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The Role of Aflatoxins in *Aspergillus flavus* Resistance to Stress

Massimo Reverberi, Marzia Beccaccioli and Marco Zaccaria

Abstract

Aspergillus section Flavi produce the aflatoxins, secondary metabolites toxic to humans and animals. Why do these fungi produce aflatoxins? They do not have a clear role in pathogenicity or in niche competition. *Aspergillus* employs a considerable amount of energy to synthesize them: more than 20 enzymatic catalyzes are needed. Within the *A. flavus* species, all opportunistic pathogens of maize, more than half of the natural population are atoxigenic, indicating that aflatoxins are not so obviously linked to an enhancement of population fitness. The perspective changes in *A. parasiticus*, pathogen to peanuts, where more than 90% of the natural population produce the four aflatoxins. In this chapter, we aim to discuss our recent hypothesis that aflatoxins act as antioxidants providing more time to *Aspergillus* to “escape” an exploited substrate, that in the meanwhile is “fully charged” with reactive oxygen species and oxylipins.

Keywords: antioxidants, oxylipins, resilience to stress, lifespan, host adaptation

1. Introduction

The species belonging to the section Flavi of the genus *Aspergillus* can produce the carcinogenic aflatoxins (AF), secondary metabolites synthesized as the final product of a very complex pathway including 25 different enzymatic activities so far [1]. Aflatoxins are detrimental for animals (humans included) since, after oxidation by cytochrome P450, their 8,9-epoxide causes DNA depurination leading possibly to carcinogenesis [2]. Notwithstanding these fungi can invade the lungs of cystic fibrosis patients, the role of AF in worsening the clinical frame remains to be demonstrated. *De facto* *Aspergillus flavus* (and *A. parasiticus* as well) are opportunistic pathogens for animals as well as for plants and a competitive soil saprophyte [3], for which the synthesis of AF appears “luxury” or not necessary. Our idea is that AF are too expensive – in terms of biosynthesis and energy devoted – to be “non necessary”. If so, why AF are produced? More than 40 years of research told us that AF are synthesized following different inputs: nutritional [4, 5], pH, light, [6], host defenses [1] and finally, oxidative stress [7, 8]. The general impression is that AF are synthesized in response to a stressful condition. In light of this, can we consider them as a part of the complex reaction set that *Aspergillus* uses for facing challenging conditions? This chapter focus on how oxidative stress can modulate (how and why modulate) AF synthesis, beginning with the description of some of the actors that switch the AF synthesis on; the following paragraph regard the important role of oxidized fatty acids in controlling several aspects of the life of these fungi and,

notably, AF synthesis; it concludes with an evolutionary point of view on the meaning of AF synthesis for the *Aspergillus* section Flavi lifestyle.

2. The role of oxidative stress in modulating aflatoxin synthesis

Oxidative stress is a condition which organisms must cope with since the process used for producing energy (namely ATP) involves a very oxidizing molecule: the oxygen [9]. Thus, aerobic organisms have evolved means for facing this stress by building up a complex antioxidant system composed of structures, proteins (enzymes) and small metabolites. The ability to control this system enables organisms to face oxidative stress and, indeed, using it to “boost” some pathways (e.g., the defense in the plants) [10]. Aflatoxins are among these: they are synthesized in response to oxidative stress conditions [7] and, as we aimed to clarify within this chapter, can act as antioxidants to enhance the survival ability of these fungi.

2.1 Reactive oxygen species (ROS)

Free radicals are, by definition, very reactive chemical species, due to their presence of one or more unpaired electrons in valence orbitals. This condition makes them highly reactive molecules, energized and unstable; free radicals will try to give up or, as more commonly happens, to acquire an electron at the expense of another to obtain a stable configuration.

