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Mutagens affect food and water biodeteriorating fungi

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Many areas of food mycology could be affected detrimentally by mutation of wild type fungi. Some of these will contact mutagens from pre-isolation to experimentation and the effect on fungi isolated from mycotoxin-contaminated food is assessed for the first time in this review. However, this mutagen issue is not considered by other authors in primary research papers, which is relevant to molecular biology techniques for gene sequencing, phylogenetics, diagnostics and mycotoxin production. The presence of mutagens is anathema to methods for DNA analysis at the experimental design level and concepts such as cryptic species and correlating anamorphs with teleomorphs are affected. Strains held in culture collections may be artifacts. Methods to ameliorate the problem are provided herein.

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Introduction

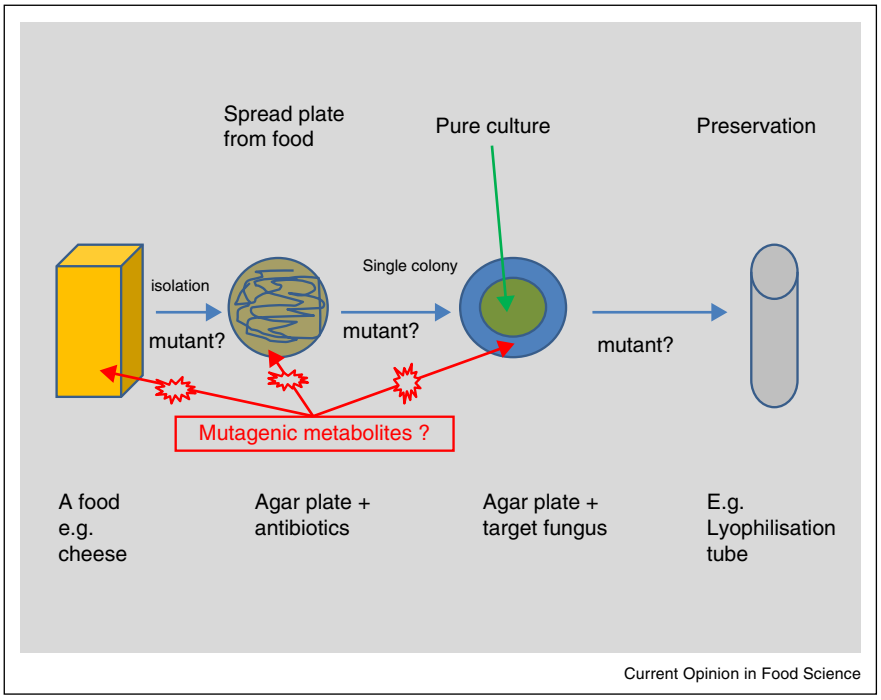
Wild type strains are employed when fungi from food and potable water are studied [1]. They are investigated to, *inter alia*, (a) determine taxonomic relationships, (b) undertake whole genome sequencing, (c) create diagnostic methods and/or (d) determine mycotoxin production. However, some of the wild types will contact mutagens, from pre-isolation to analysis, raising doubt as to the validity of data (Figures 1 and 2) [2••,3••]. The following discussion highlights this fundamental problem still unconsidered by other researchers in the primary research papers.

Certain fungi grow and deteriorate food, some of which are mycotoxin producing species (Figure 1). Many mycotoxins are known mutagens (Table 1) [2••,3••] and are tested for mutagenicity because they are found in food intended for humans and animals which may cause cancers: there are numerous other similar fungal secondary products which have not been tested as they are detected infrequently, or not at all, in food. More information is provided on the mutagenicity of these compounds in [2••,3••,4], together with appropriate references. However, it is worth discussing fusarenon X as there is some confusion as to whether it is causes only apoptosis rather than DNA breaks [4]. Nuclear DNA double strand breaks are highly deleterious because they interfere with transcription or replication: Genes are disrupted, leading to hybrid proteins or inappropriate activation of genes (see Clancy [5]). Bony *et al.* [6] mention that fusarenon X had been described by others as a potent apoptosis inducer and mention evidence for this activity in their research as being very scarce and demonstrated clear results of DNA strand breaks, although fusarenon X could cause apoptosis and DNA breaks as the two may be compatible in different systems.

