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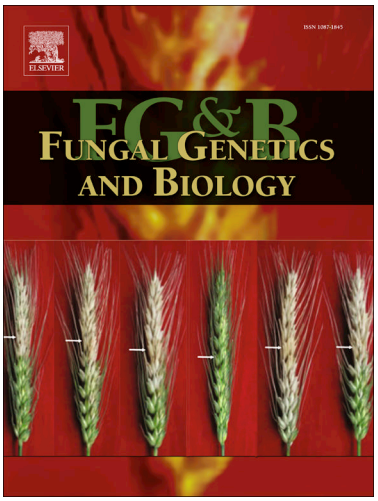

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Piperine inhibits Aflatoxin B1 production in *Aspergillus flavus* by modulating fungal oxidative stress response

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Abstract

Aspergillus flavus, a soil-borne pathogen, represents a danger for humans and animals since it produces the carcinogenic mycotoxin Aflatoxin B1 (AFB1). Approaches aiming the reduction of this fungal contaminant mainly involve chemicals that may also be toxic. Therefore, identification and characterization of natural anti-aflatoxigenic products represents a sustainable alternative strategy. Piperine, a major component of black and long peppers, has been previously demonstrated as an AFB1-inhibitor; nevertheless its mechanism of action was yet to be elucidated. The aim of the present study was to evaluate piperine's molecular mechanism of action in *A. flavus* with a special focus on oxidative stress response. For that, the entire AFB1 gene cluster as well as a targeted gene-network coding for fungal stress response factors and cellular receptors were analyzed. In addition to this, fungal enzymatic activities were also characterized. We demonstrated that piperine inhibits aflatoxin production and fungal growth in a dose-dependent manner. Analysis of the gene cluster demonstrated that almost all genes participating in aflatoxin's biosynthetic pathway were down regulated. Exposure to piperine also resulted in decreased transcript levels of the global regulator *veA* together with an over-expression of genes coding for several basic leucine zipper (bZIP) transcription factors such as *atfA*, *atfB* and *ap-1* and genes belonging to superoxide dismutase and catalase's families. Furthermore, this gene response was accompanied by a significant enhancement of

catalase enzymatic activity. In conclusion, these data demonstrated that piperine inhibits AFB1 production while positively modulating fungal antioxidant status in *A. flavus*.

Key words: Aflatoxin B1, *A. flavus*, inhibition, oxidative stress, gene expression, piperine.

1. Introduction

Mycotoxins are secondary metabolites produced by some filamentous fungi. Among the 300 reported mycotoxins, AFB1 is the most dangerous one. In fact, AFB1 is a potent carcinogenic agent in humans inducing liver cancer (IARC, 1993; Wu et al. 2014). This mycotoxin is synthesized by at least 15 species belonging to the *Flavi* section of the *Aspergillus* genus, *A. flavus* considered as the most frequent source of aflatoxins in crops (Varga et al., 2015). To date, it is estimated that at least 500 million of people living in sub-Saharan Africa, Latin America and Asia, are exposed to increasing levels of mycotoxins, including aflatoxins, through contaminated commodities (Pitt et al., 2012). In 2010, analysis of several foodborne chemicals by the Chemical and Toxins Disease Task Force reported that aflatoxin was associated with the highest number of DALYs (deaths and disability adjusted life years) (636,869) where one DALY represents the sum of years lived with disability and years of life lost (Gibb et al., 2015). Worryingly, surveys demonstrated that higher levels of aflatoxins in agricultural commodities were linked to upraising climatic conditions (Streit et al., 2013). Indeed, AFB1's contamination was recently reported on crops from other temperate regions of Europe, demonstrating an increased distribution of this mycotoxin potentially related to global climate changes (Battilani et al., 2016; Perrone et al., 2014). Even if several approaches have been developed to limit AFB1's contamination (Wild et al. 2015; Jalili, 2015), they remain insufficient to completely avoid mycotoxin contamination thus emphasizing the need to develop new

strategies to guarantee food safety. The use of natural products such as spices, plant extracts or essential oils with anti-aflatoxinogenic properties represent a complementary or alternative strategy against mycotoxin contamination (Annis et al., 2000; El Khoury et al., 2017; Holmes et al., 2008; Sakuda et al., 2016). For instance, piperine, a major active component of black and long peppers (*Piper nigrum L.* and *Piper longum L.)* has been previously demonstrated as an effective inhibitor of aflatoxin production (Lee et al., 2002; Madhyastha and Bhat, 1984). However, the molecular mechanism of action by which this molecule inhibits AFB1 production has not been studied. In the last years, aflatoxin biosynthesis has been closely linked to fungal oxidative stress status (Montibus et al., 2013; Roze et al., 2013; Amare and Keller, 2014). Indeed, studies in *Aspergilli* demonstrated several molecules with antioxidant capacities are able to inhibit aflatoxin production (Jahanshiri et al., 2012; Liang et al., 2015). Furthermore, it has been demonstrated that a regulatory network of genes coding for stress response transcription factors (AtfB, SrrA, Ap-1 and MsnA) is directly involved in aflatoxin biosynthesis (Hong et al., 2013). Within this context, the present study aimed to elucidate the anti-aflatoxinogenic mechanism of action of piperine by studying its impact on the expression of 27 genes involved in AFB1 gene cluster and 20 genes coding for oxidative stress response in *A. flavus*, by using a large-scale q-PCR approach (Caceres et al., 2016).

We demonstrated that piperine resulted in a down-regulation of almost all genes of the AFB1 cluster leading to the subsequent inhibition of the mycotoxin biosynthesis. We also showed that exposure to piperine induced an inhibition by 50% of *veA* expression levels together with an over-expression of several bZIP transcription factors (*atfA*, *atfB* and *ap-1*) and genes coding for antioxidant defense belonging to catalase and superoxide dismutase families (*sod1*, *catA* and *cat2*). Simultaneously, a significant increase in catalase enzymatic activity was also observed.