In living systems, spontaneously forming radicals are numerous, and those of greater biological interest, the so-called ROS, are those molecules in which the unpaired electron is found on O₂, such as, for example, superoxide ($\cdot\text{O}_2^-$), hydroxyl ($\cdot\text{OH}$), hydroperoxyl ($\cdot\text{OOH}$), peroxy ($\cdot\text{OOR}$) and alkoxy ($\cdot\text{RO}$) radicals. Oxygen is found in nature in the form of diatomic molecules that have two unpaired electrons of parallel spins arranged on two different orbitals (triplet), and therefore possessing characteristics paramagnetic. The fact of having uncoupled electrons makes O₂ particularly prone to forming covalent bonds but, in the case of incomplete reduction, ROS may be generated. These react quickly with other compounds to acquire the electrons necessary for their chemical stability, losing, in turn, their electrons and becoming radicals themselves, thus triggering a chain reaction. Once that process starts, it is determined in the cell a cascade of reactions that often begins with the peroxidation of lipids membrane (oxidation of the hydrocarbon chain), resulting in its destabilization, and which proceeds with the oxidation of other cellular components (such as proteins and DNA), to the point of causing the deconstruction of the entire cell.

The reactions in which the radical molecules can take part are many and vary significantly, for example, depending on: (i) the compartment or organelle cell in which they originate, (ii) of the antioxidant systems present, (iii) of the molecules that they attack, (iv) the water and nutritional conditions of the cell. Also, non-radical molecules, such as hydrogen peroxide (H₂O₂), can trigger responses that lead to the formation of ROS: the Haber-Weiss reaction, for example, produces hydroxyl radicals starting from H₂O₂ and O₂⁻. The cells of photosynthetic organisms are more subject to oxidative damage since they have concentrations of very high O₂ since, not only do they use it during breathing, but they also generate it with photosynthesis. In fact, they have membrane thylakoids composed mainly of polyunsaturated lipids (molecules subject to reactions of peroxidation) and, by means of photosynthetic pigments, absorb light energy, the excess of which favors the production of ROS. In its ground state, O₂ is relatively not very dangerous because, although it can give rise to excited states reactive

and free radicals (during photosynthesis, for example), its utilization proceeds expeditiously by means of a route in stages, in which a reduction to H₂O involving four electrons and during which intermediates can be generated partially reduced reactive species. In fungi as well as in other organisms, ROS can be produced in a tightly regulated way by the NADPH oxidase complex (NOX in fungi; [11]). This complex controls, upon stimuli, the formation of anion superoxide and controls several processes in hyphal growth and development [11, 12] and in mycotoxin synthesis too [13].

2.2 Antioxidant responses

If, as just described, the formation of free radicals can cause serious damage at the cellular level, which can sometimes lead the cell to death, it is equally true that the aerobic cells have evolved and developed efficient ROS control and detoxification systems. The latter are known as antioxidant systems and can be enzymatic and non-enzymatic in nature. The systems non-enzymatic include molecules such as: α -tocopherol, β -carotene, compounds phenolics, ascorbate, glutathione; the enzymatic ones involve: superoxide dismutases (SOD; EC 1.15.1.1), which catalyzes the dismutation of O₂⁻ in H₂O₂, together to others which eliminate the H₂O₂ such as catalases (CAT; EC 1.11.1.6), peroxidases, glutathione peroxidases (GP; EC 1.11.1.9), (which uses glutathione as an electron donor - GSH, reduced form, and GSSG, oxidized form) and ascorbate peroxidases (APX; EC 1.11.1.11; ASA, reduced form of ascorbic acid, and DHA, oxidized form). All the enzymes described are found in multiple forms (isoforms) that can be classified, for example, based on their metallic cofactor. The latter can also be found in different cellular compartments (such as cytosol and apoplast) or organelles [mitochondrion, chloroplast (in plants), peroxisome and vacuole]. Some of them catalyze the same reaction and can use different substrates as electron donors. In fungi, these antioxidant capacities are tightly controlled by transcriptional regulators. Main transcription factors that in fungi “react” to ROS are *msn2–4* [14], *skn7* [15] and *Yap-1* [16]. Notably, *Yap-1* orthologue *ApyapA* can control aflatoxin biosynthesis [17].

