REVIEW ARTICLE

Mutagens manufactured in fungal culture may affect DNA/RNA of producing fungi

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Summary

Self-produced mutagens in culture by fungi may affect DNA analysis of the same fungi. This has not been considered previously. Many fungi produce numerous mutagenic secondary metabolites (SM) in culture. There is a paradox of growing fungi in media to produce representative DNA which also support mutagenic SM. This is a crucial issue in developing diagnostic and phylogenetic methods, especially for closely-related fungi. For example, *idh* gene analysis of the patulin metabolic pathway in fungi can be interpreted as producing some false negative and positive results in terms of possession, or nonpossession, of the gene from mutated strains. The most obvious mycotoxins and fungi to consider in this regard are aflatoxins and *Aspergillus*, as aflatoxins are the most mutagenic natural compounds. Many other fungi and SM are relevant. Conditions to grow fungi have not been selected to inhibit SM production although relevant data exist. In fact, fungi repair damaged nucleic acid (NA) and are capable of removing toxins by employing transporter proteins. These and NA repair mechanisms could be inhibited by secondary metabolites. Mutagenic effects may involve inhibition of DNA stabilizing enzymes. There may be an equivalent situation for bacteria. Researchers need to devise methods to reduce SM for valid protocols. More work on how mutagens affect the NA of producing fungus *in vitro* is required. The current review assesses the potential seriousness of the situation with selected papers.

Introduction

Fungi are very important organisms with many applied aspects developed as industrial processes or under investigation. However, species concepts in relation to applied properties (Paterson *et al.* 2004; Paterson 2007a), and taxonomic research *per se*, remain unclear. This has lead to increased use of diagnostic (Seifert *et al.* 2007) and phylogenetic (Hibbett *et al.* 2007) methods employing DNA or RNA. The present review suggests strongly that the production of 'self'-produced mutagens in culture by fungi may affect DNA analysis of the same fungi. Factors such as these have not been considered previously.

Fungi produce secondary metabolites (SM) (e.g. aflatoxins) that mutate the structure of nucleic acids (NA). The mutagenic effects can be direct (e.g. changing bases) and/or indirect [e.g. inhibiting enzymes (Paterson 2008a) involved in stabilizing NA (Osiewacz and Borghouts 2000; Palma *et al.* 2007)]. Secondary metabolic antibiotics from bacteria (Robinson *et al.* 1990; von Ahsen and Schroeder 1991; Albertini *et al.* 2006) and plants (Couve'-Privat *et al.* 2007) also interact directly with DNA. Furthermore, a data base produced by Wishart *et al.* (2008) may be useful with respect to the present discussion of SM/DNA interactions. DNA repair mechanisms could be restricted as fungi produce many inhibitors and the effect of enzyme inhibitors in relation to PCR has been discussed (Paterson 2007a, 2008a). A probe for the patulin metabolic pathway using the *idh* gene was developed where a potential problem was SM production in growth media (Paterson 2007a). Indeed, Paterson (2004) first alluded to the paradox of growing fungi for DNA on

media which will support mutagenic SM and this concept was developed further in Paterson (2006a, 2007a, 2008a).

However, a mitigating factor is that organisms can be protected from such toxicity by mechanisms such as the transmembrane proteins encoded by genes located in the clusters of antibiotic biosynthesis genes, with a role in antibiotic secretion and antibiotic resistance. These proteins include efflux systems for secretion of SM. Of course these processes too may be inhibited by SM (see Paterson 2008a). The development of diagnostics and phylogenetic studies based on DNA detection can only be improved by an awareness of the issue. It is incumbent upon the present authors to discuss the two issues of (i) mutagenic metabolites and DNA and (ii) toxin removal in fungi more fully.

Mutagenic potential of mycotoxins in nonfungal systems

Living cells have been continuously exposed to UV radiation and many DNA/RNA damaging agents for more than 2·5 billion years. In addition, stable genomes are subject to chemical and physical agents in the environment (e.g. ionizing radiations, chemical mutagens, and bacterial and fungal toxins) and by free radicals or alkylating agents generated as a consequence of metabolism. Errors during replication damage DNA. The DNA lesions could be (i) altered, missing, and mismatch bases, (ii) deletion or insertion, (iii) linked pyrimidines, (iv) strand breaks and (v) intra- and inter-strand cross-links (Fig. 1) (Tuteja *et al.* 2001). DNA lesions can be removed by repair and/or replaced by recombination. Alternatively, they are retained, leading to genome instability, mutations, carcinogenesis and/or cell death.

A complex system of repair mechanisms evolved early. All organisms have developed various strategies to reverse, excise, or tolerate the persistence of DNA damage (e.g. direct reversal, base excision repair, nucleotide excision repair, photoreactivation, bypass, the double-strand break repair pathway, and the mismatch repair pathway). However, the repair mechanisms are enzyme-based and subjected to inhibition (see Paterson 2008a).