2. Materials and Methods

2.1. Chemicals and reagents

Lyophilized piperine standard (purity ≥97.0%) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Stock and work solutions were diluted in acetonitrile and stocked at 4°C until use. All solvents were analytical grade and purchased from Thermo Fisher Scientific (Illkirch, France).

2.2. Fungal strain and culture conditions

A. flavus NRRL 62477 strain, uniquely producer of AFB1, was used in this study (El Mahgubi et al., 2013). All cultures were performed on Malt Extract Agar (MEA) medium (Biokar Diagnostics, Allone, France) and maintained in the dark at 27°C during 4 days. Different concentrations of piperine were tested in order to determine the one capable to inhibit AFB1 production with a limited impact on fungal development. In all cases, vehicle solvent concentration was of 0.02 M. This concentration was previously identified as a no-effect dose on both fungal growth and AFB1 production. Control cultures were performed using the same solvent concentration. Before inoculation, media were covered with sterile cellophane layers (Hutchinson, Chalette-sur-Loing, France) as described by Leite et al. (2012) and centrally inoculated with 10^3 spores using a calibrated spore suspension prepared from a seven-day culture.

2.3. Fungal growth, morphology and spore quantification

Fungal growth was evaluated by measuring colony diameters (length and width). Macroscopic characteristics (colour of conidial areas, thallus margin and texture,

aspect of conidial heads and colony reverse) were observed under a stereomicroscope SZX9 –X12-120 (Olympus, Rungis, France).

Microscopic observation of conidial heads was performed after staining with lactophenol blue dye solution and fungal structures were observed using a microscope CX41 with a total magnification of x400 (Olympus, Rungis, France). In order to observe the impact of piperine on fungal sporulation, spore quantification was done at the end of incubation. For that, cultures were suspended in 50 mL of Tween 80 (0.05% in water) using a stomacher bag where spores were carefully scraped up off the mycelium. Samples were then homogenized with a STOMACHER Lab-Blender 400 (Interscience, Saint-Nom-la-Bretèche, France) during 90 s and filtered through sterile gauze. Then, to recover the remaining spores, three supplementary rinses followed with 20 mL of Tween solution each were made. Finally, spores were counted on a MALASSEZ cell (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and spore density was calculated as follows: $SD = SC/(\pi r^2)$ where SC is the spore count and r is the average colony radius.

2.4. Aflatoxin B1 extraction and determination by High Performance Liquid Chromatography

Culture media were mixed with 25 mL of chloroform and samples were agitated for 2 h on a horizontal shaking table at 160 rpm at room temperature. Chloroform extracts were filtered through a Whatman 1PS phase separator (GE Healthcare Life Sciences, Vélizy-Villacoublay, France), evaporated at 60°C to dryness and diluted with 500 µL of water-acetonitrile-methanol (65:17.5:17.5; *v*/*v*/*v*). Extracts were filtered through a 0.45 µm PTFE disks (Thermo Scientific Fisher, Villebon-Sur-Yvette, France). Sample analysis was performed with a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Illkirch, France) using a LC column Luna® C18 (125 × 2 mm, 5 µm, 100 Å) (Phenomenex, Torrance, CA, USA) conditioned at 30 °C. Separation conditions were adapted from Fu et al. (2008) with mild modifications.

Briefly, a 20 min isocratic mode was delivered at 82.5% of eluent A: acidified water (0.2% of acetic acid) and acetonitrile (79:21 *v*/*v*) and 17.5% of eluent B: pure methanol. Flow rate was 0.2 mL/min with a sample injection volume of 10 µL. AFB1 was detected using a fluorescent detector set at 365/430 nm excitation/emission wavelengths. Peak identity was confirmed by analyzing absorption spectrum with a diode array detector (DAD) coupled to the system. Production levels of AFB1 were calculated based on a standard calibration curve.

2.5. Isolation of fungal RNA, RT-PCR and q-PCR conditions

On day 4, mycelia were separated and grounded under liquid nitrogen. RNA was purified with a Qiagen RNeasy Plus Minikit (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop ND1000 (Labtech, Palaiseau, France) and RNA purity was confirmed by both the A_{260}/A_{280} ratio and gel electrophoresis (1.2% of agarose).

First-strand cDNA synthesis was carried out by RT-PCR. A first denaturation step was done at 70 °C for 5 min and reverse transcription was performed as follows: 5 min at 37 °C; 60 min at 42 °C and 15 min at 85 °C. All primer sets were designed based on the genomic data of the *A. flavus* strain NRRL3357 (GenBank accession number EQ963478A); primer pair sequences were adapted from Caceres et al. (2016) and sequences for complementary genes related to stress-response were as follows: *atfB* (AFLA_094010) *F:*CCCATGCGATCCCCTGAA *R:*CGACTCGGTCATAATGGACTGCT; *sakA* (AFLA_061090) *F*: ATTTCCAGGACAAGACCCCATT *R:*AGTGACCTGATGATCTCCACTGTATTC; *sskA* (AFLA_062210) F:CAATGACTTCTTGACCAAGCCTGT *R*: GTGTGGGTGTCGCTGATGACT.

A total number of 47 genes were analyzed consisting in 27 genes coding for AFB1 biosynthesis cluster and 20 genes coding for regulatory factors including:

-Enzymatic response: *cat2*, *catA*, *mnSOD* and *sod1*.

-Transcription factors: *srrA, msnA, atfA, atfB,* and *ap-1.*

-Sensing stress signal: *sakA, sskA.*

-G Protein-Coupled Receptors (GPCRs): *gprA, gprG, gprH, gprK, gprP*

-Oxylipins: *ppoA, ppoB, ppoC*

-Global regulator: *veA*

Experiments were carried out using a ViiA7 Real-Time PCR System (Applied Biosystems, Forster City, CA, USA). Plates of 384 wells were prepared by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA, USA). Each well contained a final mix volume of 5 µL and Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used as a fluorescent dye. Negative controls consisted on samples where no reverse transcriptase was added in order to verify false positives as well as water samples to control reagent contamination. Quantitative PCR steps were performed as previously described by Caceres et al. (2016).