2.3 Oxidative stress, antioxidant system and aflatoxin synthesis

Oxidants are continuously produced within and outside fungal cells. In some way they can fuel cells to switch metabolic pathways [18] or differentiation patterns [19]. Inter alia, in *A. flavus* and *A. parasiticus* ROS boost aflatoxin formation [19–21]. In the past we showed that several oxidants amended to culture as well as increase of cell ROS were able to trigger aflatoxin synthesis [20]. Intriguingly, even external oxidants augment the titer of cell oxidants. How can these oxidants turn into “aflatoxins”? which is the “mediator” of the opening of the complex aflatoxin pathway? Our group and John Linz group demonstrated that *ApYapA* can orchestrate their synthesis [20]. Notably, *ApYapA*, similarly to its orthologue *Yap-1* of *Saccharomyces cerevisiae*, is indirectly oxidized by ROS through a peroxidoxin (TSA1, [17, 22]). Once oxidized, *ApYapA* migrates into the nucleus and, during the exponential phase of growth it recognizes and binds to specific responsive elements present into the promoter of genes encoding antioxidant enzymes such as catalases, superoxide dismutases inter alia. During the stationary phase, indeed, it binds even to the promoter of *AflR*, i.e., the gene whose product controls (together with *AflJ*) the whole AF pathway and consequently, their biosynthesis (**Figure 1**). As suggested below (paragraph 4), aflatoxins are a subsidiary antioxidant response that fungal cells operate to “staying alive” as long as possible to differentiate conidia and “escape” from the spent - stressing, oxidizing – substrate [23].

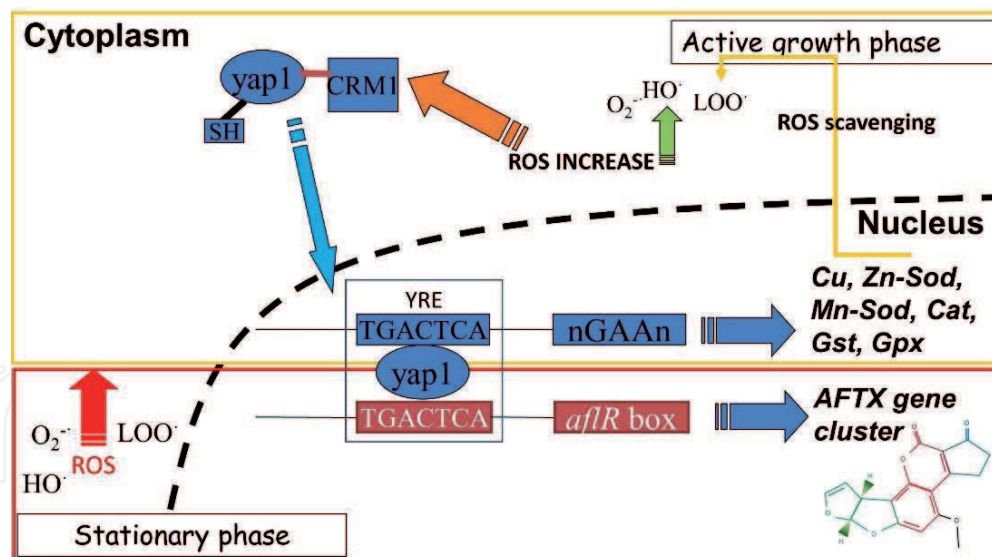


Figure 1.

Aspergillus parasiticus produces ROS during the normal course of its lifestyle. During the exponential – Active- phase of growth their production is constant but kept low by a very efficient antioxidant system that is modulated by ApyapA inter alia. In this phase aflatoxin synthesis is normally shut off or very low. Indeed, during the stationary phase (or even in consequence of external stressors – E.g., herbicides), ROS scavenging through normal detoxification system (e.g., glutathione, superoxide dismutases etc) is not efficient anymore and the oxidative stress-controlled transcription factor ApYapA recognize and bound to YRE (Yap1 responsive elements) present in the promoter of AflR, the global transcriptional regulator of aflatoxin synthesis. In this phase, AF synthesis is switched on and contribute to scavenge oxidants present in the matrix.

