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ORIGINAL PAPER

The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*

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Abstract *Aspergillus flavus* is a ubiquitous saprophyte that is able to produce the most potent natural carcinogenic compound known as aflatoxin B_1 (AFB_1). This toxin frequently contaminates crops including corn, cotton, peanuts, and tree nuts causing substantial economic loss worldwide. Consequently, more than 100 countries have strict regulations limiting AFB_1 in foodstuffs and feedstuffs. Plants and microbes are able to produce volatile compounds that act as a defense mechanism against other organisms. *Pichia anomala* strain WRL-076 is a biocontrol yeast currently being tested to reduce AF contamination of tree nuts in California. We used the SPME-GC/MS analysis and identified the major volatile compound produced by this strain to be 2-phenylethanol (2-PE). It inhibited spore germination and AF production of *A. flavus*. Inhibition of AF formation by 2-PE was correlated with significant down regulation of clustering AF biosynthesis genes as evidenced by several to greater than 10,000-fold decrease in gene expression. In a time-course analysis we found that 2-PE also altered the expression patterns of chromatin modifying genes, *MYST1*, *MYST2*, *MYST3*, *gcn5*, *hdaA* and *rpdA*. The biocontrol capacity of *P. anomala* can be attributed to the production of 2-PE, which affects spore germination, growth, toxin production, and gene expression in *A. flavus*.

Keywords *Aspergillus flavus* · Aflatoxin · Biocontrol · Yeast · Volatile compound · Epigenic control

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Introduction

Aspergillus flavus is a saprotrophic and pathogenic fungus. Many isolates of *A. flavus* are able to produce the hepatocarcinogenic aflatoxin (AF) B_1, which is often detected in agricultural crops including corn, cotton, peanuts, and tree nuts, and also in many dried fruits and spices. AF contamination is a serious and recurrent problem and causes substantial economic losses worldwide (Molyneux et al. 2007; Trucksess and Scott 2008; Amaikel and Keller 2011; Roze et al. 2013). More than 100 countries have established specific regulation guidelines limiting the allowable amounts of AFB_1 in foodstuffs and feedstuffs (van Egmond et al. 2007). Major importers of U.S. agricultural commodities have imposed threshold levels for AFB_1 below 10 μg/kg and these restrictions have had a major negative impact on exportability of a number of crops.

The toxic effect of AF on animal and human health is referred to as aflatoxicosis. Although acute aflatoxicosis in humans is rare, several lethal outbreaks have been reported. In 2004, an outbreak in rural Kenya resulted in 317 cases and 125 deaths. Contaminated maize was the culprit, and officials found AFB_1 levels as high as 4.4 parts per million (ppm), 220 times the Kenyan regulatory threshold (Azziz-Baumgartner et al. 2005; Amaike and Keller 2011). Chronic toxicity and carcinogenic activity of AFB_1 is mainly due to a double bond present in the terminal furan ring of this compound. This double bond is commonly oxidized by hepatic P-450 enzymes into an epoxide that intercalates into DNA to cause mutations (Henry et al. 2002; Amaikel and Keller 2011). Genes directly involved in AF biosynthesis are clustered on a 70-kb genome region in *A. flavus* (Yu et al. 2004; Ehrlich et al. 2005; Cary et al. 2006). Regulation of expression of these clustering AF biosynthesis genes is mediated by a complex network of interactions involving global regulators and pathway-specific

transcription factors. Recent literatures indicate that epigenetic regulation via histone acetylation and deacetylation may also play a significant role in transcriptional activation of AF biosynthesis (Roze et al. 2007; Palmer and Keller 2010; Roze et al. 2013).

Asexual spores (conidia) produced by *A. flavus* are dispersed by wind and are easily found in soil and in the air. Germinated spores form hyphae and readily infect wounded plant tissues of corn, peanuts, cotton seeds, almonds, or pistachios, resulting in production of AF (Diener et al. 1987; Horn 2005). AF contamination is aggravated by factors such as insect damage, drought, and high temperatures when the plant defense systems are weakened by these biotic and environmental stresses. Multiple strategies are being developed to eliminate harmful AF in our food chain. These include prevention of fungal infection of crops by enhancing host resistance, use of atoxigenic biocompetitive fungal strains or biocontrol yeast, and preharvest intervention of toxin production by natural products. Volatile compounds such as aldehydes, acetate esters, and alcohols of plant and microbial origin have been investigated for their inhibitory effects on fungal growth and AF production (Wright et al. 2000; Roze et al. 2007; Cleveland et al. 2009; Roze et al. 2011). Soybean volatiles, (E)-2-hexenal and (E)-2-hexenal effectively inhibit growth of *A. flavus* as well as AFB_1 production (Cleveland et al. 2009). Mixture of willow bark volatiles inhibits AF production of *Aspergillus parasiticus* by more than 90 % (Roze et al. 2011).

