Food Control 35 (2014) 329–337

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Review

Self mutagens affect detrimentally PCR analysis of food fungi by creating potential mutants

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A R T I C L E I N F O

Article history:
Received 24 April 2013
Received in revised form
10 July 2013
Accepted 16 July 2013

Keywords:
Mutagens
Mutation
PCR
Food fungi
Aflatoxins
Mycotoxins

A B S T R A C T

Analysing fungi from food by PCR is increasing rapidly. However, food fungi produce mutagens which may mutate the fungi in culture so that fungi which produce mycotoxins may have a negative PCR result for genes in the mycotoxin metabolic pathway and *vice versa*: It is impossible to state unequivocally that the current PCR results obtained are accurate. For example, food containing a mycotoxin fungus may be considered safe if the isolates from the food were mutated into being negative for the mycotoxin (or other) gene and *vice versa*. Growth conditions affect which mutagens are produced and the conditions used by authors are assessed for the first time in the current report. Previous research assumed that NA was unaffected by how fungi were grown despite no supporting evidence. Individual research groups used similar growth conditions for disparate fungi for PCR analysis which were different from methods used by alternative workers. Rationales for using particular growth methods are unexplained. The fungi will be in almost continuous contact for long periods with various biochemical mutagens at high concentrations. Only partial solutions can be provided by suggesting alternative methods. Future methods need to state why particular conditions are employed when growing fungi and what was done to avoid mutagens.

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http://dx.doi.org/10.1016/j.foodcont.2013.07.024

1. Introduction

PCR methods to detect microorganisms from food are extensive. However, a firm grounding in basic methods is required before the more novel procedures can be applied accurately (Editorial, 2013). PCR is used to analyse isolated fungi from food, or the food itself for particular fungi and much concern about fungi in food is from the production of mycotoxins which have powerful mutagenic activities causing cancers in animals and humans (Table 1) (Luch, 2006; Paterson & Lima, 2010). Interpretation of PCR results is equivocal because mutagenic secondary metabolites are produced by the target fungi in the growth media (Paterson & Lima, 2009, 2013; Paterson, Sariah, Lima, Zainal Abidin, & Santos, 2008) and, for example, fungi which are normally positive for a mycotoxin, or other, gene may be mutated to negative. This makes it possible that analysis of food by PCR may yield false results for fungi (Paterson 2012a, 2012b) and this is discussed herein.

A mutagen is a physical or chemical agent that changes the genetic material, usually DNA, of an organism and increases the frequency of mutations above the natural background level. Mutations occur due to spontaneous hydrolysis, and errors in DNA replication, repair and recombination. Mutagens are likely to be carcinogens as many cause cancer. Most genotoxic organic carcinogens require metabolic activation to exert detrimental effects on DNA. The parent compounds are considered as pre-carcinogens bioactivated into carcinogenic forms (Luch, 2006). Hence, some mycotoxins will not bind to DNA without activation. Many are not mutagenic, but can form mutagenic metabolites through cellular processes and such mutagens are called pro-mutagens (e.g. aflatoxin). Chemicals may interact directly with DNA: Others (e.g. PAHs, aromatic amines, and benzene) are not necessarily mutagenic *per se*. Mutagens may modify the DNA sequence which includes substitution of nucleotide base-pairs and insertions and deletions of one or more nucleotides in DNA sequences (Table 1). The effect of mutagens may not be obvious in, for example, fungi because mutations can (a) have minor effects, as they do not result in residue changes with significant effects on proteins and (b) be silent because they occur in non-coding or non-functional sequences, or do not change the amino-acid sequence due to the redundancy of codons (Burnett, 2003). For example, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* have particularly large genomes but small concentrations of coding DNA (Clutterbuck, 2011). Nevertheless, mutagens may act directly on DNA, causing direct damage, and most often result in replication error; others may act on the replication mechanism and chromosomal partition. Intercalating agents (e.g. ethidium bromide and proflavine) may insert between bases in DNA, causing frameshift mutation during replication (e.g. the mycotoxin alternariol (DiCosmo & Straus, 1985)). Base analogue mutagens can substitute for DNA bases and cause transition mutations (e.g. cordycepin (see later)). Finally, mutagenesis is the driving force of evolution (Burnett, 2003; Luch, 2006). How mutations within fungi are manifest is considered in the following section with relevance to our discussion.

2. Mutation in Fungi

Mutation is the sole source of variation which occurs in nDNA and mtDNA where the latter are common in fungi: Recombination mainly generates novel multi locus genotypes. Furthermore, phenotypic detection of mutations can be rapid in fungi, as in new virulent mutants (Joosten, Cozijnsen, & De Wit, 1994) and fungicide resistant mutants of crop pathogens, or in selective situations (e.g. industrial processes) and so could occur during growth of food fungi for PCR analyses. Interestingly, industrial fungal production (e.g. growth in bioreactors) is similar to the conditions employed to grow fungi for PCR analysis (e.g. pure culture, sterile growth conditions, nutrients supplied in batch form). Whether a new mutation persists and is beneficial to the fungus depends on the mutation rate, the genome in which it is located and the size of the population. A mutant or rare allele has a better chance of eventual survival if the mutation rate is high and reverse mutation is low. Thus, the potentially advantageous mutants, regarded as the most significant by some even at the molecular level, probably become established in a population only through recurrent mutation: Any gene present in a fungal population at a low frequency can be lost or fixed from (a) the inevitable random sampling of conjugating gametes or individuals as in sexual reproduction, or (b) if the population is maintained predominantly by asexual spores, when these are dispersed and germinate and persist or perish. Such stochastic changes occur regardless of whether or not the gene confers a potential selective advantage (Burnett, 2003). The present authors are considering mutants in pure culture where the spores can only disperse within the confines of the growth vessel and mutants are likely to accumulate much quicker.