The effect of mycotoxins on other fungi has been reported in terms of model systems, such as the reported mutagenicity of AFB1 on *Neurospora crassa* [7], indicating that there is not a barrier to the mutagenicity of these compounds in fungi *per se* and these studies are particularly relevant to the present discussion and are of outstanding interest. Cytochrome P-450 in the cells of *Saccharomyces cerevisiae* was investigated where cells were capable of metabolizing AFB1 to products active genetically in the same cells [8]. The formation of convertants, revertants and other types of mitotic segregants were induced in *S. cerevisiae* upon incubation with AFB1 [9]. Furthermore, AFB1, G1 and G2 were mutagenic in *N. crassa* [10,11], whereas AFB2 was not [11]. The genetic activity of PR toxin caused (a) gene conversion and mitotic crossing-over in *S. cerevisiae*, and (b) reverse mutation in *S. cerevisiae* and *N. crassa* [12] without enzymatic activation; the mycotoxin was not mutagenic in the forward mutation system of *Schizosaccharomyces pombe* [13]. Patulin was investigated in an extrachromosomal mutation system of a haploid strain of *S. cerevisiae* and mutation from wild type to petite form was observed [14], although the mechanism of mutation was not discussed.

How could extracellular metabolites interact physically with DNA in the cell [2••,3••,4]? These compounds may accumulate in the environment, to the extent that excretion could be affected and allowing them into intracellular space to interact with DNA. However, there are secondary metabolites which are already strictly intracellular, as determined most clearly within the terverticillate penicillia [15••], although they may be secured in compartments. This segregation may break down as

Figure 1



How fungi could be mutated from growth on the foodstuff, isolation in a mixture, and purification. The fungus would be subjected to further mutagens from re-growth after preservation.

the metabolites accumulate to high concentrations when growth continues. Autolysis is another factor which will allow the metabolites to interact with the DNA of the cells, or intracellular metabolites may interact directly with DNA if they are unconstrained. Finally, many of the metabolites are enzyme inhibitors which will inhibit processes such as active secretion and enzymatic degradation of toxic compounds.

Figure 2

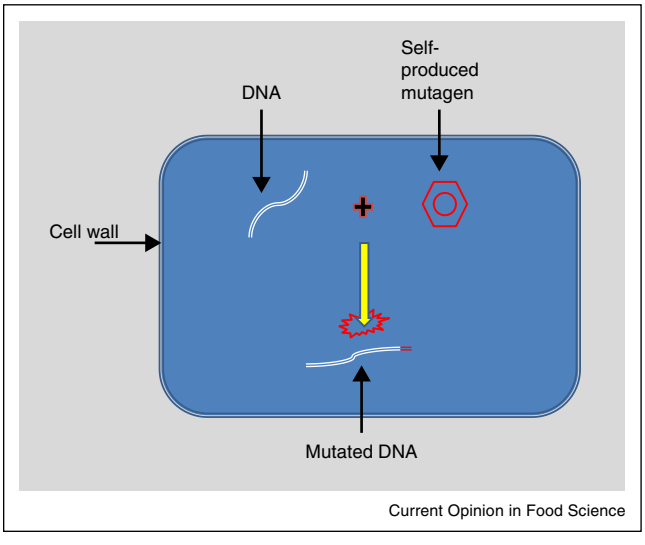


Diagram of a fungal cell containing self produced mutagens in the cytoplasm and affecting mutations in DNA.

