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MINI-REVIEW

# Biochemical mutagens affect the preservation of fungi and biodiversity estimations

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**Abstract** Many fungi have significant industrial applications or biosafety concerns and maintaining the original characteristics is essential. The preserved fungi have to represent the situation in nature for posterity, biodiversity estimations, and taxonomic research. However, spontaneous fungal mutations and secondary metabolites affecting producing fungi are well known. There is increasing interest in the preservation of microbes in Biological Resource Centers (BRC) to ensure that the organisms remain viable and stable genetically. It would be anathema if they contacted mutagens routinely. However, for the purpose of this discussion, there are three potential sources of biochemical mutagens when obtaining individual fungi from the environment: (a) mixtures of microorganisms are plated routinely onto growth media containing mutagenic antibiotics to control overgrowth by contaminants, (b) the microbial mixtures may contain microorganisms capable of producing mutagenic secondary metabolites, and (c) target fungi for isolation may produce "self" mutagens in pure culture. The probability that these compounds could interact with fungi undermines confidence in the preservation process and the potential effects of these biochemical mutagens are considered for the first time on strains held in BRC in this review.

**Keywords** Fungi · Preservation · Secondary metabolites · Biochemical mutagens · Self mutagens

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## Introduction

Microorganisms are often preserved in collections to better ensure they represent wild-type strains from nature (Paterson et al. 2012). Many are kept to maintain applied and biotechnological properties, although some may be preserved for properties unrelated to wild types such as transformed strains. There are an estimated 1.5 to 3.0 million fungal species (Hawksworth 2012), although only a small number (ca. 75,000) have been isolated and named. Some of those have high economic value, for example, in relation to antibiotic, enzyme, and organic acid production. Furthermore, internationally based culture collections are obliged to guarantee the authenticity of the fungi they hold (Santos and Lima 2001), where the aims are maintenance in a viable state without changing genes (Ryan and Smith 2007).

Fifty years can be necessary for collections to become world-recognized biological resource centers (BRC) (syn. culture collections) (Wiest et al. 2012), requiring (a) manipulation and preservation of individual isolates and (b) concomitant processing of thousands of isolates. Other collections can also take lengthy and complex paths towards development as BRC (Oh and Shin 2008; Smith and Ryan 2008). Best-practice guidelines have been developed by, for example, the Organization for Economic Cooperation and Development and the International Society for Biological and Environmental Repositories, because many fungal repositories have similar objectives. These allow the successful operation of collections and promote development and interactions. Much improved preservation of fungi can be obtained, for example, compared to that by individual laboratories preserving strains in amateurish manners (e.g., cultures stored in the "back of the fridge").

When microorganisms are isolated and preserved to determine taxonomic relationships or to assess those present in

the environment, it is assumed they are identical genetically to those in nature. Indeed, it is sometimes stated that DNA characters are preferable to others because they are "stable" and independent of cultural conditions (Dupont 2009). Paterson and Lima (2011) argued that this may not be the case and that even these characters could be affected by how the fungi are grown. Paterson et al. (2008) and Paterson and Lima (2009) detail how growing cultures of fungi may result in strains with mutated genes from the production of self-mutagenic secondary metabolites.

Mutagens are physical or chemical agents that change genetic material (usually DNA) of organisms and thus increase the frequency of mutations above the natural background level. Mutations are accidental changes in a genomic sequence of DNA and are caused by radiation, viruses, transposons, mutagenic chemicals, and errors that occur during meiosis or DNA replication. Fungi have been exposed continuously to UV radiation and many DNA/RNA damaging agents for billions of years. Errors will occur during replication which damage DNA. Lesions could be (1) altered, missing, and mismatch bases, (2) deletion or insertion, (3) linked pyrimidines, (4) strand breaks, and (5) intra- and inter-strand cross-links (Fig. 1). DNA lesions can be retained, leading to genome instability, mutations, carcinogenesis and/or cell death. Secondary metabolites are microbial products that are not essential for growth and reproduction of the producing organisms. Each 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. Concentrations of secondary metabolites 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 (Paterson and Lima 2009).