Pichia anomala WRL-076 is a biocontrol yeast that is able to inhibit *A. flavus* growth and AF production (Hua et al. 1999, 2006, 2011; Hua 2006; Isakeit et al. 2007). The underlying mechanism for its biocontrol capacity was not well elucidated. The objective of this study was to determine whether WRL-076 produced volatiles that contributed to the observed inhibitory ability. We employed solid-phase microextraction-gas chromatographic-mass spectrometric (SPME-GC/MS) analysis and identified 2- phenylethanol (2-PE) to be the major volatile of this yeast. At high concentrations, 2-PE inhibited *A. flavus* spore germination and colony growth. At low concentrations, 2-PE did not impair hyphal membrane but inhibited expression of clustering genes necessary for AF biosynthesis. Also, 2-PE altered expression patterns of chromatin modifying genes.

Materials and methods

Yeast, fungal strains and media

Yeast strain, *Pichia anomala* WRL-076 and *Aspergillus flavus* strain NRRL 3357 were maintained on potato dextrose agar (PDA). Suspensions of yeast or fungal spores were prepared in 0.05 % Tween 80 solution and counted using a hemocytometer. The nutrient broth NYDB (nutrient broth, 8 g/l, yeast extract, 5 g/l, glucose, 10 g/l) was the medium used to generate volatiles by *P. anomala* and to investigate 2-PE inhibition on *A. flavus* spore germination and AF production. The liquid medium was filtered through a Millipore membrane (pore size 0.2 μm). Solid NYDB plates were used to examine *A. flavus* colony growth. For the AF biosynthetic gene expression study, fungal spores (10^5/ml) were inoculated into 20 ml of NYDB and grown at 28 °C on a rotary shaker at 150 rpm.

Table 1 Aflatoxin biosynthetic genes and functions[a]

Old name	New name	Enzyme/product	Function in the pathway
hexA	*aflA*	FAS alpha subunit	Acetate → polyketide
aflJ	*aflS*	Transcription enhancer	Pathway regulator
aflR	*aflR*	Transcription activator AflR	Pathway regulator
cypA	*aflU*	Cytochrome P450 monooxygenase	OMST–> → AFB1/AFG1, DHOMST → AFB2/AFG2
omtB	*aflO*	*O*-methyltransferase B	DHDMST (dihydrodemethylsterigmatocystin) → DHST (dihydrosterigmatocystin)
pksA	*aflC*	polyketide synthases (PKS)	Acetate → polyketide
norA	*alfE*	NOR reductase	norsolorinic acid (NOR) → averantin (AVN)
norB	*aflF*	NOR dehydrogenase	norsolorinic acid (NOR) → averantin (AVN)
omtA	*aflP*	*O*-methyltransferase A	sterigmatocystin (ST) → O-methylsterigmatocystin (OMST)
ordA	*aflQ*	Oxydoreductase	O-methylsterigmatocystin (OMST) → AFB1 and AFG1, dihydro-O-methylsterigmatocystin (DHOMST) → AFB2 and AFG2
ordB	*aflX*	Monooxygenase/oxidase	VA → DMST
vbs	*aflK*	Versicolorin B synthase	(versiconal) VAL → VERB (versicolorin B) closes the bisfuran ring of aflatoxin
estA	*aflJ*	Esterase	versicona hemiacetal acetate (VHA) → versiconal (VAL)
nor1	*aflD*	NOR reductase	norsolorinic acid (NOR) → averantin (AVN)

[a] Clustered pathway genes in aflatoxin biosynthesis (Yu et al. 2004; Ehrlich et al. 2005; Cary et al. 2006)