2.6. Gene expression data analysis

Gene expression data were analyzed with a Quant-Studio Real time PCR software v1.1 (Applied Biosystems, Courtaboeuf, France). Housekeeping gene β-tubulin was used for data normalization after being proved as the most stable reference after analysis with the Normfinder algorithm (Andersen et al., 2004). Gene expression values of both groups (control and treatement) were determined using the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001) and means of both groups were compared to determine significant differences. Graphics are presented using normalized data where control value was fixed at 1.

2.7. Fungal enzymatic activity

2.7.1. Sample preparation

In order to observe the impact of gene expression over fungal enzymatic activities, cytosolic superoxide dismutase (SOD) and total catalase (CAT) were performed on day 4, following the same culture conditions of RNA isolation. For each assay, 200 mg of mycelium were suspended in 1 mL of cold buffer: samples for CAT measurements were placed in a 50 mM potassium-phosphate buffer (pH 7.0 containing 1 mM EDTA) while samples for SOD measurements were placed in a 20 mM HEPES buffer (pH 7.2; 1 mM EGTA, 210 mM mannitol and 70 mM sucrose). After suspension, samples were frozen in liquid nitrogen until homogenization using a Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France). The apparatus was coupled with a Cryolys system and maintained at 4°C to avoid enzyme degradation during grinding (2 x 10 seconds).

2.7.2. Superoxide dismutase and catalase tests

After homogenization, samples were centrifuged at 10,000 rpm for 15 min at 4°C and both total proteins and enzyme activities were determined in supernatants. Proteins were measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Villebon-Sur-Yvette, France) and enzymatic activity was determined using commercial kits Catalase (CAT-707002) and Superoxide Dismutase (SOD-706002) (Interchim, Montluçon, France) according to manufacturer's instructions. Sample absorbance was measured at 540 and 450 nm for CAT and SOD assays respectively using an ELISA plate reader (Spectra thermo scan, Tecan, NC, USA). All enzymatic activities were normalized according to their protein content. For CAT assays, results were expressed in nmol min^{-1} ml^{-1} per milligram of protein and for SOD assays in U ml^{-1} per milligram of protein where one unit defines the amount of enzyme needed to exhibit the dismutation of 50% of the superoxide radical.

3. Statistics

All experiments were conducted in triplicate. Gene-expression and enzymatic tests were based on six biological replicates while piperine dose-dependent effect was based on three replicates. Data analyses were done using GraphPad Prism v4 software. A Mann-Whitney test was used to determine the differences between control and treated groups and differences were considered to be statistically significant when the p-value was lower than 0.05. All results are expressed as mean ± standard error of mean.

4. Results

4.1. Effect of piperine on AFB1 production and fungal growth in A. flavus

Five different concentrations of piperine were tested for their effect on AFB1 production and fungal mycelia growth. Results demonstrated that piperine inhibits AFB1 production in a dose-dependent manner: 0.0006 mM of piperine inhibited toxin production by 30% while no detectable levels of toxin were observed using 0.17 mM (Fig. 1). Fungal mycelial growth was moderately impacted by piperine. Indeed, growth inhibition was of 12% using 0.04 mM of piperine and an inhibition of 35% was observed with 0.17 mM of piperine. Further experiments were conducted with 0.04 mM of piperine since this concentration allowed an AFB1 inhibition of 95% with only a slight impact on fungal mycelial growth.

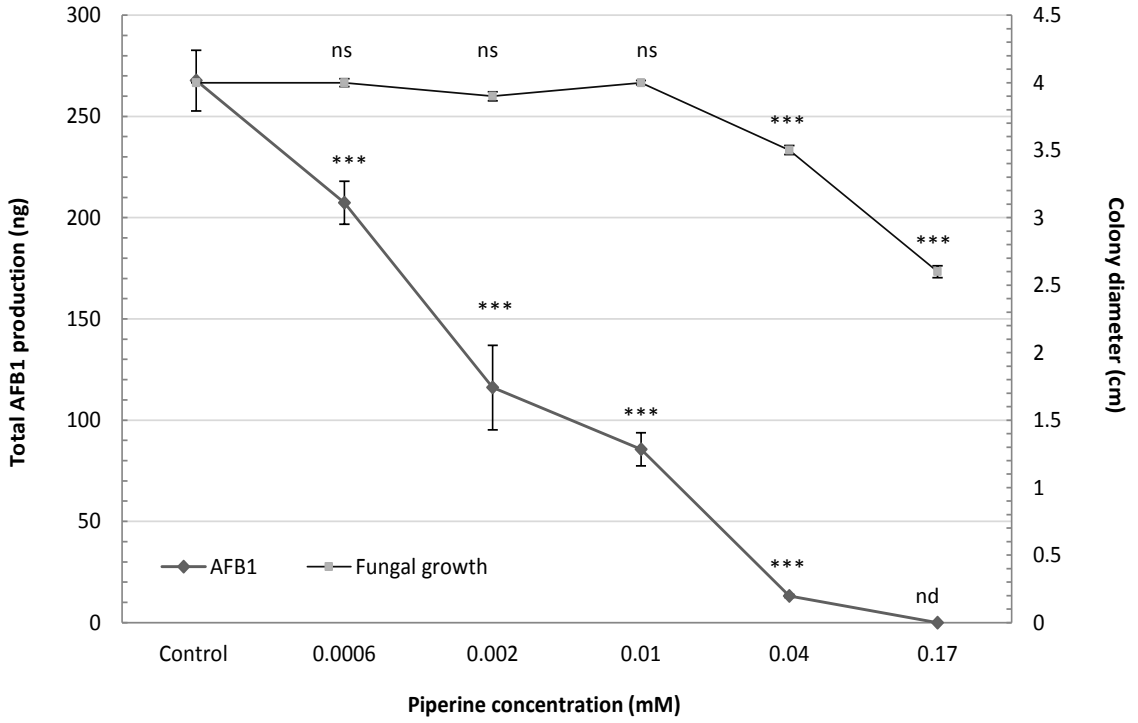

Fig. 1. Effect of piperine on AFB1 production and fungal growth in *A. flavus* NRRL 62477. AFB1 was measured by High Performance Liquid Chromatography (HPLC) and fungal growth by colony diameter. Measures were performed on day 4; ns=not significant; nd= not detectable; *** p <0.001.