It is well known that fungi can be variable in culture and spontaneous mutations occur: Wiebe (2002) discusses the situation with *Fusarium* species used to produce edible protein substitutes. Mutants occurred often in relation to growth rates and other physiological factors which could be secondary metabolites produced by the fungi. Mutants of *Aspergillus parasiticus* were obtained simply by serial transfers (Wilkinson et al. 2011). Furthermore, self-affecting compounds produced in growth media are also reported (Singh et al. 2010), and so it is reasonable to suggest that mutations may occur from the production of self-produced metabolites, with profound implications in areas such as fungal preservation, phylogenetics, PCR analysis, identifications, and taxonomy. Other factors which could affect the fungi at preservation includes the methods involved (e.g., freeze drying vs. cryopreservation (Broughton et al. 2012)), UV irradiation, and other chemical compounds encountered during preservation. However, these are beyond the scope of the present review.

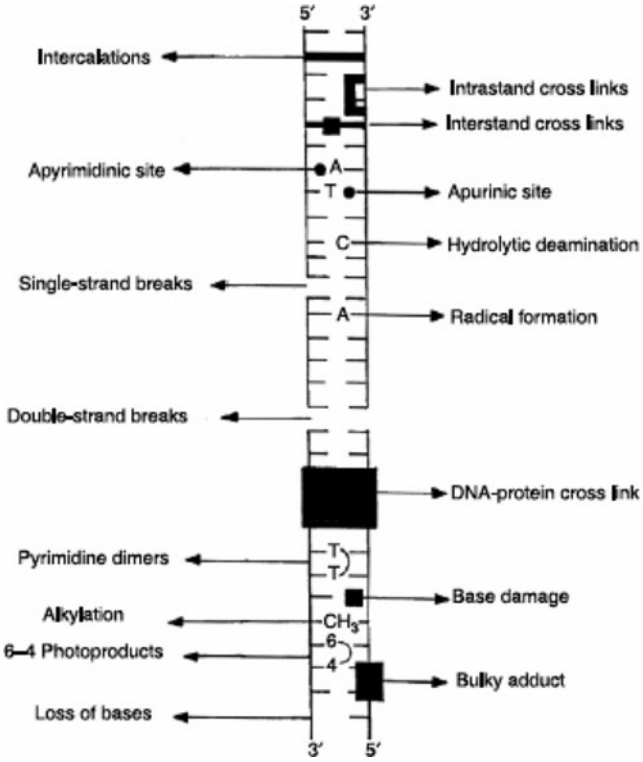


**Fig. 1** Diagrammatic representation of damage to DNA. Damage may be from mutagenic secondary metabolites from a fungus leading to changes in the DNA sequence. Epigenetic changes may occur leading to problems with determinations of DNA base sequences to phenotypic changes of the fungus (see Paterson and Lima 2009)

Consideration was given to the effects of biochemical mutagens on preservation in an abstract and poster (Paterson and Lima 2012) at the European Culture Collections' Organisation conference relating to BRC (Paterson et al. 2012). It was stated that the possibility of cultures coming in contact with mutagens is anathema to good practices in preserving fungi, as the compounds could cause the fungi to mutate, allowing them to become laboratory artifacts. This probability undermines confidence in the preservation process. However, there are no papers published in journals on the effects of these biochemical mutagens on fungi in collections whether of a hypothetical or factual nature, and this review intends to redress the balance.

## Sources of mutagens

1. Antibiotics to control contaminating microorganisms

   Antibiotics are employed routinely to control the growth of contaminating microorganisms when isolating fungi. For example, chloramphenicol or gentamicin inhibit bacterial contaminants, while cycloheximide inhibits saprobic moulds and are employed generally

in culture media during isolations. These compounds are known to be mutagenic (Martelli et al. 1991; Mitchell et al. 1980; Oliveria et al. 2011).

2. Secondary metabolites from co-isolated or target fungi
   Microorganisms on the initial isolation agar plates, and self (auto)-produced secondary metabolites by the fungal isolate of interest, may mutate the target fungi, especially after isolation in pure culture.