3. The role of oxylipins in the *Aspergillus* sect. Flavi lifestyle

3.1 Discovery of oxylipins

Understanding the evolution of fungal pathogenesis requires the treatment of some lipid molecules that mediate the fungus-host interaction. *A. flavus* preferentially infects maize seeds, which are rich in unsaturated fatty acids (UFAs). Furthermore, also *Aspergillus* species contain high levels of UFAs, including oleic ([18]: 1), linoleic ([18]: 2) and linolenic ([18]: 3) acid, which are substrates for oxygenation that converts the UFAs in oxylipins. Oxylipins are involved in the interaction-signaling of fungi, bacteria, plants and animals.

First evidence on the existence of oxylipins dates to 1987, when Champe et al., demonstrate the role of precocious sexual inducer (psi), later called oxylipins, in *Aspergillus nidulans*. Psi factors inhibit asexual sporulation and stimulate premature sexual sporulation, acting as hormone-like molecule [24].

Oxylipins derive from free fatty acids or from fatty acids present into membrane phospholipids. Fatty acids included in membranes, during the plant-pathogen interaction, are released by lipase action. Lipases are considered as virulence factors in plant pathogenic fungi [25].

Oxylipin oxidation may happen by two routes: the radical and the enzymatic. In fact, during the first steps of infection the production of radical species favors the accumulation of Reactive Oxygen Species (ROS). Superoxide anion ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$) can spontaneously oxidize the free fatty acids. The second oxylipins synthesis route, i.e., enzymatic, in fungi involves the action of dioxygenases (DOX), lipoxygenases (LOX) and cyclooxygenases (COX) that convert free fatty acids in oxylipins [26].

The crosstalk established during a plant-pathogenic fungus interaction, therefore, involves a lipases-LOX concerted activity, that carries towards the oxylipin biogenesis [27].

In *A. flavus* enzymatic set for oxylipin formation is composed of four dioxygenases, PpoA, PpoB, PpoC, and PpoD, and one lipoxygenase, LOX. PpoA encodes a 5,8-linoleate diol synthase, whereas ppoC encodes for a linoleate (10R)-dioxygenase [28–30].

3.2 Oxylipins in host-pathogen interaction

Oxylipins act as modulator of many signal transduction pathways, both in plant and fungi because the chemical structure as well as the main synthesis routes of oxylipins are common between the two kingdoms. For that reason, several authors defined the oxylipins as the common language between hosts and pathogens [31]. But why do hosts and pathogens produce oxylipins?

In *A. flavus*, oxylipins regulate dissemination, affecting the production of sclerotia and conidia, and influence secondary metabolism. In addition to having an autocrine action, oxylipins are also distinguished by their paracrine action, when *A. flavus* interacts with other organisms.

When *A. flavus* produces oxylipins, the plant recognized them and alters the expression of oxylipin synthesis genes, as the LOX, but the fungus senses the plant oxylipin gradient that promotes sporulation and mycotoxin production, this signaling exchanging define the cross-talk.

Plant can release oxylipins as 9S-HPODE (9S-hydroperoxyoctadecadienoic acid) and 13S-HPODE (13S-hydroperoxyoctadecadienoic acid) able to influence the development in the Aspergilli, in addition, but at the same time they act in the regulation of plant defense and development.

The analysis of the phenotypes derived from the mutant of ppo-genes shown that in *A. flavus* that ppoA and ppoC deletion generates strains with less conidia and more sclerotia, whereas the deletion of ppoD shown the inverted situation, or rather more conidia and less sclerotia. It was considered also the deletion mutant for all four oxylipin-biosynthesis genes (dioxygenases and lox), which shows both high levels of aflatoxin production and high levels of sclerotia production. These results shown the closely link between the oxylipin production and the asexual or sexual reproduction, underlining the role of the oxylipins in the fungal regeneration.