Mutations in *Neurospora crassa* increased by 0.3 per cent per week at 32 °C but at 0.1 per cent per week at 4 °C (indicating temperature dependent enzymatic activity (see Section 3). Presumably these reflected mutations at a number of unspecified loci on non replicating nuclei. Other spontaneous mutation rates appear in the range of ca. 1 in 10^6 to 10^7 (Burnett, 2003). Mutation frequency could be affected by conditions of starvation or stress which arise when nutrients become depleted and this can stimulate the production of secondary metabolites. Hence, the stress may increase mutations *per se* and this could be compounded by the production of mutagenic secondary metabolites.

Table 1
Known (a) mutagenicity of various mycotoxins and (b) damage to DNA (see Paterson & Lima 2013).

Mycotoxin	Mutagenicity	Known damage to DNA in general
Aflatoxins	Most carcinogenic natural compounds; induce DNA damage; affect negatively the amelioration of damage; alter DNA base composition of genes.	Intercalations, intra and inter-strand cross links; Apyrimidinic sites; Apurinic sites;
Sterigmatocystin	Covalent binding to DNA; DNA adduct formation; carcinogenic.	Hydrolytic deamination;
Ochratoxin A	Potent carcinogen; DNA single strand breaks; Forms DNA adducts; Mutagenic activity; induces base substitutions; increased mutation frequency.	Single strand breaks; Radical formation; Double strand breaks;
Patulin	Induces DNA–DNA crosslinks; mutagenicity; reactivity to DNA.	DNA-protein cross links;
Deoxynivalenol	DNA damage; genotoxic.	Pyrimidine dimmers;
Nivalenol	Direct mutagen; DNA damage.	Base damage;
Fusarenon X	DNA damage; increases DNA strand breaks.	Alkylation;
Fusarin C	Mutagenic.	6-4 photoproducts;
Altertoxin I, Alternariol, *Alternaria* extracts	Mutagenic.	Bulky adducts; Loss of bases.

Spontaneous mutants of *Aspergillus nidulans* include prototrophic white, yellow, and pale green conidium changes: the pigments are secondary metabolites, hence these are secondary metabolite mutants and relate directly to our discussion. Also, 15 auxotrophic mutants occur, e.g. 5 requiring *p*-amino benzoic acid, 2 requiring adenine, etc. and a considerable number were mutants resistant to toxic substances. All appeared to be single gene mutations and no structural changes were detected. This is important to the present discussion where only a small change in the genome could occur (e.g. metabolite production) from particular combinations of mutagenic secondary metabolites and concentrations, whilst the rest of the genome may remain unaffected and stable. Spontaneous mutations of the avirulence gene of *Cladosporium fulvum* can be from a single amino acid substitution in the elicitor protein it produces, i.e. a single base in a codon: In another mutant the loss of a single nucleotide resulted in a frame shift in the N-terminal end of the protein from 18 to 13 amino acids. The consequence is virulence to resistant tomatoes (Joosten et al. 1994). *Puccinia striiformis* f.sp. *tritici* is a clonal fungus and in 10 years ca. 15 new natural mutant pathotypes were identified almost certainly due to single gene mutations (Wellings & McIntosh, 1990). Thus very small changes in the genetic material, nucleotide substitutions or deletions, or frame shift mutations due to insertions or deletions, can result in biologically significant spontaneous mutants. In contrast, the mutagenic agents used in induced mutations can cause structural chromosomal changes (Burnett, 2003).

3. Mutagenic potential of mycotoxins

The carcinogen compounds from fungi include aflatoxins, sterigmatocystin, ochratoxin A, fumonisin, zearalenone, citrinin, luteoskyrin, patulin, and penicillic acid (Table 1) produced by a wide range of fungi. These compounds may be self-mutagenic towards fungi (Paterson & Lima, 2013) and avoiding them during growth *in vitro* is necessary as they may affect the results of PCR analyses, in that normally positive strains for a gene may mutate to negative and *vice versa*. All the above compounds are DNA damaging agents except for fumonisins which may act via disturbing signal transduction pathways (Paterson & Lima, 2009, 2010). Furthermore, DNA may sustain more than 50,000 damages per cell per day (Loeb & Nishimura, 2010) and oxidative adducts per cell generated through reactive oxidative species may be 150,000 (Beckman & Ames 1997). These can give rise to mutation if left uncorrected. The repair mechanisms are based on enzymes and many fungal secondary metabolites are enzyme inhibitors which could be involved in inhibiting DNA repair (Paterson, 2008; Paterson et al., 2008).