## Numbers of mutagenic secondary metabolites

It is impossible to give an accurate figure for the total number of these fungal compounds that exist in nature. However, approximately 600 species produced 1,200 secondary metabolites (i.e., 2 per species) listed in Cole et al. (2003). Some species are only given as the producers of one compound, although others produce many more (e.g., *Ganoderma lucidum* has 76). Hence, often more than one secondary metabolite needs to be considered in terms of potential mutagens in culture media, as does the combined effects of multiple mutagens. Cole et al. (2003) do not include fungi from which no secondary metabolites have been found and many of the other ca. 74400 of the known fungi will not have been tested for mutagenicity which is limited, in general, to a few species relevant to carcinogenicity in humans and animals. Hence, there is no information on the mutagenic potential of the vast majority of secondary metabolites, although it is predictable that some will be mutagens. For example, approximately 25 compounds are listed in this review with mutagenic activity of some manner. The current authors estimate that perhaps 100 compounds have some significance at present as mycotoxins and hence may have been expected to have been tested for mutagenicity. Thus, the rate of positive results for mutagenicity can be estimated as 25 %. The 75,000 known species may produce 100,000 secondary metabolites as a conservative figure, and so we estimate that 25,000 may be mutagenic. If this is extrapolated to all fungi, then the figures become 125,000 to 250,000 mutagenic compounds. These figures at least indicate that the number is large and the issue of self-produced mutagens affecting fungi is important.

## Mutagenic potential of mycotoxins in non-fungal systems

Mycotoxins with carcinogenic effects include aflatoxins, sterigmatocystin, ochratoxin, fumonisin, zearalenone, citrinin, luteoskyrin, patulin, and penicillic acid produced by a wide range of fungi (Table 1). Whether these compounds will be self-mutagenic towards fungi remains unknown, but it is possible: It appears axiomatic that they should be avoided when preserving fungi. All are DNA damaging agents except for fumonisins which may act via disturbing signal transduction pathways (Paterson and Lima 2009, 2010). Some evidence for mutagenicity from fungi and associated metabolites will now be considered.

**Table 1** Selected mycotoxigenic fungi with some known mutagenic mycotoxins detected from each (N.B. these are not necessarily the only species capable of producing particular mycotoxins)

| Filamentous fungi | Mutagenic mycotoxins |
|---|---|
| *Alternaria alternata* | Altertoxin II |
| *Aspergillus* | |
| *A. carbonarius, A. westerdijkiae, A. niger* | Ochratoxin A |
| *A. niger* | Fumonisins B2 and B4 |
| *A flavus, A. parasiticus* (11 other species can produce aflatoxins) | Aflatoxins |
| *A. versicolor, A. flavus* | Sterigmatocystin |
| *Chaetomium longicolleum* | Sterigmatocystin |
| *Emericella nidulans* (51 other fungal species can produce sterigmatocystin) | Sterigmatocystin |
| *Byssochlamys fulva, B. nivea* | Patulin |
| *Fusarium* | |
| *F. cerealis* | Nivalenol |
| *F. culmorum, F. graminearum* | Deoxynivalenol, Nivalenol |
| *F. equiseti* | Zearalenone |
| *F. poae* | Nivalenol |
| *F. verticillioides* (= *F. moniliforme*), *F. globosum*, *F. nygami*, *F. proliferatum* | Fumonisin B1 |
| *Penicillium* | |
| *P. expansum* | Patulin, citrinin |
| *P. verrucosum, P. nordicum* | Ochratoxin A |
| *P. islandicum* | Luteoskyrin, |
| *P. cyclopium* | Penicillic acid |
| *P. paneum*, *P. brevicompactum* | Botryodiploidin |
| Mushrooms/toadstools | |
| *Lactarius* sp., *Russulaceae* spp. | Isovelleral |
| *Lactarius necator* | Necatorin |
| *Agaricus bisporus* and related sp. | Agaritine |
| *Agaricus silvaticus*. | beta-Nitraminoalanine |

Aflatoxins are the most carcinogenic natural compounds known and avoiding contact is surely a priority when preserving fungi. The mycotoxins are well known to be produced by *Aspergillus flavus* and *A. parasiticus*. They (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. Aflatoxin B1 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 aflatoxin B1 to rats can cause a measurable increase in aflatoxin B-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. Mutagenic sterigmatocystin has a chemical structure similar to and is a precursor of, aflatoxins, although it is produced by a wider range of fungi (Table 1). Covalent binding to DNA and DNA adduct formation have been reported, and it is carcinogenic to rats and mice in which mainly liver tumors are induced.