All these may be self-mutagenic toward fungi in culture (Figure 2) and/or the environment (e.g. from food). Paterson and Lima [2••] estimated 200 000 mutagenic compounds produced from all fungi. Furthermore, DNA in general may sustain 50 000 damages per cell per day and 150 000 oxidative adducts per cell generated through reactive oxidative species, which can cause mutations if uncorrected. The repair mechanisms are enzyme-based and many fungal secondary metabolites are enzyme inhibitors which may inhibit DNA repair, creating greater mutagenic pressure. How do the mutagens occur?

Pre-isolation

Fungi may be in contact with mutagens before isolation. For example, they are exposed to UV irradiation [16•], and they can be isolated from agricultural areas contaminated with mutagenic pesticides, for example, *Aspergillus fumigatus* mutants resistant to azoles [17•]. Paterson and Lima [18,19] discussed mutants in the environment caused by increases in mutagenic fungal metabolites and UV irradiation from climate change. Fungi are often isolated from foodstuffs containing mycotoxins which may be mutagenic and the fungi could become mutated (Figure 2), and this possibility has not been considered

Table 1

Known mutagenicity of selected mycotoxins from fungi as determined from data in references [2••,3••,4].

Examples of producing fungi	Mycotoxin	Mutagenicity
Aspergillus flavus	Aflatoxins	Most carcinogenic natural compounds; induce DNA damage; affect negatively the amelioration of damage; alter DNA base composition of genes.
Aspergillus flavus	Sterigmatocystin	Covalent binding to DNA; DNA adduct formation; carcinogenic.
Penicillium verrucosum	Ochratoxin A	Potent carcinogen; DNA single strand breaks; Forms DNA adducts; Mutagenic activity; induces base substitutions; increased mutation frequency.
Penicillium expansum	Patulin	Induces DNA/DNA crosslinks; mutagenicity; reactivity to DNA.
Fusarium culmorum, F. graminearum	Deoxynivalenol	DNA damage; genotoxic.
F. culmorum, F. graminearum	Nivalenol	Direct mutagen; DNA damage.
F. equiseti	Fusarenon X	DNA damage; increases DNA strand breaks.
F. verticillioides	Fusarin C	Mutagenic
Alternaria alternate	Altertoxin I, Alternariol, *Alternaria* extracts	Mutagenic

before. Fungi can mutate quickly (i.e. from 30 min to 72 h) when treated with, N-methyl-N′-nitro-N-nitrosoguanidine, ethyl methane sulphonate or 5 aza-cytosine [20–23]; hence if wild type fungi have been growing on a foodstuff for a few weeks then mutation is possible.

Isolation and growth

Numerous microorganisms can be co-isolated from food when attempting to isolate fungi, which may produce a large number of mutagenic secondary metabolites in agar at low concentrations (Figure 1) [2••]. The mutagenic load is made higher when antibiotics in the media are considered, which are at high concentrations to ensure antibiosis: chloramphenicol, gentamicin and cycloheximide are employed and are known mutagens [24–26] and mutagenic Rose Bengal is used frequently [27]. The target fungi will be at low concentrations, tending to increase mutagenic pressure, as the effect is dependent on the amount of organism (i.e. DNA) present. Hence, mutants may have been produced already. Furthermore, cultures will be grown on agar media before preservation to check purity and identify the strains. Self-produced mutagens also may be produced at this stage and epigenetic alterations are possible [2••,3••] (Figure 1). Mycotoxins accumulate in media which contain growing cultures and in the fungi as intracellular metabolites. Secondary metabolite (e.g. mutagenic mycotoxins) production from fungi varies depending on growth conditions such as the media used, time of growth, shaken or static culture, and light or dark [28•,29]. Each individual strain produces a unique quantitative and/or qualitative profile of secondary metabolites [28•] which may cause a specific mutagenic pressure on the fungus.

