer annealing (Verma & Upadhyaya 1998). One of the main advantages of the system is that the specificity of the technique allows the detection of a number of different mRNA transcripts from mycotoxin biosynthetic genes, when the producing fungus is cultured under different physiological conditions. This allows the role of different physiological conditions on mycotoxin production to be studied. In addition, it can be used to identify phytological agents that may naturally inhibit various mycotoxin biosynthetic pathways.

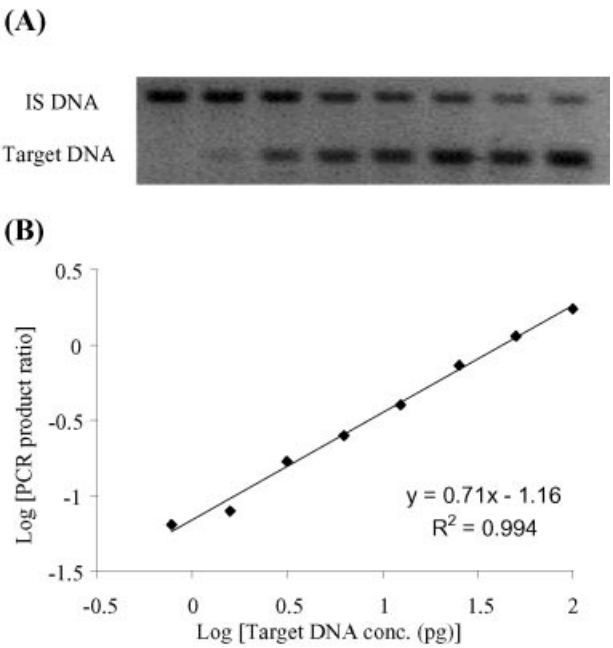


**Fig. 12.** Competitive PCR. (A) PCR products from eight two-fold serial dilutions of target DNA (200 to 0.8 pg) amplified in the presence of a fixed concentration of internal standard (IS) DNA. (B) Standard curve of PCR product ratio (target/internal standard) against concentration of target DNA.

## QUANTIFICATION OF MYCOTOXIGENIC FUNGI

The amplification rate of a PCR reaction is strongly effected by the presence of inhibitors (Wilson 1997) and attempts to use the band intensity within a normal PCR as a semi-quantitative assay can lead to highly erroneous results (Gilliland, Perrin & Bunn 1990b). However, researchers still report concentration of PCR products as a relative measure of fungal biomass. Birzele, Prange & Kramer (2000) showed a decrease in a *Tri5* PCR product concentration occurred with time for *Fusarium*-infected grain stored at 17 and 20% moisture. They concluded from this that the biomass of trichothecene-producing *Fusarium* had decreased with time, although they failed to discuss the possibility that the decrease in PCR product may have resulted from an increase in PCR inhibitors rather than a decrease in target DNA.

The two current methods developed to quantify DNA populations by PCR are competitive PCR and Real Time PCR. Competitive PCR requires the construction of an internal standard which possesses the same primer sites as the target DNA but the resulting PCR product is a different size, so the target and internal standard PCR products are distinguishable on an agarose gel after electrophoresis (Gilliland *et al*. 1990a). The internal standard is added to the PCR reaction at a fixed concentration. The target and internal standard are amplified in the same tube by the same primers under identical conditions, therefore the ratio of the two products is proportional to the amount of target DNA at the start of the reaction. A series of reactions containing a range of known target DNA concentrations are amplified in the presence of a fixed concentration of internal standard to produce a standard curve. The standard curve is then used to determine the concentration of target DNA within unknown samples (Fig. 12).