The mycotoxins with carcinogenic effects have been discussed in depth by the current authors (e.g. Paterson & Lima, 2013). However, some evidence will now be considered as these can be produced in culture during growth of food-related fungi. Aflatoxins are the most carcinogenic natural compounds known and avoiding contact is a priority when growing fungi. The metabolites are well-know to act directly in bacteria (Yourtee, Kirk-Yourtee, & Searles, 1987) rather than by epigenetic activity. The order of direct mutagenic activity compared to indirect or microsomal activity was as follows; aflatoxin Q1 much greater than G1 greater than B1 greater than aflatoxicol, which is somewhat surprising given the known high general activity of B1. The mycotoxins are produced by *Aspergillus flavus* and *A. parasiticus*. They directly or indirectly (1) induce DNA damage, (2) affect negatively the amelioration of damage, and (3) alter DNA base compositions of genes. The mutagenicity of aflatoxin B1 has been demonstrated in many systems and it induces chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, chromosomal strand breaks, and forms adducts in rodent/human cells: aflatoxins containing an unsaturated terminal furan ring can bind covalently to DNA forming an epoxide. Possible mechanisms for carcinogenicity include the induction of the cytochrome P450 system which produces mutagenic compounds from promutagens: fungi possess extensive cytochrome P450 systems (van Den Brink, Van Gorcom, Van Den Hondel, & Punt, 1998) which could be involved.

Sterigmatocystin has a chemical structure similar to and is a precursor of, aflatoxin, although produced by a wider range of fungi. The compound is mutagenic and binds covalently to DNA: DNA adducts formation has been reported. Aflatoxin biosynthetic pathway intermediates norsolorinic acid, averufin, and versiconal acetate were found to possess questionable mutagenic activity, but versicolorin A and sterigmatocystin were significant mutagens. The mutagenic activity appeared related to the bisfuran and not the anthraquinone moiety of the molecule, despite the latter being a key structure of such a potent carcinogenic mycotoxin as luteoskyrin. Furthermore, extracts of *A. nidulans* and *Aspergillus fumigatus* had mutagenic properties (Paterson & Lima, 2013).

Ochratoxin A is produced by several *Aspergillus* and *Penicillium* species, including *Aspergillus carbonarius*, *Aspergillus westerdijkiae*, and *Penicillium verrucosum*. It is one of the most potent carcinogens in rats, and is classified as a possible human carcinogen by the International Agency for Research on Cancer. Ochratoxin A induced DNA single-strand breaks in cultured mouse and CHO cells. Adducts were found in the kidney, liver, and spleen of mice treated with ochratoxin A, and the DNA adduct level was dose-dependent and time related. The strongest evidence in favour of ochratoxin-mediated DNA damage is the induction of DNA single-strand breaks and formamidopyrimidine-DNA glycosylase sensitive sites. Mutagenic activity by ochratoxin A has been reported (e.g. in murine cells). Ochratoxin-induced base substitutions were similar to those arising spontaneously, suggesting that it increases the process involved in spontaneous mutagenesis.

Patulin is produced by many species within *Aspergillus, Penicillium, Byssochlamys*, and *Paecilomyces*. DNA–DNA crosslinks are induced in the millimolar range which is a possible mechanism of the mutagenicity observed. The direct reactivity of patulin towards DNA in a cellular system has been demonstrated and mutations of cells might be from an indirect mutagenic mechanism (e.g. inhibition of enzymes that stabilise DNA) (Paterson & Lima, 2009). In addition, botryodiploidin is listed as mutagenic and produced by *Penicillium paneum* and *Penicillium brevicompactum* (Frisvad, Smedsgaard, Larsen, & Samson, 2004).

The genotoxic risk associated with the *Fusarium* mycotoxin, deoxynivalenol (Table 1) is poorly explored although dividing cells were more sensitive than differentiated. There is a genotoxic potential for deoxynivalenol at low concentrations (Paterson & Lima, 2013). Nivalenol damaged the nuclear DNA of CHO cells demonstrating that it is a direct mutagen (Paterson & Lima, 2009). DNA damage appeared in the kidney and bone marrow of mice after oral dosing, and nivalenol showed organ-specific genotoxicity in mice related to time and intensity as a direct mutagen. Nivalenol and fusarenon X 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 for even short periods. Furthermore, fusarenon X increased DNA strand breaks in dividing cells. The *Fusarium graminearum* metabolite zearalenone showed a positive DNA damaging effect in tests with *Bacillus subtilis* and treatment of mice led to the formation of DNA adducts (Paterson & Lima, 2009). Fusarin C is mutagenic and is produced from *Fusarium moniliforme*. 7-desmethyl analogues of fusarin C and (8Z)-fusarin C are mutagenic toxins of fusaria from corn which were detected from fermentation extracts of the entomopathogenic and possible biocontrol fungus, *Metarhizium anisopliae*. The metabolites

exhibited potent S9-dependent mutagenic activity (Krasnoff et al., 2006).