As previously introduced, the oxylipins produced by the plant may influence the fungal lifestyle and being chemically similar to the fungal oxylipins they can substitute them. That was demonstrated in one study, where the maize lipoxygenase Zm-LOX3 cloned in *A. nidulans* mutant strain, deficient for the two genes ppoA and ppoC, restored them functions. Oxylipins for the plant assume a protective function, in fact the inactivation of Zm-LOX3 makes the plant more susceptible to *A. flavus*, that during the infection grows more and produces more aflatoxins [32]. The increase of susceptibility is linked to the accumulation of oxylipin substrates, the fatty acids, and the decrease of the jasmonic acid, in whose biosynthesis pathway Zm-LOX3 is involved. Oxylipins acting as hormones, that means that a small concentration of oxylipins can regulate physiological processes as growth and development, but several studies show that oxylipin-mediated responses are strongly influenced by the type and the nature of interaction with the host.

The development of the fungus depends on cell densities when the cell density is high *A. flavus* produces more conidia. The cell densities also influence the secondary metabolites production, in fact the aflatoxin synthesis decreases at high cell densities. The quorum sensing, or rather the phenomenon in which the set of signaling molecules enable the single cell to sense the other cells, may be associated with the oxylipins release. The deletion of ppo and lox genes inhibits the development of the oxylipins [33, 34]. The exposure to exogenous seed oxylipins, as 9-HpODE and 13-HpODE stimulate the sporulation and the aflatoxin synthesis. The 13-HpODE

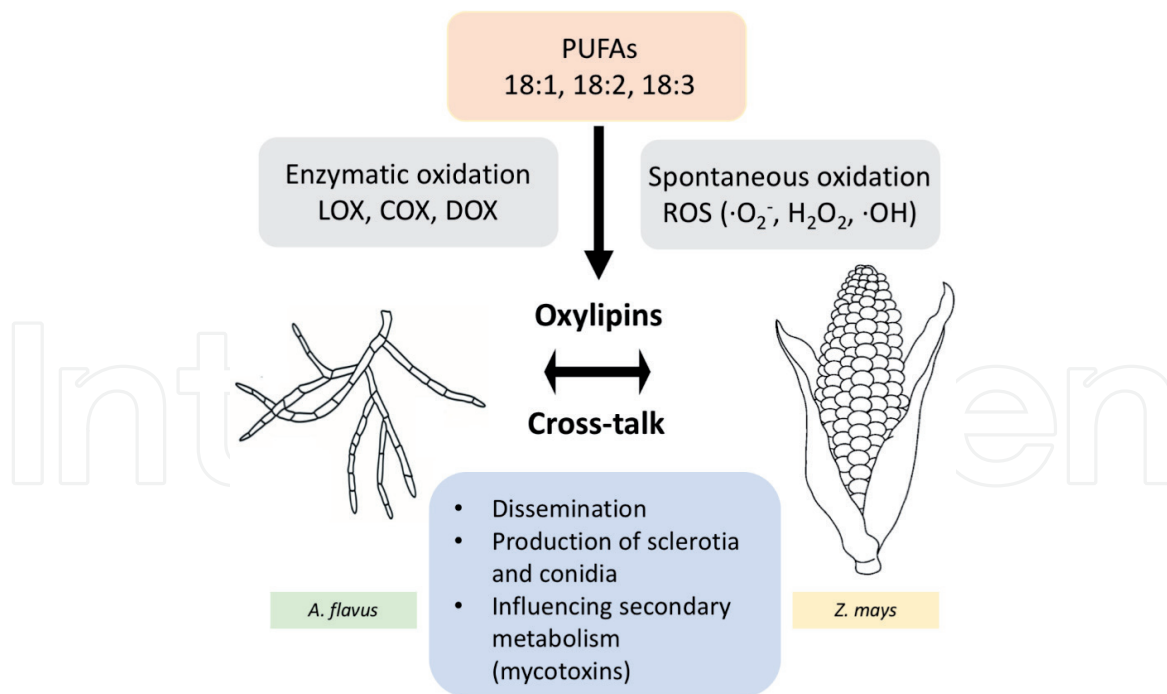


Figure 2.