Real Time quantitative PCR monitors the amplification of PCR products during each reaction cycle using fluorescent DNA probes or dyes. The cycle number at which log phase begins is unaffected by inhibitors and is predictive of the quantity of target DNA present. A standard curve can be prepared using a series of amplifications with known concentrations of target DNA (Fig. 13). Real Time PCR has several benefits over competitive PCR. Real Time PCR does not require post-PCR handling this reduces the risk of cross-contamination and handling errors, it results in a much faster throughput and is less labour intensive (Heid *et al*. 1996). Real Time PCR also has a wider range of target molecule determination of five to six orders of magnitude compared to two to three orders of magnitude commonly achieved with competitive PCR (Jordan 2000). The disadvantage of the Real Time system is the cost of the specialised equipment that is capable of real time fluorescent measurement of PCR reactions compared with cheaper gel image analysis systems required for competitive PCR. The cost of such systems can only be justified by a high throughput of samples. The amplification of the PCR product can be detected by monitoring the fluorescence of non-specific stains, such as SYBR Green, binding to double stranded DNA (Morrison, Weis & Wittwer 1998). Non-specific stains will also give a signal for primer-dimers and non-specific amplified products although this can be

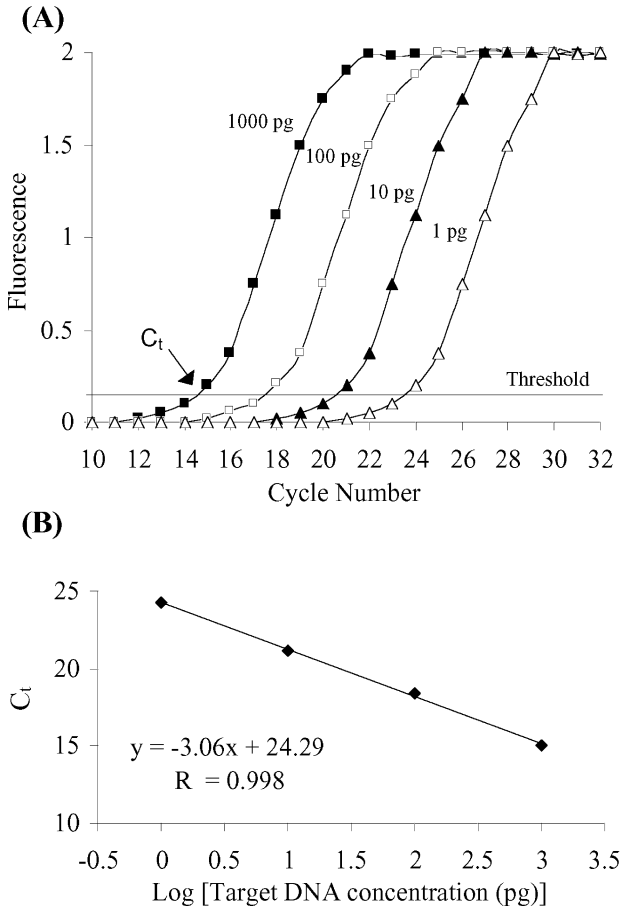


**Fig. 13.** Real Time PCR. (A) Amplification plots from four concentrations of target DNA (1 to 1000 pg). $C_t$, cycle number at which the threshold is reached. The threshold is usually set at ten times the standard deviation of the base line. (B) Standard curve of $C_t$ against concentration of target DNA.

minimised by optimisation of PCR conditions (Schnerr, Niessen & Vogel 2001). The melting profile of the PCR products can be checked at the end of the reaction to verify that specific amplification has occurred (Ririe *et al.* 1997). Various probes have been developed which are specific to the target DNA PCR product. These include, hydrolysis (TaqMan) probes, hairpin (Molecular Beacon) probes and hybridisation probes. These allow specific PCR products to be detected by probes labelled with specific fluorescent dyes. The use of different dyes with discreet emission wavelengths allows a number of target DNA populations to be quantified in a single multiplex PCR reaction.