Extracts of two aflatoxin-producing isolates of *A. flavus* and *A. parasiticus* showed pronounced mutagenic activity. Five other species (*Aspergillus heterothallicus*, *A. nidulans*, *Aspergillus terricola*, *Alternaria tenuis*, and *F. moniliforme*) which (unsurprisingly) did not contain detectable aflatoxins, were also mutagenic. *Botrytis cineria, Ceratocystis fimbriata, Cladosporium herbarum, Fusarium solani* f. sp. *pisi, Penicillium oxalicum, Thermomyces lanuginosus*, and *Verticilium albo-atrum* were possibly mutagenic (Paterson & Lima, 2013).

An extract of *Alternaria alternata* was mutagenic as were isolated compounds altertoxins I, II, and III with and without metabolic activation. Altertoxin II (ATX II) has a perylene quinone structure and is at least 50-times more potent than the common *Alternaria* toxins alternariol (AOH) and alternariol methyl ether (AME) (Fleck et al. 2012). The mutagenic effects of nitrosylation were examined with the major *Alternaria* metabolites altenuene, AOH, AME, altertoxin I (ATX I), tentoxin, tenuazonic acid, and radicinin. In the absence of nitrosylation, ATX I was mutagenic, while AOH and ATX I were weakly mutagenic in a different system. Incubation with nitrite generally increased mutagenic potencies. However, subsequent examination of three extracts made from *A. alternata* culture broth showed a different mutagenic response with broth and acetone washes being directly mutagenic in some cases (Paterson & Lima, 2013).

Cordycepin (3-deoxyadenosine) from *Ophiocordyceps* is a derivative of the nucleoside adenosine differing by the absence of oxygen in the 30 position of its ribose entity. The fungus is consumed as a food supplement extensively, and indicates the mutagenic potential of fungal secondary metabolites in general. Some enzymes do not discriminate between adenosine and this compound; hence it can participate in related reactions. For example, it can be incorporated into RNA molecules causing premature termination of its synthesis. The compound is considered to cause double strand DNA breaks and is in an inhibitor of repair enzymes of DNA (Lee, Burger, Vogel, Friese, & Brüning, 2012).

Finally, lack of information on other mycotoxins and secondary metabolites should not be interpreted as a statement on the mutagenicity of these agents. The aflatoxin literature serves as a model for subsequent research into other secondary metabolites.

4. Self mutagens in fungi

Fungi are subjected to potential biochemical mutagens during growth (Paterson & Lima, 2009, 2013; Paterson et al., 2008) and it is illogical to undertake PCR analyses under these circumstances, as the DNA may be mutated and the results affected detrimentally. The mutagens (Paterson & Lima, 2009; Paterson et al., 2008) will be present in the cells of the producing fungi when they are grown (Fig. 1): some are intracellular compounds, whereas others are detected extracellularly and intracellularly. The basic methods for fungal growth for PCR are essentially the same as those used for over a century for growing fungi and are outdated in comparison to methods employed for nucleic acid (NA) analysis. This may be satisfactory if DNA was stable and there would be little cause to doubt this if mutagens in growth media did not occur. DNA is susceptible to damage (Table 1; Paterson & Lima, 2013) and high concentrations of various secondary metabolites are produced in agars: high concentrations of mycotoxins in agars are reported in Rodríguez, Córdoba, Werning, et al. (2012), Rodríguez, Werning, et al. (2012), Rodríguez, Córdoba, Gordillo, et al. (2012), Rodríguez, Rodríguez, Luque, et al. (2012) and Rodríguez, Rodríguez, Andrade, et al. (2012). These high concentrations are indicated when undertaking the analysis of fungal cultures using the "agar plug" technique developed by Frisvad (e.g. Frisvad, Andersen, & Thrane, 2008). A high number of secondary metabolites are observed on TLC plates simply by placing small plugs from agars onto TLC plates and developing in an appropriate solvent without a concentration step.

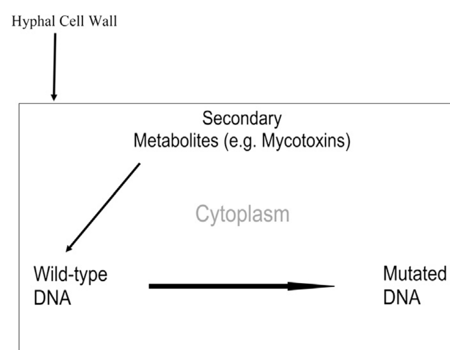

Fig. 1. Image of how mutagenic secondary metabolites from fungi can interact with fungal DNA in the cytoplasm and mutate the DNA. The mutagens can act directly or indirectly.

The priority must be avoidance of mutagens during growth for valid interpretations of PCR data (Morcia et al., 2013) and this process is itself complicated. In addition to, and based on, the current authors' published papers, some other scientists have recognized the possibility of mutated fungi in the manner described (Hawksworth, 2009; Morcia et al., 2013; Sant'Ana et al., 2010), whereas one group of researchers provided an inadequate solution as discussed in Paterson (2012c), as they indicate that internal amplification control (IAC) can validate PCR results from the generation of fungal mutants (Rodríguez, Córdoba, Werning, et al., 2012, Rodríguez, Werning, et al., 2012). Equivalent methods to these were used in a subsequent paper concerning ochratoxin A (Luque, Córdoba, Rodríguez, Núñez, & Andrade, 2013) although considerations of mutated fungi or IAC were not provided. Rodríguez, Córdoba, Werning, et al. (2012) was cited in Rodríguez, Córdoba, Gordillo, et al. (2012) for sterigmatocystin determination although an IAC was not employed, and the fungal mutant issue was not considered in this paper.