Similarly, mutagenic secondary metabolites will be produced when many fungi are re-grown from preservation for maintenance, analyses, or distribution to other organizations and the strains may be subjected to a wide range of mutagens before it is used for the analysis for which it was intended. Service culture collections have a duty to supply fungi which are unchanged from original wild types which is also important from a biosecurity point of view [30]. However, preserved strains may be laboratory artifacts rather than the desired wild type. The supply of a mutant may appear unproblematic if it is being used to produce a particular compound (e.g. citric acid production), as the genes involved in the biosynthetic pathway may not be affected by the mutations, but the possibility exists that a mutant may produce unwanted mycotoxins. If the stains are being employed in gene sequencing, taxonomy or mycotoxin determination then this would be unacceptable as important genes could be mutated.

Many papers regarding the analysis of food fungi use different conditions for growth, or in situ analysis of food, allowing an extensive range of potential mutagens [3••,31–34], further complicating the interpretation of results. (N.B. Hosoya *et al.* [32] did not use internal amplification controls (IAC) [35] for diagnostic PCR making their conclusions regarding detecting *Thermoascus* spp. and *Byssochlamys verrucosa* questionable.) Finally, fungal compounds may inhibit mechanisms involved in reducing toxicity of metabolites, such as transportation in vesicles, detoxification by enzymes, and compartmentalization [4].

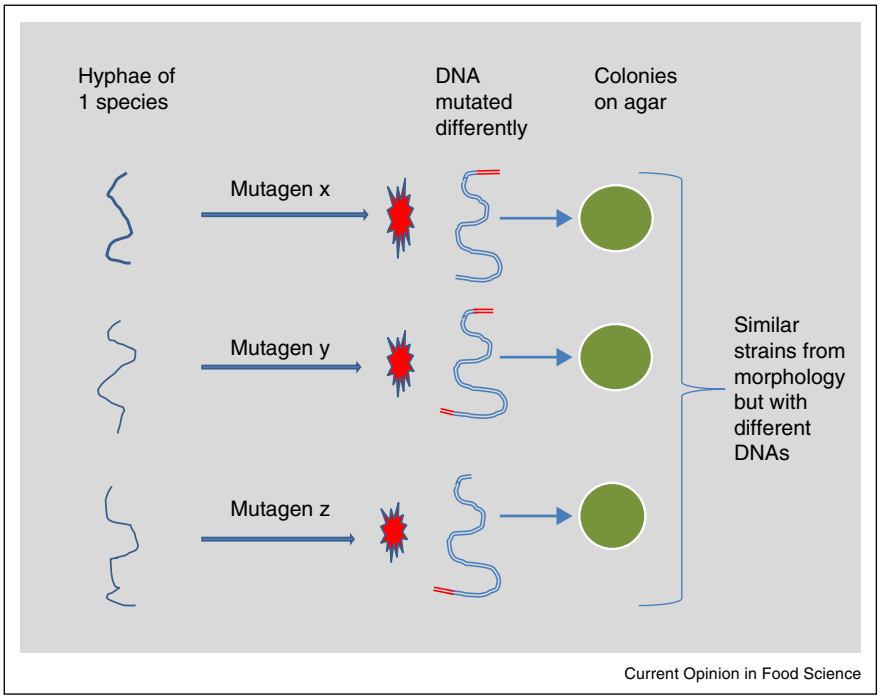
To reiterate, fungi may be mutated from the following sources: (a) foodstuffs on which they are growing; (b) isolation media; (c) co-isolated microorganisms; and (d) self produced secondary metabolites. These surely indicate there is a problem of experimental design.

Examples where interpretative problems arise

Cryptic species

Fungi may appear identical morphologically but are sufficiently different genetically for consideration as cryptic species in, for example, *Aspergillus* [36], *Penicillium* [37] and *Fusarium* [38]. Figure 3 indicates how the same species in the environment or during isolation, preservation and growth, may have similar morphologies in culture

Figure 3



Hypothetical example of how cryptic species could arise as laboratory artifacts. The mutagens x, y and z could arise from pre-isolation to re-growth as described in the text. Different mutations arise in the DNA of each strain although the morphologies are similar on agar after growth. The strains may falsely be considered as cryptic species.

but different DNAs from mutation. Alternatively, species from food with different DNAs could be mutated to have similar morphologies when grown in culture, resulting in an apparent cryptic species. Whether all cryptic species described exist in Nature is questionable from this interpretation.