4.2. Morphological changes in presence of piperine

The addition of piperine to the culture medium resulted in a marked reduction in *A. flavus* aerial mycelium that was only observed in the center of treated cultures (Fig. 2C). The appearance of pronounced ridges on the colony and a compact basal mycelium with packed conidial heads was also noted in piperine-exposed cultures (Fig. 2D). Piperine did not have any impact on other morphological aspects of *A. flavus* such as pigmentation or microscopic features. In fact, conidial heads of treated cultures maintained the characteristic morphology of this species (data not shown). The impact of piperine was also studied on fungal sporulation by analyzing quantity and density of spores. Results showed that quantity of spores was not significantly modified with $\log_{10}$ values of 9.10 ± 0.01 and 9.00 ± 0.01 for control and treated

cultures respectively (p=0.100). Similarly, no significant change was observed for spore density (p=0.100) (log_{10} values: 7.84 ± 0.01 *v/s* 7.90 ± 0.04 sp/cm^2 for control and piperine treated groups respectively).

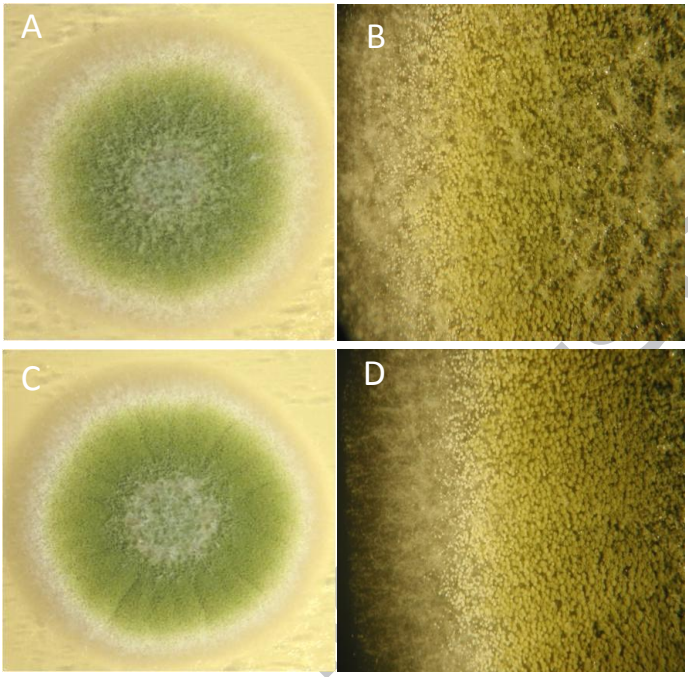

Fig. 2. Macroscopic effects of piperine at 0.04 mM on day 4 on *A. flavus* NRLL62477 grown on MEA medium. **A** and **B**: control; **C** and **D**: treated culture.

4.3. Effect of piperine on AFB1 gene cluster expression

In *A. flavus*, AFB1 biosynthesis is the result of an enzymatic cascade involving 27 genes grouped in a cluster. The impact of piperine over the entire AFB1 biosynthetic pathway is presented in Fig. 3. Following piperine exposure, 25 out of the 27 genes were significantly down-regulated compared to control. Among those, two presented decreased levels lower than 2 folds (*aflS* and *hypD*); 9 genes were mildly inhibited (2 to 4 folds decrease *e.g. aflD, aflQ, aflC*) and 13 genes presented an inhibition ranging from 4 to 8 folds. The most down-regulated genes were *aflN*, *aflW* and *aflK* with respective fold changes of 6.77, 6.82 and 7.25 and p-values lower than 0.0001. No significant changes were observed for a*flT* (p=0.565) neither for the internal cluster regulator *aflR* (p=0.404).

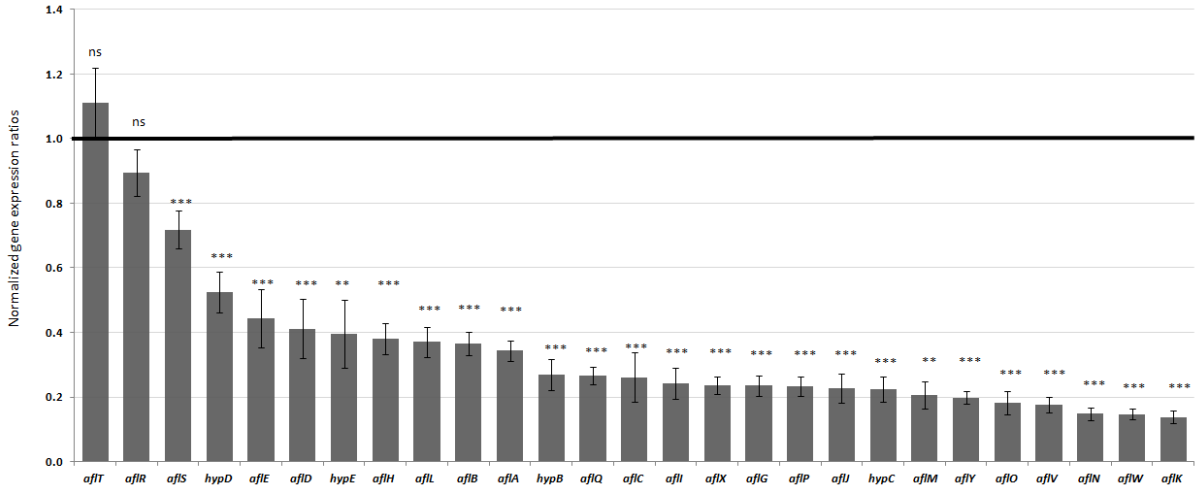

Fig. 3. Expression ratios of genes belonging to the cluster responsible for aflatoxin biosynthesis in response to 0.04 mM of piperine. Black line represents normalized control expression; error bars represent the standard error of mean; ns = not significant; ** p<0.01; *** p<0.001.