Ochratoxin A is produced by several *Aspergillus* and *Penicillium* species, including *Aspergillus carbonarius, Aspergillus westerdijkiae,* and *Penicillium verrucosum*. The secondary metabolite is one of the most potent carcinogens in rats, and ochratoxin A 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. There is still insufficient understanding of whether ochratoxin A acts as a direct genotoxic carcinogen or whether its carcinogenicity is related to indirect mechanisms. The strongest evidence in favor of ochratoxin-mediated DNA damage is the induction of DNA single-strand breaks and formamidopyrimidine-DNA glycosylase sensitive sites. Importantly, mutagenic activity by ochratoxin A has also been reported (e.g., in murine cells). Ochratoxin-induced base substitutions were similar to those arising spontaneously, suggesting that this mycotoxin increases the process involved in spontaneous mutagenesis. Finally, the metabolite induces an increase of mutation frequency at two gene loci via a mechanism that is independent of biotransformation.

The concentration necessary to induce DNA–DNA cross-links by patulin are in the millimolar range and the induction of DNA–DNA cross-links is a possible mechanism of the mutagenicity observed. The compound is produced by a wide range of species within *Aspergillus*, *Penicillium*, *Byssochlamys,* and *Paecilomyces*. However, patulin may not reach the nucleus to cause damage because of its high reactivity with proteins, and cell-free reactivity might not occur in cellular systems. 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) (Paterson and Lima 2009).

The genotoxic risk associated with deoxynivalenol (Table 1), a prevalent trichothecene mycotoxin, has not yet been explored thoroughly. The Comet assay was used to evaluate DNA damage stemming from deoxynivalenol exposure, and dividing cells were found to be more sensitive than differentiated cells. The results demonstrated the existence of a genotoxic potential for deoxynivalenol at low concentrations compatible with actual exposure situations (Bony et al. 2006). Nivalenol damaged the nuclear DNA of CHO cells demonstrating that it is a direct mutagen (Paterson and 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 recombination tests with *Bacillus subtilis*. The compound also induced (1) polyploidy in CHO cells, (2) sister chromatid exchange, and (3) chromosomal aberration in vitro. Furthermore, treatment of mice led to the formation of several DNA adducts in the liver and kidney (Paterson and Lima 2009). Fusarin C is mutagenic and is produced from *Fusarium moniliforme*. Unidentified compounds NG-391 and NG-393 from *Fusarium* spp. were also detected from fermentation extracts of the entomopathogenic and possible biocontrol fungus *Metarhizium anisopliae*. They are 7-desmethyl analogues of fusarin C and (8Z)-fusarin C, mutagenic toxins from fusaria of corn. However, like their fusarin analogues, the compounds exhibited potent S9-dependent mutagenic activity (Krasnoff et al. 2006).

Furthermore, 41 fungal isolates (one isolate per species) representing common plant pathogens and food crop contaminants were assayed for mutagenic activity. Extracts of two aflatoxin-producing isolates (*A. flavus* and *A. parasiticus*) showed pronounced mutagenic activity, as did extracts of five other species (*Aspergillus heterothallicus, Aspergillus nidulans, Aspergillus terricola, Alternaria tenuis*, and *F. moniliforme*) which (unsurprisingly) did not contain detectable aflatoxins. Seven additional isolates (*Botrytis cineria, Ceratocystis fimbriata, Cladosporium herbarum, Fusarium solani* f. sp. *pisi*, *Penicillium oxalicum*, *Thermomyces lanuginosus*, and *Verticilium albo-atrum*) revealed activity which was possibly mutagenic (Bjeldanes et al. 1978).