5. How growth conditions used for fungi vary for analysis

It is well known that secondary metabolite production varies depending on the growth conditions such as the media used, time of growth, shaken or static culture, light or dark (Frisvad et al. 2008; Schmidt-Heydt et al., 2011): hence the production of self mutagens in culture will be influenced highly by how the fungi are grown. Also, Paterson and Lima (2013) explained how strains could be mutated during isolation from substrates such as food and it is possible that the strains used for analyses may be laboratory-created artefacts even before being used for the experiments for which they are required. There is a large number of papers regarding the analysis of fungi from food and Table 2 indicates how a wide-range of different conditions are used for growing fungi relevant to food production which may allow production of a correspondingly extensive range of potential mutagens, further complicating the interpretation of results. The papers are assessed here for the first time and are commented on in more depth as follows:

5.1. Ochratoxin A

Seventy-five strains belonging to predominantly aspergilli and penicillia were obtained from large culture collections (Rodríguez,

Table 2
Growth methods described in various papers related to mycotoxigenic fungi. Isolation and maintenance data are provided as these can affect mutations (Paterson and Lima, 2013). Space indicates insufficient detail or information not relevant to particular paper. In many cases media were described which had the same name, but used different manufactures of the components in different studies (Potato Dextrose Agar (PDA) had a particularly large number of manufacturers). PDB = Potato Dextrose Broth; Malt Extract Agar = MEA; Czapeck Yeast Extract Agar CYA, Malt Agar = MA; Yeast Extract Sucrose (YES).

Related mycotoxin	Isolation/Maintenance	Genomic analysis	Toxin analysis	References
Ochratoxin A		PDA 7 days, PDB 3 days static culture		Atoui et al. (2007).
	PDA, 4–5 days	Sabouraud Broth (SB), 3 days, shaking		Gil-Serna, González-Salgado, et al. (2009)
	PDA 25 ± 1 °C, 4–5 days	SB, 28 ± 1 °C, shaken (120 rpm) 3days		Gil-Serna, Vázquez, et al. (2009)
	PDA	PDA 7 days; liquid Sabouraud medium, 3 days shaking 28 °C.		González-Salgado et al. (2009)
		Wikerham's medium, shaking		Susca et al. (2007)
		MEA, 28 °C, 6–8 days	CYA, 25 °C, 7 days	Martínez-Culebras et al. (2009)
		Pontecorvo's liquid medium, 40 h, 28 °C, shaking (150 rpm)		Morello et al. (2007)
		MEA, 4 days, 25 °C. 20 days, MEA	PDA, 15 days, 25 °C	Rodríguez, Rodríguez (2011)
		PDA, 4 days, 25 °C. PDA, 20 days.	ditto	Luque et al. (2013)
Aflatoxin	Dichloran rose Bengal chloramphenicol agar plus 3% NaCl (DRBC+), 25 °C, 5–7 days	(DRBC+), 25 °C, 5–7 days		Passone et al. (2010)
		Minimal nitrate medium, 37 °C, agitation, 2–3 days	Malt Glucose Agar (MGA): MEA, Coconut Agar medium (CAM), YES. MGA and CAM, 26 °C, 5 and 7 days. YES, 28 °C, 14 days	Criseo et al. (2008)
	PDA, 4 °C	PDA, 25 °C, 150 rpm, 2 days		González-Salgado et al. (2008)
		MEA, 4 days, 25 °C. Also 20 days.	MEA, 15 days, 25 °C	Rodríguez, Rodríguez, Luque, et al. (2012)
	MEA, dark, 7 days, 25 °C. Morphology on: MEA and CZ, 7 days, dark, 25 °C	"7 day culture"	Aflatoxin: Coconut Agar Medium, 7 days, dark, 25 °C. YES, Yeast Extract Peptone medium 25–27 °C, 7 days, dark. Cyclopiazonic acid: CYA, 25 °C, 14 days, dark	Rodrigues et al. (2009)
Sterigmatocystin		MEA, 4 days, 25 °C. MEA, 20 days	MEA, 15 days, 25 °C	Rodríguez, Córdoba, Gordillo, et al. (2012)
Fumonisin (from *Aspergillus*)	Malt Extract, Dichloran-rose Bengal medium	Malt Peptone Broth, 25 °C, 7 days	CYA20S agar, 7 days	Varga et al. (2010)
Trichothecene chemotypes		PDA, 5–6 days.		Kammoun et al. (2010)
Toxigenic fusaria	PDA, 28C, 7d. PDA and Spezieller Nahrstoffarmer Agar, 25 °C, 2–4 weeks. Sometimes Carnation Leaf Agar.	PDB, 22 °C, 6 days, 200 rpm. V8, 4 or 5d	Toxin broth, 10 days, 200rpm	Pasquali et al. (2010)
Nivalenol	Modified Dichloran-chloramphenicol-peptone agar + crystal violet,12 days, 22 ± 2 °C. PDA, 22 ± 2 °C, 6–10 days.	PDB, 22 °C, 6 days, 200 rpm. V8, 4 or 5d	Toxin broth, 10 days, 200rpm	Pasquali et al. (2010)
Patulin	PDA + vancomycin, PDA with or without streptomycin and chloramphenicol. PDA or MA. MA with penicillin G and chloramphenicol	PDA or MA. Direct from freeze dried ampoules.	YES	Paterson et al. (2003); Paterson (2004)
		MEA, 25 °C, 4 days. MEA, 20 days, 25 °C	MEA, 25 °C, 15 days	Rodríguez et al. (2011)
		ditto	ditto	Luque et al. (2011)
Toxigenic *Fusarium, Aspergillus, Penicillium*	PDA	PDB, 3–7 days, 28 °C		Suanthie et al. (2009)
Aflatoxin, ochratoxin A, patulin		PDA, 4 days, 25 °C. 20 days, PDA	PDA, 15 days, 25 °C	Rodríguez, Rodríguez, Andrade, et al. (2012)
Cyclopiazonic acid		MEA, 25 °C, 4 days. MEA, 20 days, 25 °C	MEA, 25 °C, 15 days	Rodríguez, Werning, et al. (2012)
			CYA, 14 days, 25 °C	Rodrigues et al. (2009)
Verrucosidin		MEA, 25 °C, 4 d. MEA, 20 days, 25 °C	MEA, 25 °C, 15 days	Rodríguez, Córdoba, Werning, et al. (2012)
Alternaria (e.g. toxin producers)	PDA, MEA, Potato Carrot Agar (PCA). PDA and MEA, 7 days, 25 °C. PCA alternating light/dark cycles.	Malt extract broth (MEB), 4 days, 25 °C. Or MEA, PDA and MEB		Pavón et al., 2012