Signaling oxylipin-mediated in *A. flavus*. Polyunsaturated fatty acids (PUFAs) may be converted by enzymatic and spontaneous oxidation in oxylipins, that mediate the interaction with the host (*Z. mays*).

seems to inhibit the sclerotia formation, suggesting the role of this oxylipin in the sexual/asexual reproduction in *Aspergillus* species [35]. These results proposed the oxylipins as quorum sensing mediator in *Aspergillus* species [36] (Figure 2).

Although the role of oxylipins in development and host-pathogen communication is recognized, little is known about their perception. Mammal oxylipins are sensing by G protein-coupled receptors (GPCRs), but in fungi GPCRs are not actually identified also if in *A. flavus* have been found some genes homologous to mammal GPCRs required for the high-density growth [37].

GPCR-mediated signaling seems to be linked to pathogenesis, therefore it is hypothesized that they could be potential targets for disease control [38].

4. Evolving a strategy for enhancing the resilience to stress: the role of aflatoxins

A. flavus is highly infectant, fast growing, efficient and versatile bio factory to its core [39]. Among the wide inventory of secondary metabolites it produces, aflatoxins are not the least puzzling, even after decades of dedicated research. Their physiological role has proven hard to frame unequivocally, having been linked over the years to several different purposes, including messengers for quorum sensing, facilitators of dispersal and resistance factors to UV stress [40], inhibitors of environmental competitors [41], mutagenic agents employed to compensate the limited intraspecific variability derived by a conidiogenesis-centred reproduction strategy and, finally, antioxidants.

Aflatoxins have been linked to oxidative stress since the 1980s [42], their synthesis being convincingly linked to increase in the presence of oxidants, *in vitro* and *in vivo* [43, 44]. However, the mechanism behind aflatoxins' role as direct or indirect reducing agents has also been elusive. In a general view, secondary metabolites such as mycotoxins are described by several experts as the by-product of housekeeping processes whose production is an indirect consequence of the very activity of

primary metabolism [45]. In this framework, it is possible that aflatoxins could fit the picture as the by-products of a secondary pathway aimed at channeling and exhausting environmental oxidative stress through a dedicated metabolic pathway, which only culminates in aflatoxin synthesis; aflatoxins would therefore achieve their alleged biological purpose through an indirect role. This eventuality is intriguing but, also, does not preclude the avenue of a more direct role as a ROS scavenger. As all secondary metabolites, aflatoxin production is mainly triggered after the biological switch from trophophase to idiophase, when *Aspergillus* transitions from a growth-centred lifestyle to differentiation and dispersal. At such a stage of mycelial development, aflatoxin must provide a benefit to growth/survivability to an appreciable, but not substantial extent. Given that even within *A. flavus* strains only half are aflatoxigenic, it would be sensible to hypothesize that aflatoxins in no way define or drive *A. flavus* ecology, but also in no way do they burden it beyond redemption, otherwise they would be excised from secondary metabolism altogether through selection, if nothing else, in the context of evolution.

A. parasiticus is an aflatoxin producer, and close relative to *A. flavus*, with a well-documented tolerance to intense oxidative stress, both *in vitro* and *in vivo*. Hong et al. [46] have provided precious insight into *A. parasiticus* aflatoxigenic biological triggers. In their work, antioxidant enzymes are upregulated during growth stage and into early stationary phase; it is only once stably into stationary phase that the oxidative stress-related transcription factory AP-1 like triggers aflatoxin synthesis. In this context, it is easy to see how strongly an intrinsic, direct antioxidant potential of the aflatoxin molecule would constitute a substantial clue to the proof of mechanism researchers have wondered about.