An extract of *Alternaria alternata* was mutagenic as were isolated compounds altertoxins I, II, and III with and without metabolic activation (Stack and Prival 1986). Altertoxin II (ATX II) has a perylene quinone structure and is at least 50-times more potent as a mutagen than the common *Alternaria* toxins alternariol (AOH) and alternariol methyl ether (AME) (Fleck et al. 2012). However, the mutagenic potencies of several *A. alternata* toxins seem unable to account for the levels of activity found using crude mycelial extracts of this fungus. Hence, the mutagenic effects of nitrosylation were examined with the major *Alternaria* metabolites altenuene (ALT), AOH, AME, altertoxin I (ATX I), tentoxin (TENT), tenuazonic acid (TA), and radicinin (RAD). In the absence of nitrosylation, ATX I was mutagenic, while AOH

and ATX I were weakly mutagenic. Incubation with nitrite generally increased mutagenic potencies with ATX I being strongly mutagenic, while ALT, AME, and RAD responses were enhanced in one test. 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. Thus, while nitrosylation increases the mutagenicity of ATX I, and to a lesser extent that of several other *Alternaria* toxins, the results demonstrate that *Alternaria* produces a major mutagenic activity with a *Salmonella typhimurium* response different from that found with the purified toxins (Schrader et al. 2001).

DNA repair synthesis was elicited by several compounds of unknown carcinogenicity, 5,6-dimethoxysterigmatocystin, versicolorins A and B, averufin, xanthomegnin, luteosporin, and chrysazin, and by the carcinogenic mycotoxins, aflatoxin $B_1$, sterigmatocystin, luteoskyrin, and ochratoxin A. The positive results with compounds of unknown carcinogenicity at the time of the study, suggested that they are genotoxic carcinogens (Mori et al. 1984). In addition, botryodiploidin is listed as mutagenic and produced by *Penicillium paneum* and *Penicillium brevicompactum* (Frisvad et al. 2004).

Intermediates in the aflatoxin biosynthetic pathway were screened for their mutagenic activity. Norsolorinic acid, averufin, and versiconal acetate were found to possess questionable mutagenic activity, but versicolorin A and sterigmatocystin were significant mutagens relative to aflatoxin B1. The mutagenic activity appeared to be related to the bisfuran and not the anthraquinone moiety of the molecule, even although the latter is a key structure of such a potent carcinogenic mycotoxin as luteoskyrin (Wong et al. 1977). Furthermore, extracts of *A. nidulans* and an *Aspergillus fumigatus* strains were determined to have mutagenic properties by Bubak et al. (1996).

Finally, extracts of 37 of 48 species of mushrooms exhibited significant mutagenic activity, and six metabolites reported to occur in some of the species were also tested. Mushrooms are potential foods and can be grown on agars or similar substrates in mycelial form for the purposes of preservation and so are relevant to the discussion. Significant mutagenic activity was found from (a) isovelleral from *Lactarius* sp., (b) agaritine from *Agaricus bisporus* and related sp., and (c) beta-nitraminoalanine from *Agaricus silvaticus*. Furthermore, isovelleral may be a major mutagen in some of the sharp-tasting and mutagenic *Russulaceae* species. *A. bisporus* was weakly mutagenic and agaritine was also weakly active: This fungus might contain other mutagenic material. Beta-nitraminoalanine was not found in the *A. silvaticus* tested, and the mutagenicity observed may therefore be due to other metabolites (Sterner et al. 1982). Extracts of *Lactarius necator* exhibit considerable mutagenic activity, and a highly mutagenic compound, necatorin, was isolated (Liu 2005).

Finally, lack of information on other mycotoxins and indeed secondary metabolites in general, 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 secondary metabolites (Paterson and Lima 2009).