4.4. Effect of piperine on oxidative stress genes and enzymatic activities

In order to evaluate the impact of piperine on fungal stress response, a stress-related gene-network as well as several cellular signaling receptors were analyzed. Upon piperine exposure, the expression of 16 out of the 20 analyzed genes was significantly modulated (Fig. 4). Among them, the transcription factors *msnA* and *srrA*, the global regulator *veA*, the dioxygenase *ppoC* and the cellular receptor *gprP* were down regulated with decreased fold changes of 0.74 (p=0.010), 0.84 (p=0.012), 0.5 (p<0.001), 0.80 (p=0.029) and 0.70 (p=0.001) respectively. Conversely, piperine exposure induced an up-regulation of 11 genes coding for i) oxidative stress response factors such as *sskA*, *ap-1*, *atfA*, *atfB*, *sod1*, *catA* and *cat2* ii) GPCRs (*gprK* and *gprH*) and iii) oxylipins (*ppoA* and *ppoB*). As shown in Fig. 4, the most impacted genes modulated by piperine corresponded to those involved in oxidative stress response such as several basic leucine zipper (bZIP) transcription factors and genes belonging to catalase and superoxide dismutase families. Indeed, the bZIP transcription factor *atfB* was the most impacted gene with an increased expression of 5.41 folds (p<0.0001). It was followed by *sod1* with a fold change of 3.31 (p<0.0001), *sskA* 2.76 folds (p=0.014), *atfA*,

up-regulated by 2.32 folds (p<0.0001), *cat2* by 2.06 folds (p<0.0001), *catA* by 1.72 folds (p<0.0001) and *ap-1* by 1.47 folds (p= 0.004). Genes encoding for oxylipin biosynthesis, such as *ppoB* and *ppoA*, also presented significantly higher transcript levels compared to the control with increased levels of 2.97 (p<0.0001) and 1.55 folds (p=0.001) respectively. Finally, GPCRs were slightly modulated by 1.53 folds (p=0.046) for *gprH* and 1.39 folds (p=0.0008) for *gprK*. By contrast, no significant change was observed for *mnSOD* (p=0.290), *gprG* (p=0.301), *gprA* (p=0.798) or *sakA* (p=0.212) expression levels.

Based on the previous results, we decided to investigate the impact that the over expression of catalase and superoxide dismutase genes had over the corresponding enzymatic activities. Results shown in Fig. 5A, demonstrate that catalase enzymatic activity increased by 68.3% (p=0.0165) whereas superoxide dismutase activity was not significantly impacted upon piperine exposure (Fig. 5B).

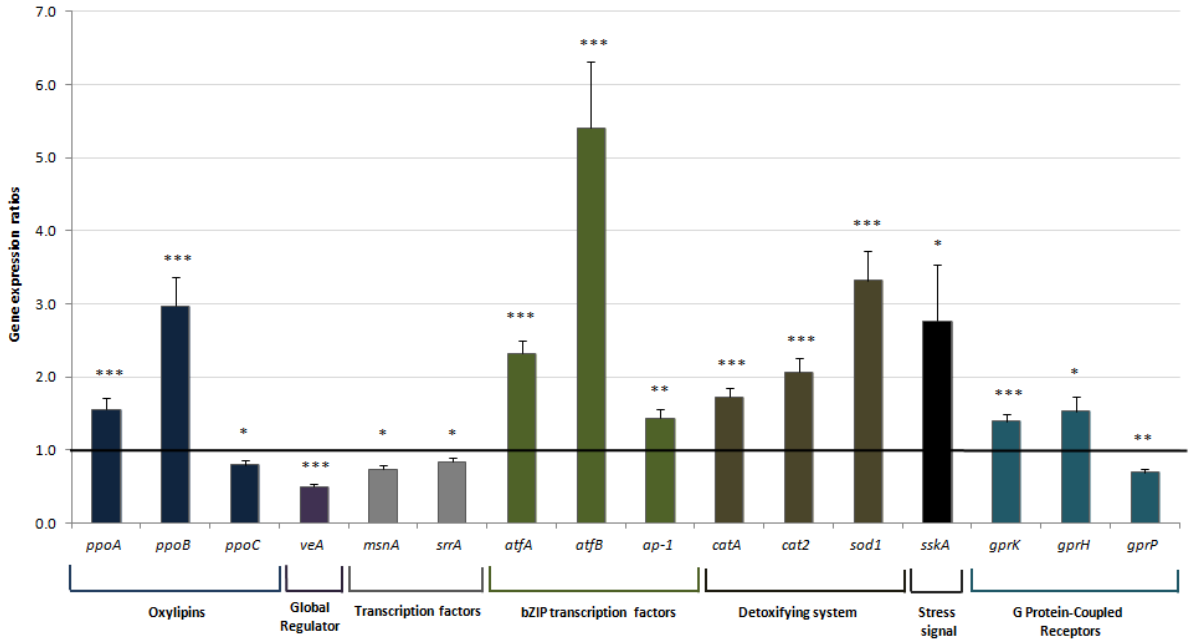

Fig. 4. Effect of piperine treatment on the expression of genes involved in oxidative stress response in *A. flavus* NRLL 62477. Black line represents control expression level;* p <0.05; ** p <0.01; *** p<0.001.