A recent research by Finotti et al. [47] aims at elucidating this aspect. Finotti explored the intrinsic potential (**Figure 3**) of the four main AFs congeners (B1, B2, G1, G2) as scavengers of reactive oxygen species (ROS). In this work, 2,2'-Azobis,

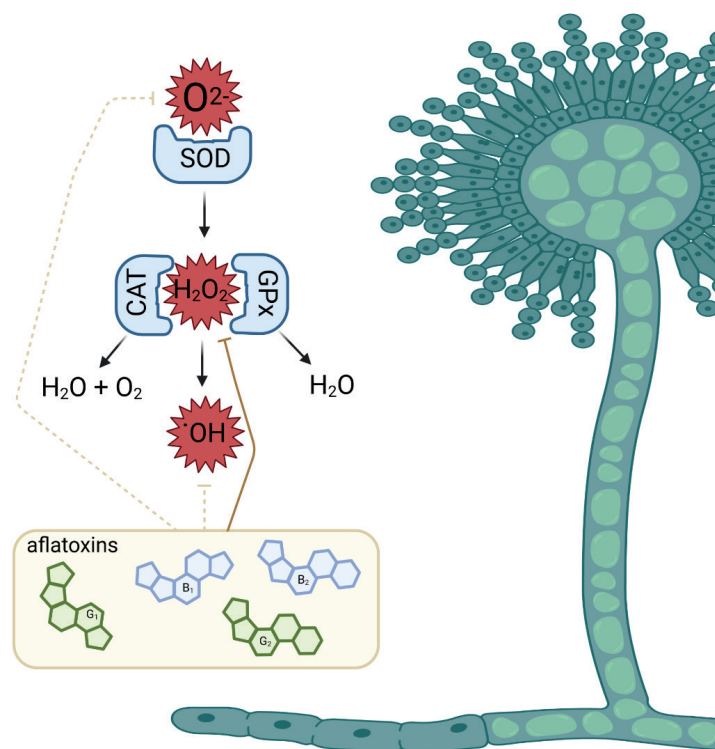


Figure 3. ROS are highlighted with a red star. Antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are adjuvated by aflatoxins' scavenging activity on H_2O_2 (observed), and on O_2^- and $\cdot OH$ (putative). Created with BioRender.com.

2-amidinopropane (APAB) was used to generate oxidants *in vitro* in hydrophilic and lipophilic environments. In the former case, all aflatoxin variants proved capable at inhibiting the oxidant-induced bleaching of crocin, each with different degrees of efficacy, namely: G1 > B2 > G2 > B1, ranked from most to least effective. Notably, AFG1 presented an antioxidant value ($K_a/K_c = 2.49$) comparable to that of the hydrophilic fraction of select polyphenols known for their remarkable antioxidant activity. A second *in vitro* test was run by Finotti and collaborators to assess survivability of *E. coli* K12 cells, when faced with hydrogen peroxide-induced oxidative stress, in the presence of aflatoxin B1. *E. coli* was selected for the test because it is non-susceptible to aflatoxin toxicity, most likely due to its lack of cytochrome p450, the enzyme whose interaction is necessary to incur into the toxic effect of AFs. 20 ug/mL of AFB1 provided increased carrying capacity to populations of *E. coli* K12 when challenged with hydrogen peroxide concentrations within 0 and 0.6 mM, and no difference with the control beyond such interval. It is of note how Finotti's data substantiate the hypothesis of AFs as active scavengers of ROS. We do not think, however, that this evidence disproves in any way the considerations on AFs' putative, ulterior biological roles. It is indeed a complicated endeavor to frame the purpose of a molecule whose ecological role is likely residual, and whose benefit to the producing organism arguably pertains more than one aspect of life, but possibly none decisively.

5. Conclusions

Fungal species belonging to the *Aspergillus* sect. Flavi synthesize the animal health-hazardous aflatoxins, the most potent natural carcinogenic on earth. Why do these fungi produce them? Our suggestion, recently supported by our findings and by other scientists, is that aflatoxins are a way for resisting an oxidizing environment; namely, they are produced to provide fungi "more time" to induce conidiogenesis and - literally - escape from the stressing environment. Considering this, oxidant stressors produced in different contexts - ranging from herbicide treated soil to host tissues - trigger aflatoxin biosynthesis that - in turn - enhance the antioxidant capacity of *Aspergillus* sect. Flavi and provide a chance to face challenging environments and exploit them.

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Conflict of interest

"The authors declare no conflict of interest."

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