SM are microbial products that are not essential for growth and reproduction of the producing organisms. Each SM is formed by a limited number of species and is encoded by sets of dispensable genes. These compounds are (often) synthesized at the end of the exponential growth phase and their formation is highly influenced by the composition of the culture medium (Martín *et al.* 2005). Concentrations of SM produced by fungi may increase beyond tolerable concentrations in culture. In addition, proliferating cells may be more mutable than quiescent because they have less time to repair damage before DNA replication.

Mycotoxins with carcinogenic effects include aflatoxins, sterigmatocystin, ochratoxin, fumonisin, zearalenone, citrinin, luteoskyrin, patulin, and penicillic acid produced by a wide range of fungi. All are DNA damaging agents except for fumonisins which may act via disturbing signal transduction pathways. Some individual mycotoxins will now be considered.

Aflatoxins

Aflatoxins are the most carcinogenic compounds known that occur naturally and avoiding production in culture is surely a priority for DNA analysis. They (i) induce DNA damage, (ii) affect negatively the amelioration of damage, and (iii) alter DNA base compositions of genes. The mutagenicity of AFB_1 has been demonstrated in many systems. AFB_1 induces chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, chromosomal strand breaks, and forms adducts in rodent/human cells. Furthermore, aflatoxins containing an unsaturated terminal furan ring that can bind covalently to DNA forming an epoxide. One dose of AFB_1 to rats can cause a measurable increase in AFB–DNA adducts that are increased after a second daily dose, and hence the effects are rapid, with relevance to the time used to grow fungi (Table 1; Paterson 2007a).

Furthermore, covalent binding of AFB_1 to adenosine and cytosine in DNA *in vitro* has been reported. AFB_1–DNA adducts can (i) form further repair-resistant adducts, (ii) undergo depurination, and/or (iii) lead to error-prone DNA repair yielding single-strand breaks, base pair substitution, and/or frame shift mutations. Mispairing of the adduct could induce transversion and transition mutations. For example, GC → TA transversion mutations were demonstrated to be induced almost exclusively in the *lac1* nonsense assay for *E. coli*. AFB_1 induced GC → TA and GC → AT mutations in a plasmid-based system with a *lacZ* mutational target when activated to its epoxide by microsomes, or as the 8,9-dichloride. Hot spots for AFB_1 mutagenesis in the plasmid-based *lacZ* assay were found predominantly in relatively GC-rich regions of DNA. AFB_1–8,9-epoxide in human fibroblast cells transfected with a shuttle vector plasmid, caused base substitutions mutations at G:C pairs with only approximately 50% GC → TA transversion.

An oligonucleotide containing a single AFB $-N^7$- 1 Gua adduct was inserted into the genome of bacteriophage M13. Replication in SOS-induced *E. coli* yielded a mutation frequency of 4%. The predominant mutation was G → T, identical to the principal mutation in human liver tumours induced by aflatoxin. Finally, the effect of aflatoxins from fungal cultures to produce G → C
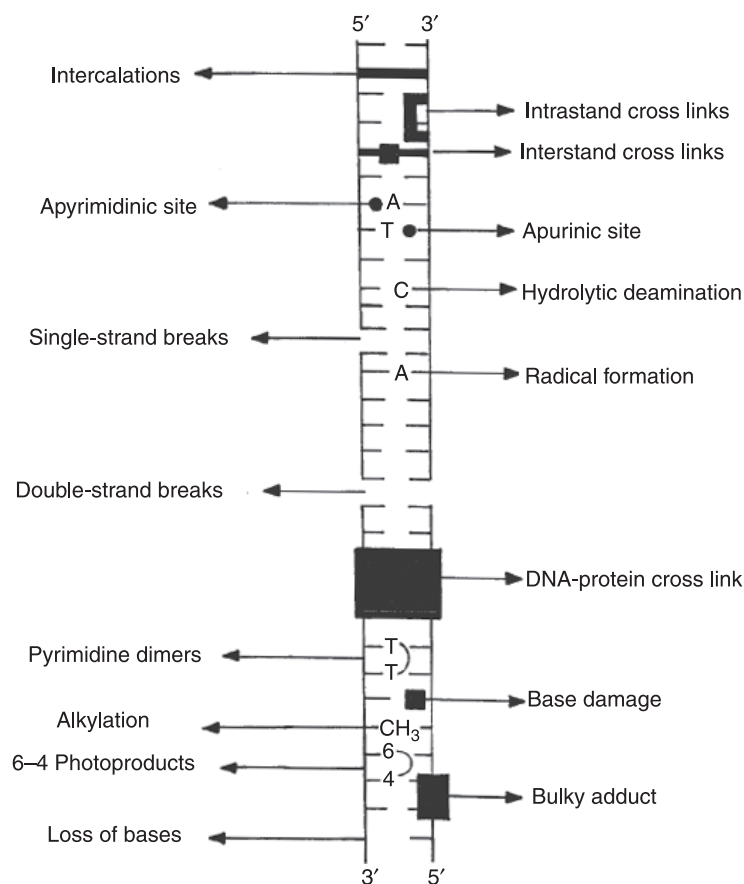
Figure 1 Some of the changes encountered by DNA in general (Tuteja *et al.* 2001).

tranversion mutations at codon 249 in DNA has been suggested to screen for aflatoxin activity (CAST 2003).