Rodríguez, 2011) (Table 2). It is worth pointing out that conidia were obtained from 20 day old cultures grown on MEA to test the sensitivity of the PCR on food, which is inordinately long, and allows for more time for mutations to occur. This represents a general procedure employed by these authors in other papers (Table 2). Atoui et al. (2007) employed a polyketide synthase gene for *A. carbonarius* quantification using real-time PCR. A wide range of different taxa were obtained from culture collections. Gil-Serna, González-Salgado, et al. (2009) used ITS-based analysis of a wide range of fungi enabling the detection and quantification of *Aspergillus ochraceus* and *A. westerdijkiae* in grapes and green coffee beans by real-time quantitative PCR. Gil-Serna, Vázquez, et al. (2009) analysed the predominant ochratoxin A-producing species in *Aspergillus* section *circumdati* by PCR. González-Salgado et al. (2009) reported specific detection of *A. carbonarius* by quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. Various aspergilli, *Botrytis* and *Saccharomyces* strains were analysed by Susca et al. (2007) to identify *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. Fifteen isolates collected from Brazilian coffee bean samples were used to design a species-specific primer pair to amplify a region of the β-tubulin gene from *A. westerdijkiae* (Morello et al., 2007). Finally, Martínez-Culebras et al. (2009) obtained isolates of black aspergilli from various vineyards and the growth conditions employed by the above authors are provided in Table 2.

5.2. Aflatoxins

Aspergillus section *Flavi* strains were analysed by Passone et al. (2010); Criseo et al. (2008) tested *Aspergillus flavus* from food, feed and plants and *A. flavus* cultures were analysed by González-Salgado et al. (2008). The sensitivity of qPCR for aflatoxin-producing fungi on artificially inoculated food matrices was tested with 20 day old conidia (Rodríguez, Rodríguez, Luque, et al., 2012) and again 20 days is particularly long. Finally, 31 isolates of *Aspergillus* Section *Flavi* from almonds were analysed by Rodrigues et al. (2009) (Table 2).

5.3. Patulin

Fungi were grown on MEA for 20 days (which is a long time) at 25 °C after which conidia were collected for various analyses of foods (Luque et al., 2011). Paterson (2012a,b) commented on this paper in relation to patulin production from *A. flavus*, *Aspergillus oryzae* and *Aspergillus tameri* as being unprecedented. However, (Luque et al., 2011) also detected the gene and patulin in *Penicillium camemberti*, *Penicillium commune*, *Penicillium melanoconidium*, *Penicillium polonicum* and *P. verrucosum* (Table 3) and none of these species are considered normally as patulin producers. This data was reproduced in Rodríguez et al. (2011) where a wide range of particularly penicillia and aspergilli, were tested in relation to patulin production. Hence, the strains in Rodríguez et al. (2011) and Luque et al. (2011) may have mutated to "patulin positive" in a manner consistent with the current deliberations. (A paragraph on "gain of function" is provided in Section 6. General Discussion.) The details of the patulin production of Type and typical strains from, for example, the Centraalbureau voor Schimmelcultures are available in the literature (e.g. Frisvad & Samson, 2004) and it is surprising that Rodríguez et al. (2011) and Luque et al. (2011) did not refer to them, as they indicate clearly which species (and actual strains) produce patulin.