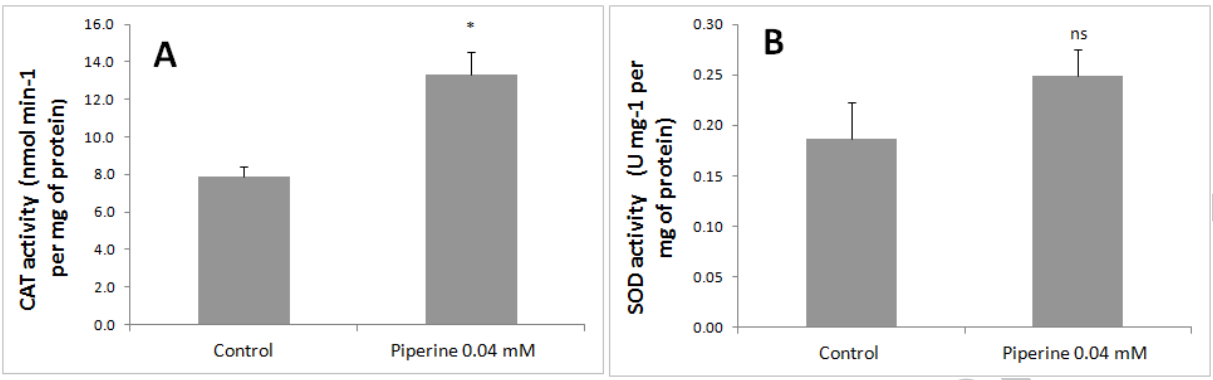

Fig. 5. Effect of piperine on antioxidant enzymes activities: **A**) total catalase activity; **B**) cytosolic superoxide dismutase activity; * $p<0.05$; ns= not significant.

5. Discussion

Oxidative stress is an unbalanced status occurring when reactive oxygen species overcome the scavenging capacities of antioxidant mechanisms. This causes alteration of cellular functions by inducing damages to DNA, proteins or lipids (Montibus et al., 2013). Studies performed in *Aspergilli*, have demonstrated that several aflatoxin inhibitors can modulate the antioxidant system in fungi (Grintzalis et al., 2014; Reverberi et al., 2005; Sun et al., 2015). Thus, in the present study, we investigated the molecular impact of the anti-aflatoxigenic molecule piperine over the expression of the entire AFB1 gene cluster as well as its impact on a gene-network involved in oxidative stress response and cellular signalization. In addition to this, CAT and SOD enzymatic activities were also measured. The fact that piperine can display antioxidant or pro-oxidant properties depending on its concentration, is of particular interest. Indeed, *in vitro* studies demonstrated that piperine concentrations ranging from 0.004 mM to 0.014 mM present scavenging properties while at higher doses (0.035 mM to 0.35 mM), this molecule increases hydroxyl radical generation (Mittal and Gupta, 2000). Herein, 0.04 mM of piperine which corresponds to a pro-oxidant dose, was chosen since it greatly inhibited AFB1 production with only a slight impact on fungal growth.

5.1. Effect of piperine on AFB1 gene cluster

Aflatoxins are polyketide-derived furanocoumarins synthesized by a 21 step enzymatic cascade (Bhatnagar et al. 2003). In *A. flavus*, this process is governed by a gene cluster where *aflR* and *aflS* act as internal regulators (Georgianna and Payne, 2009; Georgianna et al. 2010). Their corresponding proteins interact to form a functional complex required for aflatoxigenesis (Kong et al. 2014; Chang 2003). Upon piperine addition, mRNA levels of *aflS* were significantly down regulated. This finding suggests that the unbalance AflR-AflS ratio led to a down-regulation of most of the genes belonging to the AFB1 cluster and consequently to lower levels of toxin production. A recent study using another piperine-like synthetic compound (1-(2-methylpiperidin-1-yl)-3-phenylprop-2-en-1-one) also revealed an inhibition of *aflS* gene expression while production of AFB1 was no longer detected (Moon et al. 2016). During piperine exposure, *aflT* was not modulated which is in agreement with the fact that this gene is not regulated neither by *aflR* nor by its co-activator *aflS* but by other external regulators (Chang et al. 2004). Structural genes such as *aflD* and *aflM*, respectively coding for the first stable intermediate norsolorinic acid and versicolorin A transformation (Yu et al., 2004) were also significantly decreased. Interestingly, promoter regions of several aflatoxin genes carry binding motifs that are recognized by several of the bZIP transcription factors analyzed in this study. For instance, in *A. parasiticus*, AtfB binds to the promoter regions of seven genes intervening in aflatoxin synthesis process such as *aflC*, *aflD*, the intergenic region of *aflA-aflB*, *aflP*, *aflQ*, *aflM* and *aflR* (Roze et al., 2011).

5.2. Effect of piperine on stress-related and cellular response regulatory factors

The impact of piperine was analyzed by targeting a regulatory network of 20 genes including: *atfA, atfB, ap-1, msnA, srrA, sskA* and *sakA* coding for stress response transcription factors; genes involved in fungal antioxidant defense (*catA, cat2, sod1*

and *mnSOD*); genes coding for oxylipins (*ppoA, ppoB and ppoC*); genes belonging to GPCRs (*gprK, gprH, gprG, gprA and gprP*) as well as the global regulator *veA*. In the present study, piperine significantly induced the over-expression of 11 genes (*ppoA, ppoB, atfA, atfB, ap-1, catA, cat2, sod1, sskA, gprK and gprH*) while 5 others (*ppoC, veA, msnA, srrA* and *gprP*) were down-regulated.

5.3. Piperine impacts cellular signaling receptors and the global regulator veA in A. flavus

Receptors of the cell surface play an important role on how fungi overcome environmental changes. In *Aspergilli*, this signal transduction is activated by cellular receptors such as G Protein-Coupled Receptors and oxylipins (Affeldt et al., 2014). Indeed, GPCRs are involved in oxylipins response and it is established that the proper regulation of these signaling pathways play an important role in secondary metabolite production (Yu and Keller, 2005). After piperine addition, three of the five analyzed GPCRs and the three analyzed oxylipins genes were significantly modulated which demonstrates a perturbation of the cellular signalization pathway. Studies on piperine in mammalian cells have reported passive diffusion and alterations in cell permeation characteristics (Khajuria et al., 1999, 2002) suggesting that piperine may have the same behavior in fungal cells. In addition to this, several of the GPCRs studied in this work have been linked to aflatoxin production and fungal stress. Nevertheless, little is known today about the exact role that GPCRs have in fungi, although deletion of different GPRCs in *A. flavus* strains such as Δ*gprK*, produced higher levels of aflatoxin when exposed to the AF inhibitor methyl jasmonate (Affeldt et al., 2014). This seems to be in agreement with our results since inversely; over expressed *gprK* levels were associated with AFB1 inhibition by piperine. As mentioned, GPCRs are responsible of oxylipins modulation. Within the analyzed oxylipins, *ppoB* shown to be the most impacted gene upon treatment. In *A. nidulans*, *ppoB* deletion resulted in a precocious production of sterigmatocystin, a precursor of the AFB1 metabolite (Tsitsigiannis and Keller, 2006). This suggests that