Volatile analysis

For volatile analysis yeast cells or spores of *A. flavus* were inoculated into 5 ml of NYDB in flasks screw capped with rubber septa, to a final concentration of 10^5 cell /ml and incubated at 28 °C on a rotary shaker at 150 rpm for 72 h. Headspace volatiles generated by the yeast cultures were adsorbed onto 100 μm solid-phase microextraction (SPME) polydimethylsiloxane fibers (Supelco, Bellefonte, PA) for 45 min. The SPME fiber was then removed and the adsorbed volatiles desorbed onto a J & W Scientific (Folsom, CA) DB-1 column (60 m × 0.32 mm i.d. × 0.25 μm) installed on a HP6890 gas chromatograph (GC) coupled to HP-5973 mass selective detector (MS; Palo Alto, CA). Desorbed volatiles were analyzed via the following method: injector temperature, 200 °C; splitless mode; inlet temperature, 200 °C; constant flow, 3.0 ml/min; oven settings, initial temperature, 40 °C; hold time, 0.0 min; ramp 1, 4 °C/min; final temperature, 250 °C; hold time, 5.0 min. MSD parameter: source temperature, 230 °C; MS source temperature, 150 °C; El mode, 70 eV; solvent delay, 1 min. NIST, Wiley, and internally generated databases were used for fragmentation pattern identification. Volatile peak identities were verified by injection of authentic samples and comparison of retention times and fragmentation patterns.

A calibration curve was generated (R^2=0.9973) from the headspace detection and peak surface area of 2-PE (Sigma-Aldrich). The compound was added to each 5 ml of NYDB in flask at 0.25, 0.5, 1 and 2 mg respectively and incubated at 28 °C on a rotary shaker at 150 rpm for 72 h. Headspace volatiles were analyzed as described above. Based on the calibration curve, the amount of 2-PE in yeast cultures was determined.

Effect of 2-PE on fungal growth

A. flavus spores were inoculated into 20 ml of NYDB to give a final concentration of 10^5 /ml. To NYDB, 2-PE was added to a final concentration of 0, 1, 2 or 3 μl/ml and cultures were grown at 28 °C on a rotary shaker at 150 rpm. Fungal growth was sampled daily and examined using a Zeiss Axioskop Microscope (Carl Zeiss, Thornwood, New York). The inhibitory activity of 2-PE on *A. flavus* colony growth was evaluated by placing membrane discs spotted with different amounts (1 μl or 2 μl each disc) of 2-PE, on solid agar plates pre-seeded with fungal spores.

Fluorescent imaging of fungal hyphae

$DiBAC_4(5)$ [Bis-(1,3-dibutylbarbituric) pentamethine oxonol] and CFDA-AM (5-carboxyfluorescein diacetate, acetoxymethyl ester) were chosen as the vital stains to create a two-colored scheme by employing both the red fluorescent cell membrane potential sensitive oxonol stain and the esterase dependent green fluorogenic probe. Fungal hyphal maintaining a normal potential gradient exhibited intense green fluorescence when visualized by epifluorescence microscopy (Hua et al. 2011). CFDA-AM and $DiBAC_4(5)$ were purchased from Ana Spec (San Jose, CA, USA). Stock solutions of these stains were prepared in DMSO: 2.5 mg/ml for CFDA-AM and 1 mg/ml for $DiBAC_4(5)$. Staining was performed by addition of 1:1 of each stain per 1 ml of sample for 30 min at room temperature in the dark. Samples were thereafter kept on ice until microscopic observation. The stained samples were viewed through a fluorescein filter (FTIC-Long Pass filter) in a Zeiss Axioskop epifluorescent microscope (Carl Zeiss, Thornwood, New York). Images were captured with a Sony AxioCam MR (Sony Electronics, Tokyo, Japan).

Table 2 Real-time RT-qPCR primers used in this study

Gene	F/R primer	Primers (5' to 3')
18s	F	TTCCTAGCGAGCCCAACCT
	R	GCCCGCCGAAGCAACT
aflR	F	GCCGCGCCCGAAA
	R	GCACTTTTGAGCTGGCACAA
cypA	F	TCGGGAAGCCAGGAGGTT
	R	CGTCACGGGCCTCGAGTA
estA	F	GGGCACTATCGCGCTATGA
	R	GCGGACGTGCCCATCA
hexA	F	CGTGAGGTCAAGGCATTCCT
	R	GACTTGGCCCCCCTTCTGT
aflJ	F	CCGAAGATTCCGCTTGGA
	R	TGAAGACATGCAGCAAAAGGA
omtB	F	TGCTGTGGCATCCATTCAAA
	R	GGACTGCGTCTTCCAAAAGG
nor1	F	ACTGCGACTCGGAAACTGATG
	R	TGCTCCTCCCGCAATGTC
norA	F	TCTAGCGCCGGTGTTCGT
	R	CATTGCCGAAGCTCATCGTT
norB	F	AAGATGCTGGGCACGTTTG
	R	CATGGGTGAGGACGAATTGG
omtA	F	TGTGTCGAGTGATGTGGGACTAG
	R	GCCACCCAGCTCAACCTACA
ordA	F	TTGCTGGGCTTGTGGATTC
	R	GAGGAGGACGCGTGTCTTTG
ordB	F	ACCGCGTTGCACATCGT
	R	TGGGTGTCCACAACCTTCGT
pksA	F	TCACAAGCGATGCACAGTTG
	R	AACTGACGAATGTGGGTCTTGTACT
vbs	F	GAGTCTACCGCCGCCGATA
	R	GAAAAGGTCGGCCAGTCATC