P. hirsutum and some strains of *P. brevicompactum* freshly isolated from orchards (Paterson et al., 2003); Paterson (2004), were positive for the *idh* gene but they are not generally considered as patulin producers. Paterson et al. (2003) reported on the analysis of a large number of *Penicillium* species isolated from orchards, stores and pack houses in apple growing areas to test patulin production. These isolates were processed at CABI Bioscience (Egham, UK) for identification, accession with IMI strain numbers and maintenance. Freeze dried ampoules of fungi were obtained from the culture collection of CABI Bioscience for direct analysis without re-growth (Paterson, 2004) (Table 2). The use of these samples is a potential method for reducing exposure to mutagenic compounds from growth on media, despite no PCR product being obtained and more work is required.

Table 3
Fungal species positive for the various mycotoxins which were previously not considered as producers and may be mutants due to mutagen production from the growth conditions employed.

Mycotoxin	Species	Reference
Aflatoxin	*Penicillium aurantiogriseum, P. griseofulvum , P. commune, Aspergillus oryzae, A. tamari, A. tubingensis Rhizopus oryzae*	Rodríguez, Rodríguez, Luque, et al. (2012)
Ochratoxin A	*A. tamari, P. carneum, P. melanoconidium, P. aurantiogriseum*	Luque et al. (2013)
Patulin	*A. flavus, A. oryzae, A. tameri, P. camemberti, P. commune, P. melanoconidium, P. polonicum, P. verrucosum*	Luque et al. (2011)

5.4. Aflatoxins, ochratoxin A and patulin

Production of aflatoxins, ochratoxin A and patulin was tested in fungi by Rodríguez, Rodríguez, Andrade, et al. (2012). Strains were grown for 20 days on PDA to test the robustness and effectiveness of the qPCR method and again 20 days is unusually long, allowing more time for mutations to occur.

5.5. Verrucosidin, cyclopiazonic acid and sterigmatocystin

For verrucosidin determinations, 22 strains (predominantly penicillia and aspergilli) belonging to 14 species were obtained from various culture collections (Rodríguez, Córdoba, Werning, et al., 2012). Again, a long period of 20 days was taken to grow the fungi to produce conidia for PCR sensitivity testing. Thirty-two strains commonly found in foods and belonging to 16 different fungal species, were obtained from various BRC in the case of detecting strains for cyclopiazonic acid production (Rodríguez, Werning, et al., 2012). Growth conditions were identical to those used for verrucosidin production and similar methods were used for sterigmatocystin (Rodríguez, Córdoba, Gordillo, et al., 2012). Finally, cyclopiazonic acid was also determined in Rodrigues et al. (2009).

5.6. Fumonisin (from Aspergillus)

Varga et al. (2010) analysed black aspergilli using sequence analysis of the partial calmodulin gene. Fumonisins have been detected only recently from aspergilli and were an unexpected result: it is conceivable that production is a trait not associated with wild type strains corresponding to the general discussion herein.

5.7. Others

Various fusaria were analysed by Kammoun et al. (2010), Sampietro et al. (2010) and Pasquali et al. (2010). Suanthie et al. (2009) tested one or more taxa within *Fusarium, Aspergillus,* and *Penicillium*. Toxigenic *Alternaria* spp. were analysed by Pavón et al.

(2012). The various growth conditions employed are provided in Table 2.

Finally, the names of some ochratoxin A, aflatoxin and patulin producing species, which would not normally be considered as producers, are presented in Table 3 to provide evidence of mutations from the original wild type strains which may be from contact with self produced mutagens as described herein.

6. General discussion

The general theme of this paper is that the interpretation of results of the PCR of genes of fungi could be that strains negative for a gene may have been mutated from positive and *vice versa*, because of the presence of self mutagens in the growth media. Hence, when a foodstuff is contaminated with a fungus which is positive for a mycotoxin, a false result may be obtained when the fungus is isolated due to mutagenic secondary metabolite production in the culture causing the gene to mutate to one that does not yield a PCR product upon analysis. Hence, a contaminated food may be considered safe which represents the worst result for a diagnostic method.

The growth conditions were similar for each strain used in the individual studies described above and this standardization would be unnecessary they were unaffected by the conditions, i.e. almost any method could be used. The only criterion in the choice of methods appears to be that enough biomass is produced for extraction of sufficient DNA. The qualitative and quantitative production of mutagens will be different between strains and even between different growth runs of each strain when the same conditions are used for growing fungi. The same conditions do not produce the same growth environment for each strain as might be expected. In addition, methods were different between papers even for similar taxa. Overall, conditions employed for PCR analyses require to ensure minimal mutagen production and authors are required to state what the rationale was for the particular growth conditions employed.