in *A. flavus* the over-expression of *ppoB* could also have a negative impact in aflatoxin production. Fungal secondary metabolite production is also controlled by global regulators such as *veA*. In fact, in *A. flavus*, *veA* represents an important element since it governs the production of several secondary metabolites including AFB1, but is also involved in oxidative stress response (Duran et al., 2007). Following piperine addition, we reported that the expression level of *veA* was inhibited by 50%. Studies in *A. flavus* showed that disrupted *veA*-strains resulted in the absence of *aflR*, *aflD*, *aflM* and *aflP* genes expression and consequently in the absence of AF production (Duran et al., 2007). In *A. flavus*, *veA* is also necessary for oxidative stress tolerance since it controls genes intervening in the HOG (high-osmolarity glycerol) signaling pathway. Indeed, *veA* deletion resulted in decreased transcription levels of several genes intervening in stress response such as *srrA*, *msnA* or *atfA* (Baidya et al., 2014). According to our results, the down-regulation of *srrA* and *msnA* by piperine seems to be directly related to the decrease of *veA* expression levels. However, this was not the case for the other genes such as *atfA* suggesting that other regulatory factors may also be involved in piperine's mechanism of action.

5.4. Piperine inhibits AFB1 production and modulates bZIP transcription factors

The bZIP transcription factors AtfB, SrrA, AP-1 and MsnA were demonstrated as co-regulators of aflatoxin biosynthesis and oxidative response in *A. parasiticus* (Hong et al., 2013). According to our results, genes belonging to bZIP-type family stand as important elements in the mechanism of action of piperine. As previously mentioned, these bZIP elements play a critical role in modulating secondary metabolite production in response to different types of stresses, thus standing as key elements in fungal adaptation to different environmental stimuli (Hong et al., 2013; Temme et al., 2012; Van Nguyen et al., 2013; Yin et al., 2012, 2013). For instance, Ap-1, belonging to a redox-state sensor protein family, maintains highly conserved functions in mammalian, yeast and fungi (Toone et al., 2001). In fungi, the Ap-1-like

protein possesses *N*- and *C*-terminal cysteine rich domains that might act as a sensor-target of ROS such as H_2O_2 (Sies, 2014). Interestingly, activation of Ap-1 occurs either in anti- or pro-oxidative conditions via the signalization of different phospho-relay HogA/SakA cascades (Gomez del Arco et al., 1997). As a matter of fact, while an over-expression of *ap-1* was observed upon piperine addition, the response regulator *sskA* (involved in the HogA/SakA pathway) was also positively modulated. In *Aspergilli*, homologous and orthologous of the *ap-1* gene have already been characterized and associated with mycotoxin production. For instance, the over-expression of *napA* in *A. nidulans* resulted in a decreased in sterigmatocystin production (Yin et al., 2013). Similarly, the over-expression of *ApyapA* in *A. parasiticus*, led to a decrease in aflatoxin production suggesting that the latter is a negative regulator of aflatoxin production (Reverberi et al., 2006). Indeed, the *ApyapA* deletion displayed an earlier mRNA expression of AF cluster genes such as *aflR* and *aflE* and subsequently, a precocious aflatoxin production (Reverberi et al., 2008). According to our results, the over-expression of *ap-1* upon piperine exposure is related to the aflatoxin decrease and it suggests that this transcription factor could also act as a negative regulator of AFB1 production in *A. flavus*.

The *atfB* gene was the most highly modulated gene following piperine exposure. AtfB, belonging to the ATF/CREB protein family, is a well-characterized transcription factor in *A. parasiticus* (Roze et al. 2011). This regulatory factor plays an important role coupling oxidative stress response and secondary metabolite production by binding to promoters of genes carrying CRE sites. The fact that promoter regions of seven genes belonging to the aflatoxin cluster as well as antioxidant genes, such as catalases and superoxide dismutases, carry CRE-binding sites recognized by AtfB is of high relevance. Moreover, it was suggested that AtfB could form regulatory complexes with Ap-1, SrrA and AtfA contributing AtfB to bind to CRE sites (Hong et al. 2013). A recent study performed in *A. fumigatus* suggested that AtfB might be regulated by SakA pathway and also demonstrated that *atfB* expression was dose-dependently increased by using different concentrations of the antifungal agent

caspofungin (Pereira Silva et al., 2016). In paralell, AtfB was demonstrated to be positively regulated by VeA (Baidya et al., 2014). By contrast, in our study, *atfB* was over expressed while *veA* levels were decreased suggesting that other factors may influence *atfB* expression. Finally, *atfA*, another gene mainly involved in oxidative-stress response (Balázs et al., 2010), was also up-regulated. AtfA, belonging also to the ATF/CREB family, permanently resides in the nucleus until activation as a response to oxidative or osmotic stress signals (Lara-Rojas et al., 2011). To date, the specific interaction between AtfA and aflatoxin biosynthesis lacks information. Since it has been demonstrated that almost all genes regulated by AtfB are also regulated by AtfA, a link between AtfA and aflatoxin production was suggested (Sakamoto et al., 2009). Herein, we demonstrated that the over-expression of the bZIP transcription factors *atfA, atfB* and *ap-1* is directly involved in the inhibition of aflatoxin production by piperine.