## Concentrations of secondary metabolites in media

In general, mycotoxins accumulate to a high level in growth media. Aflatoxin B1 ranged from 1.4 to 4 $\mu g ml^{-1}$ in *A. parasiticus* or *A. flavus,* and 60 $\mu g ml^{-1}$ of aflatoxin B1 was detected from *A. flavus* in yeast extract sucrose (YES) broth. Yeast extract peptone was the non-inducing medium and would be a logical choice for preserving these fungi by avoiding the mutagen. Ochratoxin A was estimated at 0.5 mg20 $ml^{-1}$ in YES from *Aspergillus alliaceus,* and paxilline accumulates at very high levels in growth media from *Penicillium paxilli*. *Penicillium 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, and approximately 3 $mg ml^{-1}$ was detected from *P. expansum* on Czapeks Dox agar (Paterson and Lima 2009). All *Fusarium culmorum* strains tested produced DON/3-AcDON at levels ranging from 21 to 11,000 $\mu g g^{-1}$ of dry biomass on Mycotoxin Synthetic medium (Kammoun et al. 2010). Paterson (2007) even found 15 $ng l^{-1}$ of ZEN produced in water by *F. graminearum*. Hence, it is well known that fungi grown on standard growth media can produce high levels of mycotoxins.

## Egigenetic alterations

In addition, epigenetic alterations are possible when preserving fungi (Fig. 1) as these also have been reported in other non-fungal systems: More work is required on the epigenetic effects of fungal metabolites. For example, the mechanisms of fumonisin carcinogenicity do not appear to involve interaction with DNA and the carcinogenic activity of fumonisins may be mediated via epigenetic mechanisms (Coulombe 1993). Fumonisin B1 induced hypermethylation of DNA (Mobio et al. 2000) and may be involved in histone modifications (Pellanda et al. 2012). Ochratoxin A also has epigenetic effects (Marin-Kuan et al. 2008) and is likely to act through a network of interacting epigenetic mechanisms, including protein synthesis inhibition, oxidative stress, and the activation of specific cell signaling pathways, which are responsible for carcinogenicity. Low, but sustained levels of chronic oxidative stress can result in cell proliferation ultimately converting DNA damage into mutations, leading to cell transformation, and, in relation to our discussion, to mutated fungal strains. Finally, aflatoxin B1 preferably binds to methylated lysines or modified histones leading to DNA damage via these epigenetic mechanism (Herceg

2007). How are the above discussions relevant to preservation and biodiversity estimations?

## Mutagens may affect preservation and biodiversity estimations

Figure 2 indicates how isolates may be affected by mutagens during initial isolations from the natural environment, where many different organisms can be co-isolated which may produce secondary metabolites. For example, aflatoxins are the most carcinogenic natural compounds known, and fungi that produce these compounds are isolated quite frequently in nature. Admittedly, the concentrations of secondary metabolites may be low as the target isolates are removed quickly from the media; on the other hand, there could be a complex mixture of compounds from all the organisms isolated on the agar. The mutagenic load is higher when the antibiotics included in the media are also considered, which are at high concentrations. Hence, a mutant may already have been isolated instead of the desired wild type.

Isolates are grown on media before preservation to check purity and to obtain identifications when self-produced mutagens or epigenetic compounds may also be produced (Paterson and Lima 2009; Paterson et al. 2008). These compounds can be produced at high concentrations and may inhibit mechanisms involved in reducing toxicity such as transportation in vesicles, detoxification by enzymes, and compartmentalization (see Paterson and Lima 2009). Specifically, enzymes involved in detoxification (Hansen et al. 2011) may be inhibited by enzyme inhibiting secondary metabolites produced by the fungi. The strains will be preserved subsequently but may be laboratory artifacts.