Sterigmatocystin

Sterigmatocystin has a chemical structure similar to afla-toxins. Also, the compound is a precursor of these notori-ous mycotoxins. Sterigmatocystin was mutagenic in various assays with relevance to the present topic. It is carcinogenic to rats and mice and mainly induces liver tumours. Finally, covalent binding to DNA and DNA adduct formation have been reported.

Ochratoxin A

Ochratoxin A (OTA) is produced by several *Aspergillus* and *Penicillium* species. The SM is one of the most potent carcinogens in rats. OTA is classified as a possible human carcinogen by the International Agency for Research on Cancer. OTA induced DNA single-strand breaks in cul-tured mouse and CHO cells and was positive for unsche-duled DNA synthesis in primary hepatocytes from mice and rats. Adducts were found in kidney, liver, and spleen of mice treated with OTA and the DNA adduct level was

dose-dependent and time related. There is still insufficient understanding of whether OTA acts as a direct genotoxic carcinogen or whether its carcinogenicity is related to indi-rect mechanisms (e.g. inhibition of stability enzymes; Osiewacz and Borghouts 2000). However, direct DNA-binding of OTA and DNA adducts formation have been reported. The strongest evidence in favour of OTA-mediated DNA damage is the induction of DNA single strand breaks and formamidopyrimidine-DNA glycosylase sensitive sites. In agreement is the unscheduled DNA synthesis in primary human urothelial cultures, primary hepatocytes, and rat and mouse cell lines. Importantly, mutagenic activity by OTA has also been reported (e.g. in murine cells). OTA-induced base substitutions were similar to those arising spontaneously, suggesting that this myco-toxin increases the process involved in spontaneous muta-genesis. Finally, the metabolite induces an increase of mutation frequency at two gene loci via a mechanism that is independent of biotransformation (Palma *et al.* 2007).

Patulin

The concentration necessary to induce DNA–DNA cross-links by patulin are in the millimolar range.

Patulin may not reach the nucleus to cause damage because of its high reactivity with proteins, and reactivity observed cell-free might not occur in cellular systems. However, if enough was present the protein would be 'overwhelmed' leaving the nucleus exposed. Mutations of cells by patulin might be from an indirect mutagenic mechanism [e.g. inhibition of enzymes (Paterson (2008a)]. The induction of DNA–DNA cross-links is a possible mechanism of the mutagenicity observed. Finally, the direct reactivity of patulin towards DNA in a cellular system has been demonstrated (Schumacher *et al.* 2006).

Table 1 Conditions used to grow fungi for DNA analyses including those which affect aflatoxin production. Conditions for pregrowth are provided if known

Authors	Fungi	Preproduction conditions	Production media	Temperature	Time
Price *et al.* (2005)			Aflatoxin Enhancing conditions: Organic nitrogen, simple sugars (pH 4·5 is conducive)	28°C permits production	
			Aflatoxin Inhibiting conditions: inorganic nitrogen, complex carbohydrates (pH 8·0 inhibits)	37°C completely inhibits, although it is growth optimum	
Klich *et al.* (2003)	*Aspergillus ochraceoroseus*		liquid YES	28°C agitation	48 h
Quirk and Kupinski (2002)	*Aspergillus* section *Flavi*		liquid defined mineral medium with acid hydrolysate of casein and sucrose	22–25°C	1–2 days
Geiser *et al.* (2000)	*Aspergillus flavus* and *Aspergillus oryzae*	Potato dextrose agar	Yeast extract– glucose or PDA	37°C (or 30°C for *A. oryzae*) without agitation	1–3 days
Cary *et al.* (2005)	*Aspergillus* section *flavus*		Yeast extract – sucrose liquid		7 days
Peterson (2000)	*Aspergillus*	Agar slants (Czapek's and malt extract agars?)	Usually Czapek's broth amended with 20% sucrose 'when needed', or M40Y when that was 'most satisfactory'.	25°C shaker	2–3 days or approximately 1 g biomass
Peterson *et al.* (2005; 2008)	*Aspergillus*	Yeast-malt agar with antibiotic (Peterson *et al.* 2005). Dichloran Rose Bengal Chloramphenicol agar medium at 25°C for one week (Serra and Peterson 2007).	Malt extract agar.	25°C	7–10 days
Sung *et al.* (2007)	*Cordyceps*		Not mentioned directly, and difficult to trace details.		
Hong and Jung (2004)	*Ganoderma*		MEA (malt extract, peptone, agar) covered with cellophane.	25°C	7 days
Paterson *et al.* (2000; 2003; Paterson 2004, 2006b)	Producers of patulin and others closely related		GY medium* (Glucose, Yeast extract, minerals)	25°C	72 h
Kristensen *et al.* (2005)	*Fusarium*		'General fungal medium'	shaking at room temperature (approximately 20°C)	3–7 days
Samson *et al.* (2004)	Penicillia	Malt extract agar, 24 h	Malt peptone broth	24°C	7 days
Dombrink-Kurtzman (2005)	*P. griseofulvum* and *P. expansum*		Yeast extract Peptone Glucose liquid medium	25°C gyratory shaker (200 rev min^{-1})	48 h
Dombrink-Kurtzman (2007)	Penicillia		PDA	25°C.	approximately 14 days