Table 3 Primers used in this study for RT-PCR

Gene	F/R primer	Primers (5' to 3')	Size (bp)	Accession number
β-*tubulin*	F	GTTGACCCAGCAGATGTTCGA	257	XM_002380380
	R	TGGAGGTGGAGTTTCCAATGA		
pksA	F	AGGGATCCCATCGACAAG	321	XM_002379910
	R	GGCTGGGATTTGAAGGAC		
aflR[a]	F	TGAGAACGATAAGGACGAC	556	XM_002379905
	R	CATCCTCAATCGAATCAAC		
aflJ	F	CTTCAACAACGACCCAAGGTT	376	XM_002379904
	R	AGATGAGATACACTGCCGCA		
omtB	F	ACAGACGATGTGGGCAAACG	440	XM_002379892
	R	ACGCAGTCCTTGTTAGAGGTG		
ver-1[a]	F	TTGTATCGTTCGGTCACC	388	XM_002379900
	R	GGTTCAAAGGAGAGAGCC		
vbs	F	AACGAGCAGCGTAAGGGTCT	629	XM_002379889
	R	TCAGCCAGAGCATACACAGTG		
gcn5[a]	F	GCGCGACAGCTTTGTTGTCC	247	XM_002383227
	R	CAAATGCGCACCATAACCCTTC		
hdaA[a]	F	TATCAGGATGGGAAATTC	282	XM_002373982
	R	CATAAGCATGTGTGTCATG		
rpdA[a]	F	ACGTTGGTAACTATGCCTAC	331	XM_002381015
	R	TTATTGCGATTAAGTCGG		
MYST 1[a]	F	TACCTCCTCACATTCACGGACC	333	XM_002373399
	R	CTTCTGCTGTGACGAATCCTCC		
MYST 2[a]	F	GGGCTCCGTTACATGCAGTTGC	356	XM_002374160
	R	TGGGCGTCGTTTGACAACAGG		
MYST 3[a]	F	GTCTAAGGCAGGCGGGAAAG	322	XM_002382120
	R	CGAGAACGAGGCAGGATAAGG		

[a] Primers are from Roze et al. (2011)

High performance liquid chromatography (HPLC) analysis of AFB_1

AFB_1 was extracted from the liquid fungal culture by chloroform and analyzed by high performance liquid chromatography (HPLC) on an Agilent model 1260 Infinity ChemStation (Agilent, Palo Alto, California, USA). HPLC was performed on a Supelcosil LC-C18 reversed-phase column (150 mm× 4.6 mm i.d., 5 μm particle size) at a flow rate of 1 ml per minute. The mobile phase was methanol/acetonitrile/H_2O