5.5. Effect of piperine on antioxidant defense genes and fungal enzymatic activities

Exposure of *A. flavus* cultures to piperine resulted in a modulation of several bZIP transcription factors. Most of the targets of these transcription factors are antioxidant-related genes such as catalase and superoxide dismutases, which also mediate cellular defense against oxidative stress. As expected, mRNA levels of *sod1*, *catA* and *cat2* were significantly up regulated. Studies in *Aspergilli* have demonstrated that both, *atfA* and *atfB,* are essentially required to activate *catA* expression (Balázs et al., 2010; Hagiwara et al., 2016; Lara-Rojas et al., 2011). Herein, we observed higher expression levels of *catA* due to the over-expression of the *atfB* gene. In fact, in *A. oryzae*, *catA* expression was not observed in *atfA*-overexpressing strains (Sakamoto et al., 2009). By contrast, an over-expression of *atfB* induced a large up-regulation of *catA* (Sakamoto et al., 2008). Superoxide dismutase transform superoxide radicals into H_2O_2 while catalase convert H_2O_2 into H_2O molecules and oxygen within the antioxidant defense system (Weydert and Cullen, 2011). Since

genes belonging to catalase and superoxide dismutase families were strongly up regulated, we further investigated whether this modulation impacted fungal enzymatic activities. Actually, a significant increase in CAT activity was observed whereas no significant effect was detected for SOD. Previous reports have demonstrated that the use of several products capable to inhibit aflatoxin production in toxigenic *Aspergilli* also resulted in a modulation of the antioxidant enzymatic activities. However, it appears that the impact on this enzymatic defense may vary depending on the type of aflatoxin inhibitor. For instance, dithiothreitol, dimethyl sulfoxide as well as β-glucans from *Lentinula edodes* greatly inhibit AFB1 production with an increase in SOD enzymatic activity (Reverberi et al. 2005; Grintzalis et al. 2014). Conversely, ascorbic acid and cinnamaldehyde resulted in AFB1 modulation going with an increase in CAT activity (Grintzalis et al. 2014; Sun et al. 2015). Our results demonstrate that piperine enhances CAT enzymatic activity (at least via the over expression of *atfB*) as part of the mechanism of action occurring during AFB1 inhibition. In order to summarize the molecular events following exposure to piperine, a hypothetical molecular mode of action is proposed (Fig.6).

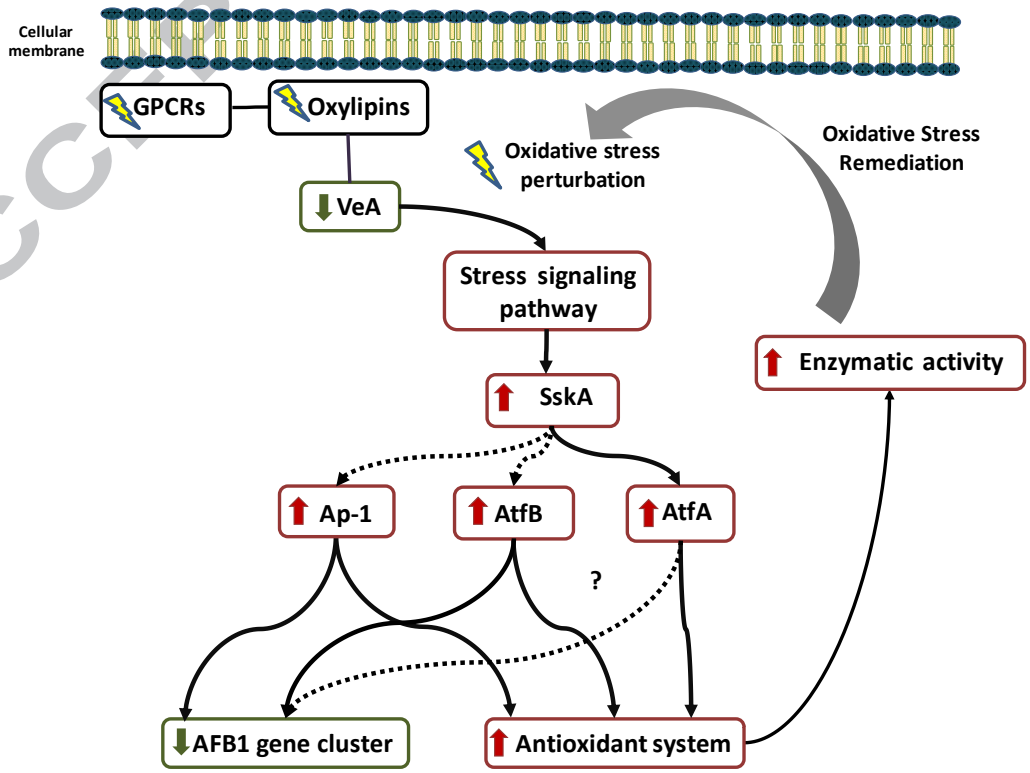

Fig. 6. Hypothetical mechanism of action of piperine. Piperine perturbs cellular signaling pathway by modulating GPCRs and oxylipins expression levels. Simultaneously, decreased levels of *veA* might make fungus less tolerant to oxidative stress response which could trigger an activation of several genes involved in the stress signaling pathway such as bZIP transcription factors *ap-1*, *atfA* and *atfB*. Final targets of these modulators correspond to fungal antioxidant system consisting in genes coding for catalases and superoxide dismutase defenses. Thus, the over expression of the antioxidant defense genes results in increased CAT enzymatic activity as a fungal strategy to remediates the imbalance stress levels. The down regulation of genes belonging to the AFB1 cluster may then be a final consequence of the repressive modulation caused by the over expression of bZIP transcription factors.

6. Conclusion

Taken together, our results demonstrate that (i) piperine inhibits almost all genes of the aflatoxin biosynthetic pathway leading to an inhibition of the mycotoxin biosynthesis, (ii) piperine's mechanism of action involves the inhibition of the global regulator *veA* together with enhanced levels of the stress response system including the over expression of bZIP transcription factors Ap-1, AtfA, AtfB and genes coding for antioxidant defense such as catalases and superoxide dismutases and iii) gene stress response was accompanied by an enhancement of catalase enzymatic activity. In conclusion, this strongly suggests that piperine inhibits AFB1 production by *A. flavus* via the perturbation of the oxidative stress balance.

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Highlights

- Piperine inhibits AFB1 production by transcriptional regulation of cluster genes.
- Piperine inhibition of AFB1 production goes with an enhancement of antioxidant status in *A. flavus*.
- Over-expression of bZIP-type factors are involved in AFB1 inhibition by piperine.