*Brevianamides were detected from this medium after growing *P. brevicompactum* (Paterson, unpublished data).

Nivalenol and fusarenon X

Nivalenol (NIV) damaged the nuclear DNA of CHO cells demonstrating that it is a direct mutagen (Tsuda *et al.* 1998). DNA damage appeared in the kidney and bone marrow of mice after oral dosing. NIV showed organ specific genotoxicity in mice related to time and intensity as a direct mutagen. NIV and fusarenon X (FusX), caused DNA damage after 24 and 72 h exposure in Caco-2 cells and damage was observed dose-dependently with relevance to growing fungi in culture. Furthermore, FusX increased DNA strand breaks in dividing cells (Bony *et al.* 2007).

Zearalenone

The *Fusarium* metabolite zearalenone (ZEN) showed a positive DNA damaging effect in recombination tests with *B. subtilis*. The compound also induced (i) polyploidy in CHO cells, (ii) sister chromatid exchange, and (iii) chromosomal aberration *in vitro*. Treatment of mice lead to the formation of several DNA adducts in the liver and kidney (Wang and Groopman 1999).

Fusarin C

Fusarin C is mutagenic and is produced from *Fusarium moniliforme* (Gelderblom *et al.* 1984). Finally, lack of information on other mycotoxins should not be interpreted as a statement on the mutagenicity of these agents. In fact, the aflatoxin literature at least serves as a model for subsequent research into other SM (Wang and Groopman 1999).

'Self'-protection (auto-protection)

DNA (and RNA) is employed with organisms to determine phylogenies (Hibbett *et al.* 2007) and produce diagnostics (Seifert *et al.* 2007): A stable genome is a prerequisite. However, it is in the fields of DNA analyses especially that reducing mutagens can benefit the analyses, because growing fungi in a milieu of inhibitory and mutagenic SM is illogical (Paterson 2004, 2006a, 2007a, 2008a). Fungi can tolerate toxins under normal conditions, although what occurs in pure (batch) liquid culture or agar where the toxins are not removed?

Obviously, toxin levels accumulate to a high level. AFB$_1$ ranged from 1·4 to 4 μg ml^{-1} in *A. parasiticus* or *A. flavus* under similar conditions used to produce biomass for DNA (Cotty 1997; Molina and Giannuzzi 2002; Sánchez *et al.* 2005). Scherm *et al.* (2005) detected 60 μg ml^{-1} of AFB$_1$ from *A. flavus* on yeast extract sucrose (YES) broth. Yeast extract peptone (YEP) was the noninducing medium and would be more logical for NA

analysis (see later). Abrunhosa *et al.* (2007) determined for OTA approximately 0·5 mg 20 ml^{-1} in YES from *A. alliaceus*. Young *et al.* (2001) state that paxilline accumulates at very high levels in growth media from *Penicillium paxilli*. *P. griseofulvum* produced nearly 2 mg ml^{-1} of patulin after 96 h in a potato dextrose broth, which was much higher than for the other species tested (Dombrink-Kurtzman and Blackburn 2005). Paterson (2007c) observed approximately 3 mg ml^{-1} from *P. expansum* on Czapeks Dox agar. Paterson (2007b) even found 15 ng l^{-1} of ZEN produced in water by *Fusarium graminearum*. So how might the fungi cope with such high concentrations of mutagenic SM?