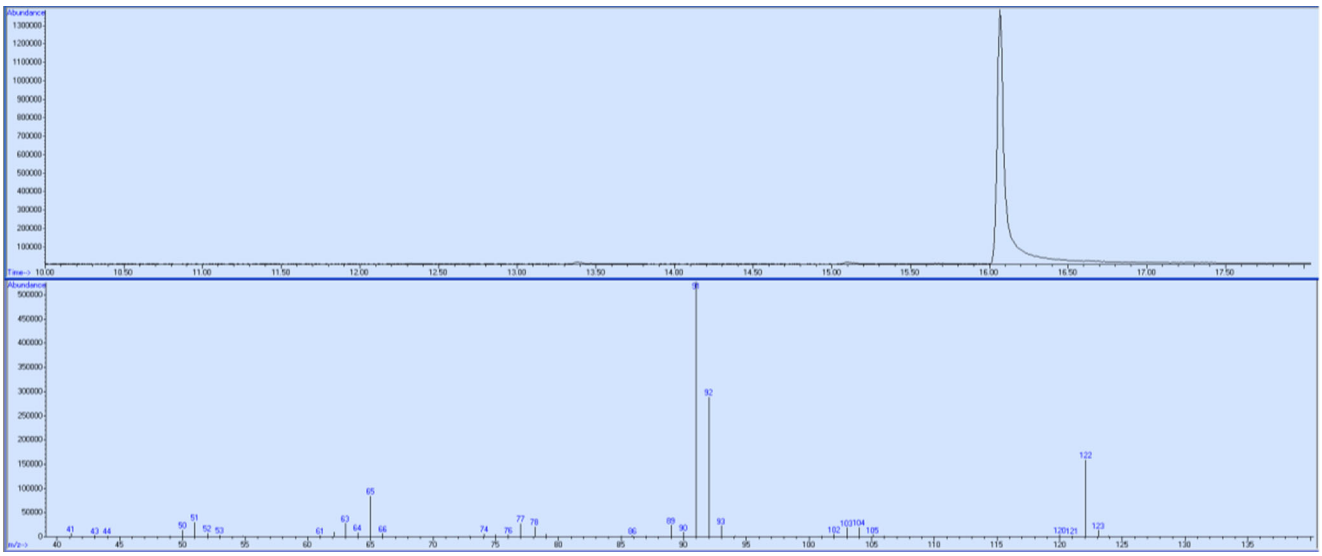

Fig. 1 GC/MS total ion chromatogram (TIC) detection of 2-phenylethanol in the headspace of the yeast *P. anomala* cultured on NYDB. Details of procedure were described in the method section. The only volatile detected had a retention time of 16.7 min. The corresponding fragmentation (electron impact) pattern of 2-phenylethanol is shown directly below the trace.

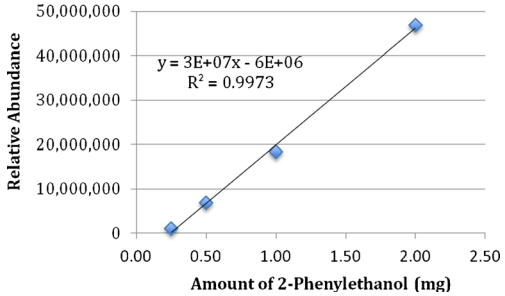

Fig. 2 Calibration graph to determine 2-PE concentration. The compound was added to 5 ml NYDB in flasks at 0.25, 0.5, 1 and 2 mg respectively in triplicates. Detail of the analysis procedure is described in the "Materials and methods" section

(20:20:60). AFB_1 were quantified by a fluorescent detector with excitation at 365 nm and emission at 455 nm.

Primer design

Gene sequences used for primer design were from NCBI, NRRL 3357 genome database for the 70 kb AF biosynthesis gene cluster. Primers were designed with ABI Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) and were ordered from Invitrogen. The AF genes and their functions are summarized in Table 1. Primers used in this study were listed in Tables 2 and 3.

RNA extraction and reverse transcription

Total RNA isolation was carried out by using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RNA samples were treated with Ambion TURBO DNA-free DNase (Ambion, Austin, TX, USA). Samples were stored in a −80 °C freezer. The purity and concentrations of RNA were examined by measuring the absorbance of samples at 260 nm and 280 nm using a ND-1000 Spectrophotometer (NanoDrop Tehnologies, Wilmington, DE, USA). A GeneAmp RNA PCR Core Kit (Applied Biosystem) was used for reverse transcription to obtain cDNA according to the manufacturer's procedure. For negative control, the same reactions were performed in the absence of the enzyme.

Real-time RT-PCR analysis of AF biosynthestic genes

Quantitative PCR reactions were carried out in an ABI 7300 Real Time PCR System. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), which increases florescence upon binding to double-stranded DNA product, was used as the amplification detector. Triplicates of each reaction were performed. The final primer concentration was 500 nM in the 25-μl reaction mixture. Final cDNA quantities in the reaction mixture were within the recommended 100 ng. PCR cycles were programmed as follows: 2 min at 50 °C for AmpErase UNG Activation, 10 min at 95 °C for AmpliTaq Gold DNA Polymerase Activation, followed by 40 cycles of 15 s at 95 °C and 1 minute at 60 °C for both primer annealing and product extension. Melting curves were performed using Dissociation Curves software (Applied Biosystems) to ensure only a single product was amplified.