Similarly, mutagenic secondary metabolites may be produced when fungi are grown from preservation for maintenance, analyses, or sale leading to further mutations (Fig. 3). Hence, the strain could have been subjected to a wide range

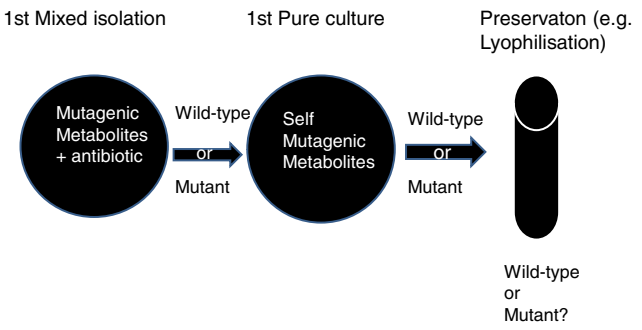


**Fig. 2** Exposure to mutagenic compounds during isolation. The initial isolation from nature involves spreading a mixed culture onto an isolation medium. In this situation, many secondary metabolites may be produced by the various organisms present and the isolate of interest may be subjected to self-produced metabolites

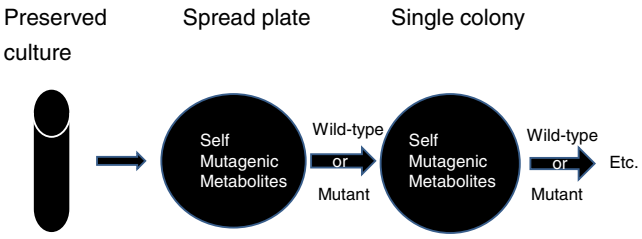


**Fig. 3** Exposure to mutagenic metabolites from re-growth. When an isolate is re-grown from preservation, it may come in contact with self-produced mutagenic secondary metabolites leading to undesired changes to the wild-type fungus

of mutagens before it is even used for the analysis for which it was intended. This may be unproblematic if it is being used to produce a particular compound (e.g., citric acid), as the genes involved in the biosynthetic pathway may not be affected by the mutations. However, if the stains are being employed in large scale gene sequencing (Andersen et al. 2011) or used in a taxonomic study involving DNA analysis, then this could indeed be problematic.

### Biodiversity

A corollary of the above argument is that estimations of fungal biodiversity (see Hawksworth 2012) may be inaccurate from mutations of strains during isolation and culture. If isolates of different species from nature are subjected to mutagens, then they may result in more similar strains which could be mistakenly identified as the same species (Fig. 4). Conversely, different isolates from the same species in nature may mutate to become increasingly different because of the effects of mutagens (Fig. 5). Hence, there would be an underestimation and overestimation of diversity respectively, and care needs to be taken to ensure that these two scenarios do not occur.

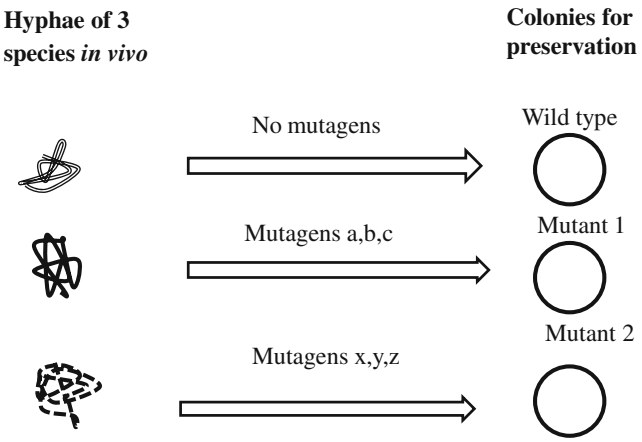


**Fig. 4** Artificial decrease in biodiversity from mutagens. This is represented by three different species in the environment being made more similar by mutation when cultured in vitro and appearing to be three isolates of a single species

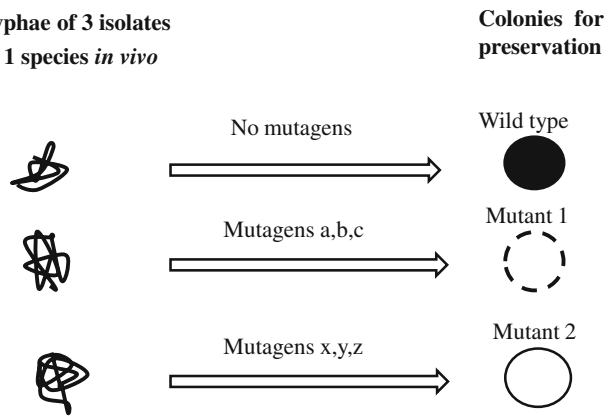


**Fig. 5** Artificial increase in biodiversity from mutagens. This is represented by three different isolates of the same species in nature appearing to give three different species when cultured in vitro