It is common to suggest that the existence of fungi in nature is favoured by the production of metabolites toxic to competing organisms. How do the producing organisms resist such compounds? Presumably, it is both that allow the fungi to compete in ecosystems. The genes for the biosynthesis of SM are usually organized in clusters and include genes for resistance to and secretion of, the SM. Examples of these include antibiotics and mycotoxins. The SM are secreted to the extracellular medium and interact with other organisms. The presence of active toxin secretion mechanisms lowers intracytoplasmic concentration, contributing to self-protection. Active toxin transport systems for fungal toxins and their encoding genes have been suggested for HC-toxin in *Cochliobolus carbonum*, cercosporin in *Cercospora kikuchii*, and trichothecenes in *Fusarium* spp. Similar transporters are present in virtually all species of toxigenic fungi (e.g. *Fusarium*, *Alternaria*, *Aspergillus*, *Penicillium*) and are likely to operate in secretion of a wide array of toxins (Sorbo *et al.* 2000).

Transmembrane proteins encoded by genes located in the clusters of antibiotic biosynthesis genes have been cloned and their role in antibiotic secretion and antibiotic resistance is becoming clearer. The proteins include efflux systems for secretion of antibiotics, such as penicillins and cephalosporins, and several other SM. These 'antibiotic pumps' belong to the multiple drug resistance (MDR) protein class. Several studies have indicated that the expression levels of multidrug transporters can be enhanced by the addition of the compounds that are exported by these pumps. An interesting question is whether these transporters respond primarily to the internal SM being synthesized or whether external inducers have the same effect. However, the answer to this question requires detailed molecular genetic studies (Martín *et al.* 2005). Mechanisms for self-protection include: (i) the development of membrane permeability barriers coupled with efficient efflux mechanisms for removal of drug molecules from the cells; (ii) inactivation or sequestration of intracellular drug molecules and any biologically active precursors thereof and (iii) modification or replacement

of the target site(s) at which specific drugs act (Martín et al. 2005).

The ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters are the main transporter proteins. Both classes can have broad and overlapping substrate specificities for natural toxic compounds and are first-line defence barriers for survival. Families of integral membrane proteins can mediate transport of natural toxic compounds over biological membranes. The ABC and MFS of transporters play the major roles. ABC transporters (primary active transporter systems) bind and hydrolyze mainly ATP and use the energy to transport solutes across cell membranes which can occur against an electrochemical gradient. They comprise the largest number of efflux pumps and account for transport of a great number of endogenous toxicants. On the other hand, MFS transport (secondary active transport systems) compounds over membranes driven by the proton-motive force, which is composed of the membrane potential and electrochemical proton gradient. Some transporters actively secrete nonhost-specific toxins (Sorbo et al. 2000). In all instances, toxin concentrations in certain cell compartments or the cytoplasm are lowered by these mechanisms apart from the autolysis scenario. Also, concentrations at the target site will be reduced resulting in protection of the organism and transporters are involved in secretion of antibiotics, host-specific and nonhost-specific toxins, and mycotoxins.

The protection mechanisms may have a (i) qualitative or (ii) quantitative action, e.g. (i) the absence of sensitive target sites and (ii) a differential sensitivity of the target site in different organisms and to differences in the concentration of the toxin that can be built up at the target site respectively. The toxin concentration at the target site is determined by factors such as uptake, transport, storage, and metabolism of the toxicant. Fungi have membrane efflux systems that can transport toxic compounds over the plasma membrane or membranes that separate different cell compartments (Sorbo et al. 2000).

For example, *Fusarium sporotrichioides* secretes trichothecene mycotoxins. Strains disrupted in *Tri12,* which encodes a MFS transporter, produce less trichothecene and are more sensitive to the compound. When expressed in *S. cerevisiae, Tri12* confers ability to secrete trichothecenes. Hence, *Tri12* encodes a transporter involved in secretion of mycotoxins. Other genes encoding MFS transporters have been studied, such as *aflT* from *A. flavus. AflT* encodes a MFS transporter, and is located adjacent to *pksA,* encoding a polyketide synthase involved in aflatoxin biosynthesis. Multidrug transporters can bind a range of structurally unrelated drugs. Transcription of drug transporter genes can also be induced by a wide variety of compounds. Finally, some transcription regula-

tors have drug sensor motifs, which can bind chemically unrelated drugs (Sorbo et al. 2000).

AFB_1 is well known for its DNA damaging effect that can lead to cancer development in animals. To examine whether *aflT* has a role in self-protection against aflatoxins (presumably to stop DNA damage of the fungus), the total amounts of aflatoxins produced and the amounts of aflatoxins secreted into the medium by *aflT*-deleted mutants, *aflT*-non-deleted transformants, and the recipient strain were determined. However, *aflT* does not have a significant role in aflatoxin secretion and another system may be involved (Chang *et al.* 2004). Furthermore, the ABC transporter gene in the sirodesmin biosynthetic gene cluster of *Leptosphaeria maculans* is not essential for sirodesmin production but facilitates self-protection (Gardiner *et al.* 2005) (N.B. the authors state erroneously that *aftT* is a significant MFS transporter in *Aspergillus* spp.) However, the ABC transporter in *Leptosphaeria maculans* facilitates 'self'-protection. *P. paxilli* posseses a *paxT* transporter gene identified in the production of paxilline which can accumulate in growth medium at a high concentration (Young *et al.* 2001).