Amplification of *A. flavus* 18S ribosomal RNA was used as the endogenous control (reference gene) due to its relatively stable expression level. Plates and quantification assay documents were created in SDS Software 1.3.1 (Applied Biosystems). The relative quantification of gene expression changes were computed by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

RT-PCR assessment of HAT and HDAC expression under the influence of 2-PE

Parallel RT-PCR assays were performed to assess changes in expression levels of AF biosynthesis genes and chromatin modifying genes. The primers used are listed in Table 3. PCR products were separated on a 1 % agarose gel and stained with GelRed. Gel images were viewed under UV light with Bio-Rad Universal Hood Gel Doc XR system. Band density was determined using the Bio-Rad Quantity One 4.6.3 software. The relative expression was calculated based on the

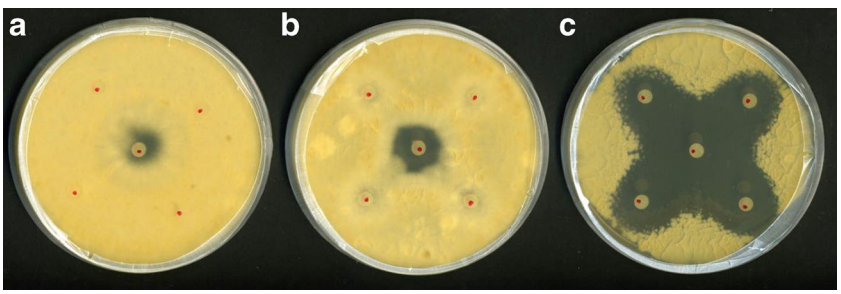

Fig. 3 Growth inhibition of *Aspergillus flavus* by 2-PE. Five membrane discs containing 2-PE (0, 1 or 2 μl/ml) were placed on agar plates pre-seeded with spores at 1×10^5/ml. **a** Control; **b** 1 μl/ml 2-PE; **c** 2 μl/ml 2-PE. Clear zone of inhibition was observed after 5 days of incubation when the concentration of 2-PE in membrane disc was 2 μl/ml

ratio of the band density of 2-PE treated samples divided by the controls.

Results and discussion

Identification of dominant volatile compound from yeast

The major volatile from *P. anomala* WRL-076 was identified by SPME-GC/MS analysis to be 2-PE (Fig. 1, *upper panel*). GC/MS total ion chromatogram (TIC) showing the detection of 2-PE corresponding fragmentation (electron impact) pattern of 2-PE shown directly below the trace panel. The chromatogram of the volatile shows a peak with the retention time of 16.07 min. A calibration curve (Fig. 2) was generated to determine the concentration of 2-PE produced by *P. anomala*. It was found to be 1.83 mg in 5 ml of liquid culture. No 2-PE was detected in cultures of *A. flavus NRRL3357*

Spore germination and growth of *A. flavus* in response to 2-PE

The inhibitory effect of 2-PE on *A. flavus* depended on the concentration of the compound applied. In liquid culture with shaking, spore germination was not observed in NYDB containing 3 μl/ml of 2-PE even after 7 days of incubation. When the concentration decreased to 2 μl/ml, germination was delayed for 24 h and limited hyphal elongation was observed after that. *A. flavus* grew well in the presence of 1 μl/ml of 2-PE. The inhibitory activity of 2-PE to *A. flavus* was most likely at the germination stage and is dosage dependent.

We tested the inhibitory effect of 2-PE on solid agar plate with five membrane discs on each Petri dish as shown in Fig. 3. A clear zone of inhibition was observed after 5 days of incubation when the concentration of 2-PE in the membrane disc was 2 μl/ml (Fig. 3c). However the clear zone was invisible when 1 μl/ml of 2-PE was applied Fig. 3b). We used Autoplate 4000 (Spiral Biotech, Boston, MA, USA) to spread the spores to the agar plates, the clear zone in the center of the agar plates of the control and the 1 μl/ml 2-PE was caused by the instrument.

Table 4 Fold of change in AF biosynthetic gene expression of *Aspergillus flavus* in response to 2-phenylethanol based on relative qRT-PCR

Genes	24 h	48 h	72 h
aflJ	−6.10±0.73	−1.38±0.08	1.71±0.57
aflR	−1.63±0.09	1.35±0.08	2.50±0.21
cypA	−15.38±6.79	−1.44±0.18	1.45±0.09
omtB	>−10,000	−4.98±0.66	−166.67±12.48
pksA	−11.49±3.79	−7.87±0.53	−3.73±0.31
hexA	−3.64±0.72	−1.90±0.12	−6.90±0.70
norA	−1,000.00±640.21	−3.02±0.46	>−10,000
norB	−10.20±8.60	−1.14±0.31	−3.13±0.74
omtA	−1,689.19±24.83	−8.13±2.32	−142.86±69.26
ordA	>−10,000	−6.62±0.96	−31.25±3.45
ordB	−142.86±78.26	−3.45±0.40	−111.11±43.99
vbs	>−10,000	−7.63±2.30	>−10,000
estA	−166.67±30.91	−2.73±0.04	−200.00±55.00
nor1	−18.87±3.16	−2.16±0.05	−10.10±1.55