Several PCR assays have been developed for the quantification of toxigenic fungi. The first systems developed were species-specific competitive PCR assays for fusarium ear blight pathogens (Nicholson *et al.* 1996, Nicholson *et al.* 1998). These techniques have been used to study the role of trichothecene mycotoxins in the virulence of *Fusarium graminearum* on wheat (Smith 1997) and maize (Harris *et al.* 1999). More recently these assays have been applied to the study of the varietal resistance of wheat to fusarium head blight (Mentewab *et al.* 2000) and the fungicidal control of fusarium head blight pathogens (Doohan, Parry & Nicholson 1999a, Jennings, Turner & Nicholson 2000, Simpson *et al.* 2001). The development of a competitive PCR assay for the *Tri5* gene allowed all trichothecene-producing *Fusarium* spp. to be quantified in a single assay (Edwards *et al.* 2001). This assay also allows analysis of the relationship between the amount of trichothecene-producing *Fusarium* and the amount of trichothecene mycotoxins within a sample. This provides information as to whether differences in mycotoxin levels are due to differences in the biomass of trichothecene-producers present or due to the stimulation or suppression of mycotoxin synthesis. An alternative pair of assays have been developed which will quantify all *Fusarium* spp. commonly associated with wheat and *M. nivale* (Glynn *et al.* 2000). These assays allow the two main components of fusarium head blight, namely toxigenic *Fusarium* spp. and the non-toxigenic *M. nivale* to be quantified. As the visual symptoms of the species involved are not distinguishable these assays provide valuable information regarding the proportion of toxigenic fungi present within the disease complex. All the above assays systems have shown that there are important differences in the efficacy of fungicides against ear blight pathogens. Namely, the triazoles, metconazole and tebuconazole are effective against *Fusarium* spp. and not effective against *M. nivale* and the strobilurin, azoxystrobin is effective against *M. nivale* and not *Fusarium* spp. (Jennings *et al.* 2000, Edwards *et al.* 2001, Simpson *et al.* 2001, Pirgozliev *et al.* 2002). A recently developed Real Time PCR assay for the *Tri5* gene showed there was a high correlation ($R = 0.83$) between the amount of trichothecene-producing *Fusarium* and the amount of DON within field samples of wheat (Schnerr, Niessen & Vogel 2001). This correlation is higher than maybe expected when it is considered how trichothecene B-producing *Fusarium* are either DON or nivalenol producers and the amount of DON produced can vary greatly among different DON produces *in vitro* (e.g. more than a 1000-fold range between isolates recorded by Chelkowski *et al.* (1999). It will be very interesting to determine how the relationship between *Tri5* and DON concentration varies between geographical region, wheat cultivar, and year.

Messenger RNA can be quantified in two ways using RT-PCR. By inclusion of an internal standard in the amplification of cDNA, the absolute quantity of a specific mRNA can be quantified (Pfaffl & Hageleit 2001). By multiplexing the PCR of the cDNA with that of a housekeeping gene, such as the β-tubulin gene, which has a fixed rate of expression, the relative expression of a specific mRNA can be quantified (Pfaffl 2001). Doohan *et al.* (1999b) were able to detect changes in the level of *Tri*5 expression in the presence and absence of fungicides in *in vitro* cultures and during infection of wheat by *F. culmorum*. The two methods of quantifying mRNA therefore can be used to quantify

the total amount of a mycotoxin gene being expressed which would indicate the amount of mycotoxin being produced or the relative rate of a mycotoxin gene expression. This allows the factors affecting expression of the specific gene to be studied.

## DISCUSSION

The need to know what fungal contamination is present within a foodstuff will vary for different situations. The need simply to detect what is present or to quantify what is present will also vary for different situations. It may only be necessary to have an initial screen to measure the total fungal content of a foodstuff as an indication of the potential presence of mycotoxigenic fungi. PCR assays have been developed using universal fungal primers for rRNA genes that will detect the presence of any fungi within samples (Borneman & Hartin 2000, Jaeger *et al*. 2000). A reverse transcription (RT-)PCR assay using universal fungal primers has been designed for the elongation factor gene that allows the detection of viable fungi within samples (Vaitilingom, Gendre & Brignon 1998). This review has shown that different levels and types of specificity have been used in the development of assays for mycotoxigenic fungi ranging from genera and species specific assays which distinguish these taxonomic groups and mycotoxigenic gene-specific assays which distinguish fungi able to produce a specific mycotoxin or group of mycotoxins.