However, during growth of a pure culture compartmentalization is likely to break down from autolysis encouraging mixing of toxins and nucleic acids. The situation is completely different where (i) there are no competitors and (ii) toxins will accumulate to a maximum value for a substantial period as would occur in the media in Table 1 and in those listed in Paterson (2007a). Reducing SM in culture at least contributes to the logic of protocols for NA analysis and can only benefit the analyses.

Fungal diagnostics and phylogenetic studies

The rationales for undertaking the current review were to (i) make DNA protocols more logical, (ii) improve analyses in the future and (iii) explain results obtained by using *idh* to screen fungi for the patulin metabolic pathway. Unpredictable results occurred on some occasions with *idh*, such as (i) presence of the gene in *Penicillium brevicompactum* and (ii) *P. expansum* very occasionally lacking the gene. A reason may have been mutations of *idh* because of mutagens in the growth medium as discussed herein. Furthermore, a discussion of various PCR methods for mycotoxigenic fungi is provided in Paterson (2006a, 2007a) including details of growth media. None of the growth media were designed to inhibit SM production to avoid inhibitors. Equally it can be assumed that mutagenic compounds will be produced – an unsatisfactory position for NA analysis. Of course, there are numerous reports on phylogenetic analysis of fungi (Hibbett *et al.* 2007). Hence only a limited number have been

selected herein to illustrate how this field of study could be affected by mutagen production in the media used to grow the test fungi.

It is essential to focus on the aflatoxin producing fungi: aflatoxins are the most mutagenic natural compounds and a great deal of work has been undertaken with respect to diagnostics and phylogenetic studies. It is reasonable to suggest that any problems regarding damaged NA would be most apparent within this group. The current authors find it surprising that it is known that organic sources of nitrogen and simple sugars favour aflatoxin production, and inorganic nitrogen and complex carbohydrates retard biosynthesis, and yet this information has not been applied to DNA analysis. The growth-optimal temperature of 37°C inhibits aflatoxin biosynthesis completely, while 28°C allows production. Conversely, the growth-optimal of pH 4·5 is conducive for aflatoxin biosynthesis and production is inhibited at pH 8·0 (Price *et al.* 2005). Of course, these data not being applied is a serious omission. Sánchez *et al.* (2005) used YES to produce and YEP to inhibit aflatoxins as mentioned. These are surely minimal requirements to consider when growing aflatoxigenic fungi for NA analysis.

A. flavus is not a stable species concept. Some isolates produce abundant small sclerotia ('S') where B- and G-aflatoxins were detected (cf. *A. parasiticus*); others with fewer and larger sclerotia ('L') produced only detectable B aflatoxins. However, some S isolates were named *A. flavus* var. *parvisclerotigenus,* although the type isolate was a 'nonproducer' of G aflatoxins. *A. flavus* have diverse (i) vegetative compatibility groups, (ii) isozymes, (iii) electrophoretic karyotypes, (iv) randomly amplified polymorphic DNA (RAPD), (v) nuclear and mitochondrial DNA RFLPs, (vi) RFLPs of nuclear coding genes, and (vii) DNA sequences. RAPD showed S isolates formed a single clade derived from within the L. Furthermore, RAPD analysis was undertaken among toxigenic and nontoxigenic isolates and it was found that neither was monophyletic. DNA sequences from five different coding gene regions in isolates found two clear groupings. Also, a close phylogenetic association between *A. flavus* and *A. oryzae* has been demonstrated. *A. oryzae* is used to produce soya sauce and concerns about mycotoxin production persist. Obviously, it is important to use methods which establish accurately the similarity of such taxa, and to avoid mutagens in media. Molecular methods, including isozyme analyses, DNA/DNA hybridization studies, and DNA sequencing, have confirmed a very strong phylogenetic connection between the two species. In fact, 100% DNA/DNA hybridization between *A. flavus* and *A. oryzae* has been demonstrated. Some of the genes in the aflatoxin biosynthetic pathway appear to be present in *A. oryzae* isolates, although large deletions have been