Dual fluorescent stains indicating intact hyphal membrane

Live *A. flavus* hyphae grown in NYDB without 2-PE had green fluorescence when visualized by epifluorescence microscopy because of the presence of CFDA-AM (Fig. 4a). Heat inactivated hyphae showed only red fluorescence because the loss of membrane potential permitted $DiBAC_4(5)$ fluorescence (Fig. 4b). Hyphae of *A. flavus* grown in NYDB with 2-PE at the concentration of 1 μl/ml showed green fluorescent, suggesting that the hyphae were intact (Fig. 4c).

Inhibition of AF biosynthesis by 2-PE

AF production in liquid medium by *A. flavus* either untreated or treated with various amounts of 2-PE were determined. Compared with the control AFB_1 production was decreased by 30, 35, and 96 % at the 2-PE concentration of 0.2, 0.5, and

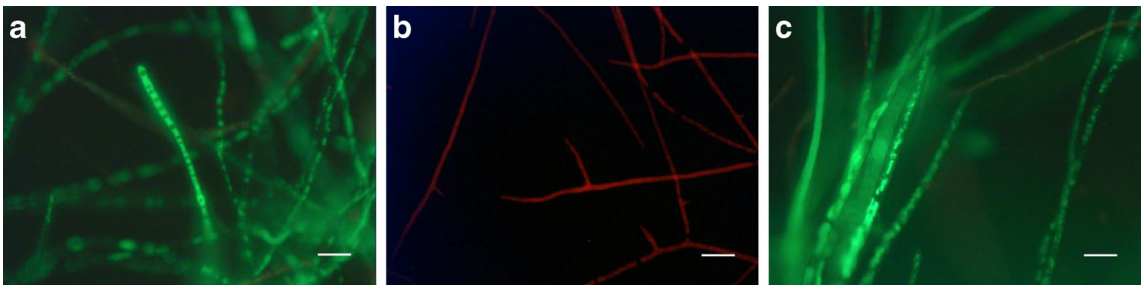

Fig. 4 Epifluorescence micrographs of fungal hyphae. Details of staining procedure using $DiBAC_4(5)$ and CFDA-AM were described in method section. **a** Live hyphae showed intense *green* fluorescence; **b** dead hyphae displayed *red* fluorescence; **c** hyphae treated with 2-PE (1 μl/ml). Magnification, 400×. *Bars* 20 μm

1.0 μl/ml respectively. At the concentration of 2 μl/ml of 2-PE, AFB_1 was not detected in fungal cultures.

Down-regulation of AF biosynthetic cluster gene

For a better understanding of the effect of volatiles on aflatoxin production, the expression of genes in the AF biosynthetic gene cluster was analyzed. Fourteen genes and their functions in AF biosynthesis were selected for this study and listed in Table 1. Genes that play an important role, such as *aflR* (a positive aflatoxin pathway regulator), *pksA* (polyketide synthase, an early gene of AF pathway), *nor1* (norsonorinic acid reductase, a middle gene in the AF pathway) and *omtB* (*o*–methyl-transferase B, a late gene in AF pathway), were evaluated by real-time qRT-PCR. The results are summarized in Table 4. All the genes were down-regulated by 2-PE in the range from several fold to more than 10,000 fold in experimental samples collected at 24, 48 and 72 h. Except for *aflR*, the AF pathway regulatory gene, at 48 and 72 h and *cypA*, which is exclusively involved in the G-type AF production. The results suggest that the 2-PE treatment reduced the transcript levels of all AFB_1 structural genes, which in turn resulted in reduction of AF biosynthesis.

Changes in expression of chromatin modifying genes are associated with down-regulation of AF biosynthesis genes