The ability to develop taxon-specific PCR assays is dependant on the availability of a large collection of verified type cultures of the fungi of interest and of a phylogenetic classification of these fungi. The USDA has made a major contribution towards this goal for *Fusarium* species (O'Donnell 1996). Development of taxon specific assays for *Penicillium* and *Aspergillus* spp. would undoubtedly benefit from similar studies. The development of mycotoxigenic assays has had varying success. The single *Tri*5 assay has been shown to readily distinguish between trichothecene-producing *Fusarium* spp. and non-producing species (Niessen & Vogel 1998). In comparison, multiplex mycotoxin gene assays have not been able to distinguish between aflatoxin- (Geisen 1996) and ochratoxin- (Geisen 1998) producing and non-producing species or isolates. Results from Niessen & Vogel (1998), and various previous studies indicate that the trichothecene genes are conserved within the *Fusarium* genus and homologues of these genes involved in the production of other secondary metabolites do not exist. In contrast, genes responsible for ochratoxin and aflatoxin appear to be less conserved between producing species and homologues appear to be present in non-producing species. Extensive sequencing of mycotoxin genes from producing isolates and their homologues in non-producing isolates are required to develop primer pairs to distinguish between such isolates.

If these assays are to be used within industry as a screen for the detection of foodstuffs that are potentially contaminated by mycotoxigenic fungi or indeed mycotoxins then they must be tested against the principles of valid analytical measurement (VAM) (Saunders 1999). As well as having the required specificity, they must also have the required sensitivity and a range of quality control and assurance measures to ensure the correct facilities and procedures are followed and that controls are in place. Once PCR conditions have been optimised the sensitivity of an assay is dependent on the copy number of the target DNA. For this reason, PCR assays developed for rDNA can be 100 times more sensitive than assays developed towards single copy DNA. The sensitivity of assays can be increased by a short preliminary culture of the sample (Chang *et al*. 1995) or by nested PCR (Green *et al*. 1998), either of these techniques results in the inability to use quantitative PCR.

Many PCR assays within research are performed with a positive and negative control, however few diagnostic assays are assessed for amplification efficiency, which in turn effects the sensitivity of an assay. Quantitative PCR is performed with a range of standard concentrations of target DNA down to the level of sensitivity. As a result they have an accurate measure of amplification efficiency. A negative result in a PCR assay indicates three possibilities: the target DNA was not present at a detectable concentration within the sample DNA; or no sample DNA was in the reaction mixture due to being lost during extraction or pipetting error; or inhibitors co-extracted with the sample DNA resulted in no amplification of the target DNA. Many of the necessary controls against the possible false negatives occur within competitive PCR. The presence of the internal standard PCR product acts as a control against false negatives which may result from inhibition. Total genomic DNA is quantified, against which the target DNA is measured (ng target DNA $\mu g^{-1}$ total DNA), which determines any loss of sample DNA during extraction. Pipetting errors can still occur, as in any molecular technique, although this can be controlled for by using standardised procedures and replicated samples. When analysing samples such as processed foods which are a diverse organic matrix, then the level of PCR inhibition will vary between samples (Bickley & Hopkins 1999) and the sensitivity of an assay should be assessed for each individual sample (Reiss & Rutz 1999).

A number of PCR and RT-PCR assays have been described in this review. The advantage of RT-PCR is that it will only detect mRNA, which is short-lived. If the mRNA targeted is constitutively produced then the method will detect viable target fungi. If the target mRNA is an inducible mycotoxigenic gene then the method will only detect fungi actively producing that mycotoxin. To detect *Fusarium* pathogens at harvest a PCR assay would be more appropriate, as these pathogens will have produced the mycotoxins prior to

harvest and harvesting may be done under non-permissive conditions for toxin production, when target DNA but not mRNA would be present. A RT-PCR test would be more appropriate to detect the presence of mycotoxigenic fungi on foodstuffs during storage and processing, when under adverse storage conditions, mRNA of storage fungi and *Fusarium* would be present and detectable. RT-PCR is also an important aid to understanding the influence of various biotic and abiotic factors on the production of mycotoxins. Quantitative PCR will provide a valuable aid to understanding aspects of the epidemiology of mycotoxigenic fungi.

Both PCR and RT-PCR assays will be valuable aids within the research and monitoring of good agricultural practice (GAP) and good manufacturing practice (GMP) directed towards reducing the levels of mycotoxins within food products.

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