detected in the cluster in different *A. oryzae* isolates. Toxin production is known to vary greatly among *A. flavus* isolates, with aflatoxin levels ranging from undetectable to 'quite' high. However, Geiser *et al.* (2000) has provided evidence of genetic distinctiveness between *A. oryzae* and most aflatoxin-producing strains of *A. flavus*. Furthermore, Cary *et al.* (2005) analysed fungi external to the *Aspergillus* section *flavus* taxon and which produced aflatoxins. Again the media used for growth may have supported aflatoxin production and affected results as discussed herein. Mitochondrial DNA characterization was useful in determining the taxonomic status of isolates in *Aspergillus* section *Flavi*. RFLPs allowed a clear differentiation between the *A. parasiticus–A. sojae* and *A. flavus–A. oryzae* groups. The medium used is provided in Table 1. Additional DNA sequence analyses will be necessary to determine whether *A. oryzae* represents a distinct organismal lineage within the *A. flavus/parasiticus* species complex or whether it simply possesses a divergent class of alleles in the aflatoxin gene cluster, although one hopes that conditions will be employed to minimize mutagenic SM production. Growth media employed in Geiser *et al.* (2000) are provided in Table 1, and it is reasonable to suggest that aflatoxins would be produced. Variation, as described above, may be explained by aflatoxin production in the growth medium affecting the resulting phenotypes, indeed the DNA of a different taxon could become more similar to another by mutation rather than less similar.

Peterson (2000) undertook a phylogenetic analysis of *Aspergillus* using rDNA sequences which included aflatoxigenic strains. The growth conditions used are provided in Table 1 and do not appear to be designed to inhibit SM. Finally, Peterson *et al.* (2008) presented a novel taxonomic classification of the *Aspergillus* sections based on multilocus sequence data where the SM production of each taxon is also presented. The methods for growth were provided in Table 1. Again, it is entirely possible that SM would be produced in the medium for biomass production, including aflatoxins within the appropriate groups. Finally, Klich *et al.* (2003) described the atypical aflatoxin producer *A. ochraceoroseus* and used YES to grow the fungus (Table 1).

Samson *et al.* (2004) investigated the *β*-tubulin sequences for the phylogenetic analysis of *Penicillium* subgenus *Penicillium*. The authors are also very familiar with SM production from this taxon, some of which are suspected mutagens (e.g. patulin and ochratoxin A). The growth and conditions are listed in Table 1 and it would be expected that SM would be produced by these fungi. The growth conditions used in some other phylogenic studies are also provided in Table 1 and see Paterson (2007a) for the equivalent data on diagnostic development.

Furthermore, the partial translation elongation factor 1 alpha gene and intron sequences are reported from *Fusarium*: The isolates were inoculated to a growth medium as described in Table 1 for extraction and SM may have been produced in line with what is being considered herein. It is emphasized that the objective of this review is to point to the paradox of growing fungi for NA analysis in media that may support mutagenic SM with a view to improve future procedures.

Paterson (2006a, 2007a) and Niessen (2008) have reviewed the literature concerning fungal diagnostic methods using PCR. This is relevant to the patulin metabolic pathway, which has been investigated by the current authors. Indeed, the use of the genes as a diagnostic method was the motivating factor to consider the topic now discussed (Paterson 2004, 2006b). Results were obtained which were not predictable. These included the detection of the gene in unexpected species, and occasionally not obtaining a positive result from all members of some species. One explanation for not obtaining a positive result from all members of a species was the occurrence of false negative results from inhibitors (Paterson 2007a, 2008a). The various media listed by Paterson (2007a) were not designed to inhibit SM except for one (Sánchez *et al.* 2005).

Furthermore, Dombrink-Kurtzman (2005) investigated the differences between the sequences of the *idh* of *P. griseofulvum* and *P. expansum*. The growth conditions for extraction are provided in Table 1. Both species produced patulin in culture with *P. griseofulvum* producing much more under the conditions employed (Dombrink-Kurtzman and Blackburn 2005). The analysis of the sequences revealed that the three strains of *P. griseofulvum* tested had two introns located at nucleotides 2086–2139 and 2383–2435 based on the *idh* sequence provided in GenBank; there were nine differences among the 54 bp in intron 1 and five differences among the 53 bp in intron 2 for the three strains of *P. griseofulvum*. However, all three strains had been correctly identified because they had the same D1/D2 sequence as the neotype of *P. griseofulvum*. Furthermore, sequences of ITS1 and ITS2 from two strains were identical to that of the neotype of *P. griseofulvum*. However, a third strain of *P. griseofulvum* had one nucleotide difference in ITS1 and ITS2. The three *P. expansum* strains were identical for introns 1 and 2, but there were nine additional bases in intron 1 compared to the *P. griseofulvum* idh gene deposited in GenBank. *P. expansum* had more nucleotide differences in both introns than did strains of *P. griseofulvum*. The *P. expansum* strains had the same D1/D2 sequences, and ITS1 and ITS2 sequences, as the neotype *P. expansum*. The majority of the differences were in the third codon position of the amino acids, when compared with the reference *P. griseofulvum*.

For example, one *P. griseofulvum* had nine bp differences and another five differences compared to the standard. Differences in the idh sequence were found between *P. griseofulvum* and *P. expansum* strains were considered likely to influence the ability to produce patulin.