In Table 3 we indicate that some fungi may have gained the ability to produce particular mycotoxins. Although “gain of function” mutations may appear unlikely upon first consideration, it should be noted that the Ames test uses *Salmonella typhimurium* strains deficient in histidine biosynthesis and checks for mutants that revert to wild-type (i.e. gain a function). The resistance to 8-azaguanine in the *S. typhimurium* test checks for forward mutation that confers resistance to 8-Azaguanine in a histidine revertant strain. Systems similar to the Ames test have been developed in yeast and *Saccharomyces cerevisiae* resistance to 5-methyltryptophan may be used for forward mutation. Furthermore, it was at first believed that point mutations happened by chance, without regard to their effects on organisms. Now it is suggested that these mutations occur in response to environmental challenges and are more likely to occur when they are advantageous to the organism, rather than when they are neutral or disadvantageous. When *Escherichia coli* cells were deprived of tryptophan for prolonged periods of time, point mutations in the trp operon reverted to tryptophan, leading to an advantageous result, more frequently than under normal conditions when the mutations were neutral (Hall 1990). This may occur in the case of secondary metabolite production where production may confer an advantage, such as greater virulence on a plant host or the ability to sporulate more efficiently. A considerable number of spontaneous mutations of *A. nidulans* were resistant to toxic substances. Spontaneous mutations of the avirulence gene of *Cladosporium fulvum* made the mutants virulent to resistant tomatoes and *Puccinia striiformis* f.sp. *tritici* had 15 new natural mutant pathotypes almost certainly due to single gene mutations. Thus very small changes in the genetic material can result in advantageous mutants as discussed in the Mutation in Fungi section of this paper.

Mutations may affect obviously a small part of the genome that is involved in mutagenic secondary metabolite production without necessarily affecting other parts of the genome. Many mutations (a) have minor effects as they do not result in residue changes that have significant effect on the structure and function of the proteins and (b) are silent mutations causing no visible effects at all because they occur in non-coding or non-functional sequences, or do not change the amino-acid sequence due to the redundancy of codons. Another useful example is that of *A. flavus* (the aflatoxin producer) and *A. oryzae* (employed in soy sauce production) which are very similar fungi except for aflatoxin production (Chang & Ehrlich, 2010), because most of the genome has remained almost unchanged.

Furthermore, only some (a) self-mutagenic secondary metabolites, (b) mixtures of compounds and (c) concentrations of compounds are suggested here as being capable of mutating genes of the producing fungi. The other factor is being in contact with the compounds for significant periods of time during growth. Hence, some genomes will be stable because mutagenic combinations of secondary metabolites were not encountered, but what these are remain unknown. These points need addressing at the experimental design level, or by minimising secondary metabolite production *a priori*.

7. Solutions

There are no absolute solutions to the problem of mutagens in growth media, and some procedures to ameliorate the problem were discussed (Paterson & Lima, 2009; 2013). However, it is important that work is undertaken to determine the effect of self mutagens on the genomes of fungi as a priority rather than assuming that there is no effect, which is the current situation. Preservation of the original substrates from which fungi were isolated may ensure the wild type is preserved, although re-isolation from the substrate may be impossible. More work is required on analysing directly the preserved culture (e.g. freeze dried ampoules (Paterson, 2004) without re-growth but whether the preserved culture was a wild type isolate would remain unknown (Paterson & Lima, 2013).

Growing cultures on different media for analysis may be useful and then undertaking PCRs. However, which of these cultures would be representative of the wild type? Variously mutated strains may be obtained as the secondary metabolites produced on each medium would be different. To test for the effect of secondary metabolite production on NA of the producing fungi the fungi could be grown for various periods (e.g. 3, 5, 7, 11, 14 days) and the results of the PCR determined. This may indicate if the sequence had changed. Analysis of the growth media would be required to determine if there was a correlation between the production of metabolites at the particular concentrations and the PCR result. Finally, incubating fungi with pure secondary metabolites would be worth undertaking as there is a chance that the correct compound, concentration and time could be chosen to affect the PCR.

Most suitable would be growing fungi in such a manner to avoid secondary metabolite production. These compounds are often produced when nutrients become limited in growth media during the idiophase in batch culture. Fungi could be harvested when all nutrients are in excess in trophophase then secondary metabolites may be avoided. Unfortunately, all secondary metabolites are not produced in this manner and could occur during exponential growth. Separating the growth phases is more difficult when solid agar substrates are employed. DNA is a valuable and changeable resource and more care is required when growing fungi for its

production. Much greater control of growth and secondary metabolism is possible using bioreactors and these may be required to produce the best quality DNA. It is the responsibility of the individual researcher to determine if the growth conditions employed could affect the results obtained.

Repeated PCR of cultures is potentially one way of determining if a gene has mutated, with no reports of this actually having been carried out. But was the strain a wild type and unaffected by the isolation process and subsequent subculturing (Paterson & Lima, 2013)? The secondary metabolite profile can change for each strain in each run for a PCR analysis. If there was a negative result after a positive this would require correlation with multimycotoxin analysis of each culture to at least provide leads as to what caused the changed result. This is only for one gene of course and no information on the remainder of the genome would be given.

8. Conclusions

Much more work is required on the effects of mutagens on fungi. There is a fundamental discrepancy in current methods based on incorrect experimental design from the assumption that DNA integrity is independent of growth conditions. Future papers need to state why particular growth conditions were employed and what steps were taken to avoid biochemical mutagens in growth media. The use of bioreactors may be advisable to produce the best quality DNA and understanding the physiology of growth of fungi is essential. After all, the analysis of NA cannot be seen in isolation from the physiology of the whole fungus.

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