## Patulin-producing potential—an example of the problem

The mutagenic effect of secondary metabolites on the DNA of the producing fungi in pure culture was first postulated by Paterson (2004), when it was realized that inhibitory and mutagenic secondary metabolites could determine whether isolates were positive or negative for possession of the *idh* gene of patulin biosynthesis (a mycotoxin and antibiotic). For example, it was impossible to state with certainty that the isolates represented those in nature because some had been in contact with patulin in culture, which is mutagenic: these and the other isolates tested, had also been subjected similarly to different self-produced secondary metabolites (Paterson and Lima 2009; Paterson et al. 2008). The work was undertaken at a large public BRC where the strains had been accessed, given strain numbers and preserved as detailed in the paper. Furthermore, the fungi employed had been in contact with antibiotics to control other microorganisms during isolations, hence increasing the mutagenic load on the target organisms. It was impossible to state that the preserved strains were representative of the fungi in the orchard and may have been laboratory artifacts (Paterson and Lima 2012). Equivalent methods to those described above have been employed routinely by many other workers.

## Solutions

Solutions to this problem of experimental design are somewhat difficult to conceive. A literature search is at least required as to which secondary metabolites are known to be produced by the taxa of interest, as some are known to produce many secondary metabolites whilst others produce few. Some precautions can sensible be employed such as decreasing the concentration of antibiotics in isolation media if this is possible without overgrowth by contaminants. Also, the time period in which isolates are grown could be perhaps be reduced, but often the growth periods are part of standard methods, and changing these times could have detrimental consequences. The number of subcultures needs to be minimized. A consideration of which growth media are employed is required to ensure that the secondary metabolites are minimized (see section Concentrations of secondary metabolites in media). However, often particular media are prescribed for characterizing taxa, and changing media may cause unforeseen problems. The use of physical barriers to the media (e.g., cellulose-based, pressure sensitive adhesive tape (e.g., sellotape) may be useful, which separate the isolates from the media, although the production of intracellular metabolites will remain a problem and metabolites may be able to cross the barrier. It would be desirable to preserve the substrates from which the isolates were obtained to enable the re-isolation of the fungi, although this would increase the space requirements of BRC considerably, and the target fungus may not be able to be re-isolated from certain substrates.

A logical approach would be to grow the fungi in such a manner that the metabolites are not produced. This is classical fungal physiology where growth (i.e., trophophase) is distinct from the secondary metabolite production phase (i.e., idiophase) (Paterson and Lima 2009). Trophophase is usually linked to exponential growth of the fungus where biomass increases rapidly. Secondary metabolite production can commence often when a nutrient such as the nitrogen source is depleted. These phases are defined in stirred batch bioreactors using liquid media rather than agar plates of course. However, mutagenic metabolites could be avoided, at least in some cases, by choosing an appropriate growth phase at which to harvest mycelium.

Finally, fungi should be grown for various time periods and on different media to determine the effects on DNA sequences, and it is surprising that this type of work has not been performed routinely. The cultures would require analysis for secondary metabolites simultaneously, and there are numerous methods for analyzing secondary metabolite profiles of strains ranging from TLC to HPLC UV/MS (Nielsen and Smedsgaard 2003). All the above areas require further work to obtain data which unequivocally demonstrates that mutagenic metabolites are reduced or eliminated. Also, data from individual strains will probably not be applicable to all fungi, and work on the particular fungi of interest will be required.

## Conclusions

Steps require to be taken to ensure strains from nature do not become laboratory artifacts and the present, unprecedented collaboration between culture collections (Paterson et al. 2012) provides a unique platform to ensure that authentic microorganisms are obtained and preserved. Work is required on (a) the mutagenic effects of such compounds on

strains and (b) how to avoid them. Finally, it is essential to be able to state with confidence that fungi in BRC are not laboratory artifacts.

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