Dimorphic fungi

Deciding which anamorphs belong to teleomorphs by employing DNA sequencing is another area affected by the current authors' interpretation. Anamorphs are fungi disseminated by propagules other than from cells in which meiosis has occurred [39], and these are correlated with fungal states that produce spores from cells where meiosis has, or is inferred to have, occurred, that is, the teleomorphs(s). Furthermore, the International Code of Nomenclature for algae, fungi and plants demanded recently that dimorphic fungi with sexual and asexual names bear a single name [40•]. DNA sequences may be mutated as described herein causing false relatedness between the DNA of the two forms and this situation tends to make more unreliable the nomenclature of food fungi.

Others

Finally, Paterson and Lima [3••] discuss PCR used in identifying food fungi, where problems may occur if the genes chosen as the target are mutated to give false negative or positive results. Mycotoxin production may be lost by strains which have been mutated in the genes of the biosynthetic pathway for particular mycotoxins. Alternatively, false positive results could be obtained if genes are mutated so that they react with the PCR primers.

Solutions

Solutions to this problem of experimental design are difficult to conceive. A record is required of the location where fungi were isolated to indicate if they could have been in contact with mutagens (e.g. a highly rotted apple compared to a perfect one). Decreasing the (a) concentration of antibiotics in isolation media, (b) growth period for isolates and (c) number of subcultures are essential. Growth media should be selected to allow minimum secondary metabolite production and the use of physical barriers to the media (e.g. cellulose-based, pressure sensitive adhesive tape) may be useful, which separate isolates from media. The substrates from which the isolates were obtained could be preserved to enable the re-isolation of the fungi. In addition, fungi can be grown in such a manner that the metabolites are not produced (i.e. trophophase) as distinct from the secondary metabolite production phase (i.e. idiophase) [2••,3••]. 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. All the above areas require further work to obtain data which unequivocally demonstrates that mutagenic metabolites are reduced or eliminated.

Discussion

It is instructive to consider how equivalent problems are dealt with in other areas of biochemistry. Contaminating proteases may degrade target enzymes when undertaking experiments to assess the reactions of the target enzymes. Similarly, contaminating DNAase may degrade DNA polymerase during PCR reactions and DNA polymerase inhibitors can affect the polymerase reaction. Steps are taken to ensure that these problems cannot occur and inhibition mechanisms may be investigated (e.g. [41]). For example, (a) unwanted enzyme activity is removed by heat, (b) the presence of inhibitors is reduced by additional purification steps, and (c) inhibition is detected by IAC [35]. One would realize there are potential problems and take steps to ameliorate them, even without direct evidence, because it is common sense. In addition, the effects of UV irradiation are already controlled when fungi are protected by indoor cultivation. Mutagens certainly would not consciously be included in a growth medium, unless this was to intentionally induce mutations [20–23].

Conclusions

Steps are required to ensure strains from nature do not become laboratory artifacts. Work on (a) the mutagenic effects of such compounds on strains and (b) how to avoid them can be devised. Furthermore, it is essential to state with confidence that fungi in culture collections are not laboratory artifacts. The concept is in the form of a hypothesis; however, there is no doubt that current procedures are contentious from an experimental design basis, where the need to avoid mutagens, a priori, is analogous to procedures taken to avoid contaminants in other areas of biochemistry. In our opinion, the (a) validity of previous research and (b) authenticity of strains held in culture collections are undermined because of this problem. We consider that future experimental designs devised for isolating, preserving and experimenting upon strains, need to include protocols for eliminating mutagens.

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- •• of outstanding interest

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