Epigenetic regulation is mediated by chromatin modifications. Genes encoding histone acetyltransferases (gcn5, MYST1, MYST2, and MYST3), and two histone deacetylases (hdaA and rpdA) have been found in the genome of *A. flavus* (Roze et al. 2011, 2013). Histone acetyltransferases decrease the bind of histones to DNA. This decrease causes chromatin to expand, allowing transcription to be initiated. Histone deacetylases counteract the activity of histone acetyltransferases. We found that, compared with the respective controls, the transcript abundance of *MYST1*, *MYST2*, *MYST3*, *gcn5*, *hdaA* and *rpdA* in 2-PE treated *A. flavus* changed differently (Fig. 5). The transcript levels of *MYST1* and *MYST3* increased slightly at 24 h but decreased gradually from 48 to 72 h. The *MYST2* transcript level decreased tenfold consistently at 24, 48 and 72 h. The *gcn5* transcript levels of increased nearly twofold at 24 h but decreased fivefold at 48 h. The *hadA* transcript level increased greater than twofold at 24 h but decreased thereafter. The *rpdA* transcript level was the same as that of the control but decreased from 48 to 72 h. In the parallel analyses, all six AF biosynthesis genes, *pksA*, *aflR*, *AflJ*, *omtB*, *ver1* and *vbs*, examined were down regulated by 2-PE treatment with the exception of *aflR* at 72 h. The data were consistent with the qRT-PCR results (Table 4). Volatiles from willow bark are able to inhibit AF production of *A. parasiticus*; the inhibition is associated with an increase in *gcn5* expression by threefold but also with a decrease in

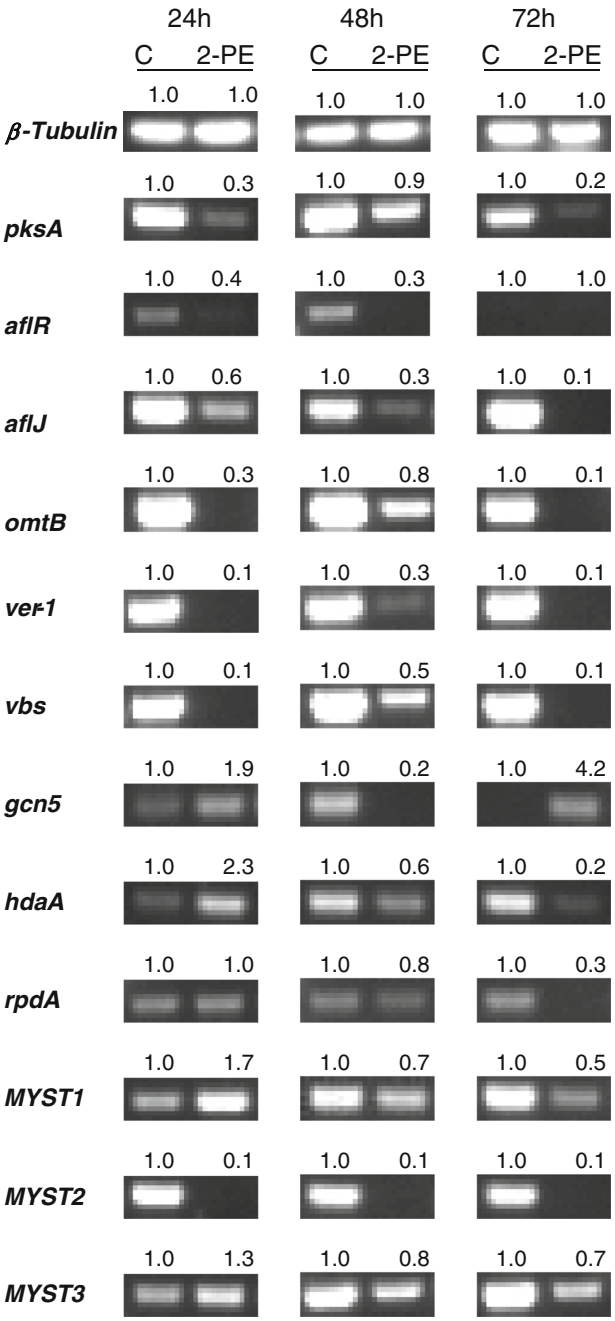

Fig. 5 RT-PCR assessment of HAT and HDAC transcript levels. Details of experimental sample collections, RNA extraction and RT-PCR were described in methods. Numbers above the band are the relative expression values (RE) in comparison with control without 2-PE treatment

MYST3 expression by twofold (Roze et al. 2011). In theory, decreasing acetyltransferase activity or increasing deactylase activity can down-regulate AF gene expression, but the final outcome may depend on the net effect of both types of activities. Future research is needed to determine whether 2-PE is indeed able to illicit epigenetic regulation of genes involved in AF biosynthesis. In conclusion, we have demonstrated that 2-PE is the major volatile produced by *P. anomala* WRL-076. It is likely that 2-PE, which can inhibit spore

germination, growth, and AF production of *A. flavus*, is the major attribute to the yeast's biocontrol capacity.

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Conflict of interest The authors declare that there are no conflicts of interest.

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