Dombrink-Kurtzman (2007) extended the work to many other patulin producing species and demonstrated good correlation with species concepts, although *P. griseofulvum* 3523 was considerably different from other strains of the species. The growth conditions were changed from those in the earlier study (Table 1). In addition, results of a maximum parsimony analyses indicated that *P. carneum* and *P. paneum* were closely related. These have been reported to be indistinguishable using traditional morphological and physiological characteristics, although they have a 12 nucleotide difference in the ITS regions (Boysen *et al.* 1996).

Furthermore, the potential of *Byssochlamys nivea* and *Byssochlamys fulva* to produce patulin (for example, in canned food) was discussed in relation to polyketide synthase (*pks*) and *idh* involved in the patulin biosynthesis pathways in the genomes. The strains were also characterized by ITS rRNA and *β*-tubulin fragment amplification and sequencing. All of the *B. nivea* and none of the *B. fulva* produced detectable patulin. However, it is noted that two strains (25% of those tested) were renamed *B. nivea* from *B. fulva*, and one was the lowest patulin producer of those tested. The *pks* and *idh* were cloned from *B. nivea*: The deduced amino acid sequence of *pks* was 74% identical to the 6-methylsalicylic acid synthase gene of *P. griseofulvum* and had the five functional domains characteristic of fungal type I *pks*. The complete coding sequence of *idh* gene displayed 88% and 85% identity with *P. griseofulvum* and *P. expansum idh* respectively. The presence of these genes was determined in the genome of the stains by PCR and all *B. nivea* and no *B. fulva* strains displayed the genes. The authors concluded that the absence of 6-MSA and *idh* can explain why *B. fulva* cannot produce patulin (Puel *et al.* 2007). All isolates were inoculated in YES broth for DNA extraction, and placed on a shaking incubator at 200 rev min^{-1} at 25°C for 4 days. In contrast, Paterson (2007e) found *idh* was expressed in two strains each of *B. fulva* and *B. nivea* and hence these points require further investigation, particularly in relation to DNA polymerase inhibition and mutagenesis.

Paterson (2007d) discusses the issue of diagnostic methods for the oil palm pathogen *Ganoderma*, and again the growth conditions may support SM production, as the fungus is a prolific SM producer (Paterson 2006c). Hong and Jung (2004) provided an assessment of the classification of *Ganoderma* and the conditions used for growth are provided in Table 1. Paterson (2008b)

mentions that the phylogenic analysis of *Cordyceps* (Sung *et al.* 2007) is difficult to assess from this point of view, as the methods of growth of the fungi tested are not able to be determined readily in the paper.

Studies which are carefully linked to the other characters used in classification such as morphology, could assist to ameliorate problems that may occur with altered NA. However, mutations may be genetically determined in so far as the SM which cause the mutations are; indeed SM have been employed in classifications as mentioned. It is absolutely crucial that mutagenic molecules are avoided in such studies. Indeed, SM are produced in cultures at high concentrations, and can be used as characters in fungal identification or classification (Frisvad *et al.* 2008).

Future work

Growing fungi in media which do not stimulate SM production need to be investigated and these should be employed for NA analysis. SM can be controlled by employing continuous culture where the fungus can be maintained in trophophase (i.e. growth) rather than idiophase (i.e. SM production). In this way, SM can be reduced. However, this may not be practical for analysing many cultures for DNA. Growing fungi for the minimum amount of time may be useful, and in minimum nutrients as this will limit SM. The use of water to grow fungi may be appropriate in this regard (Paterson 2007b). However, the ratio of SM to NA may be more significant in terms of mutagenesis rather than simply low SM concentrations. Obviously, experiments are required to determine if fungal SM affects NA in fungi. For example, pure NA of *A. flavus* could be incubated with various concentrations of aflatoxin over time and then sequenced. The next step could be to grow the fungus in various concentrations of additional aflatoxin and the NA extracted and sequenced. Similar experiments without additional aflatoxin would be desirable. Other work can surely be devised.

Conclusions

It is conceivable that strains that possessed the *idh* gene of the patulin metabolic pathway were mutated so that negative or positive results were obtained in relation to the present authors' work. The mutations could be conceived of as being from (i) direct effects on NA and/or (ii) enzyme inhibition causing decreased stability of NA. This may be another form of false negative result. In addition, a false positive reaction is possible whereby the gene is mutated to the *idh* sequence in strains which, in the unaffected state, would be negative. This has ramifications for other diagnostic methods used in other fungi and for phylogenetic studies, with aflatoxigenic fungi being of prime concern. Fungi have evolved mechanisms to remove toxins such as transporter proteins. However, these mechanisms may not cope in pure culture where concentrations of SM can be large. Finally, confidence in data will be improved by employing media which do not support the production of mutagens when growing fungi for DNA analysis.

Acknowledgements

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