

Potential involvement of *Aspergillus flavus* laccases in peanut invasion at low water potential

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Aspergillus flavus accumulates carcinogenic aflatoxins in peanuts, mainly in immature kernels during drought. *Aspergillus flavus* invasion induces accumulation of phytoalexins, mostly stilbenoids in peanut, as a plant defence mechanism. Because fungal laccases are often related to pathogenicity and can degrade stilbenoids, this study reports for the first time the expression of *A. flavus* laccases in the presence of kernels, hulls and low water potential in relation to the accumulation of phytoalexins in peanut kernels. Packed-cell volume (PCV) of *A. flavus* biomass was significantly higher ($P \leq 0.01$) in the presence of mature kernels, dead kernels, and mature and immature peanut hulls than the control. The presence of kernels and hulls lowered the level of expression of three *A. flavus* laccases by 4–6-fold ($P < 0.01$), whereas 3% sucrose up-regulated them by 35–304-fold, and low water potential (-1.1 MPa) up-regulated them by 85–248-fold ($P < 0.01$). Phytoalexins that accumulated in peanut kernels in the presence of *A. flavus* and were quantified by HPLC-DAD-MS were primarily the stilbenoids: 3'-isopentadienyl-3,5,4'-trihydroxystilbene (IPD), chircanine-A, arachidin-2, arachidin-3 and arahypin-1. Apparent degradation of phytoalexins was observed when using a priori induction of phytoalexins in seeds in combination with a priori induction of laccases in *A. flavus*. The up-regulation of laccase expression observed at -1.1 MPa and at high sucrose concentration could be contributing to peanut invasion in immature kernels under drought conditions.

Keywords: aflatoxin, *Arachis hypogaea*, drought, groundnut, phytoalexins, stilbenoids

Introduction

Peanut plants have an indeterminate growth habit, which results in pods (shells with in-shell seeds) of several maturity stages present at harvest (Stalker & Wynne, 1983). For reasons not well understood, immature kernels during drought are more susceptible to accumulation of the carcinogenic aflatoxins resulting from infection by the fungal pathogens *Aspergillus flavus* and *Aspergillus parasiticus* (Blankenship *et al.*, 1984; Sanders *et al.*, 1985; Dorner *et al.*, 1989). Maturity stages in peanuts are classified by the colour of their mesocarp: yellow-1, yellow-2, orange, brown and black, where black is the most mature (Williams & Drexler, 1981; Fig. 1). Contamination of peanuts by maturity groups and number of days of drought is shown as a contour representation by levels of aflatoxin in Figure 1, from numerical values published by Dorner *et al.* (1989). During droughts, it is common to find up to $1000 \mu\text{g kg}^{-1}$ of aflatoxin in peanuts (N'Dede, 2009), whereas the limit for these mycotoxins established by the Food and Drug Administration for edible peanuts in the USA is $20 \mu\text{g kg}^{-1}$ (FDA, 2012).

Plants respond to pathogen invasion and abiotic stress by producing chemical compounds called phytoalexins

(Hammerschmidt, 1999; Ahuja *et al.*, 2012). In peanut plants, phytoalexins are mostly stilbenoids (Sobolev *et al.*, 1995, 2006, 2009, 2010), many of them exhibiting antifungal activity (Sobolev *et al.*, 2011). Accumulation of phytoalexins by various peanut cultivars has shown direct correlation with disease resistance (Sobolev *et al.*, 2007); however, these compounds are present, both in mature and immature kernels, only at high water potentials (Wotton & Strange, 1987; Dorner *et al.*, 1989). Thus, the prevalence of aflatoxin in immature kernels during prolonged drought cannot be solely explained by phytoalexin accumulation (Dorner *et al.*, 1989).

To counter the effect of plant phytoalexins, pathogens can produce enzymes that detoxify these compounds (Pedras *et al.*, 2011; Rodriguez-Bonilla *et al.*, 2011). One group of these enzymes are laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2). These are multi-copper oxidases that reduce oxygen to water while oxidizing phenolic compounds (Thurston, 1994). Additional substrates of laccases include lignins and tannins, although laccases also participate in fungal morphogenesis and pigmentation (Baldrian, 2006; Desai & Nityanand, 2011). Fungal pathogens can use laccase enzymes to degrade stilbenoids, and that enzymatic activity has been directly correlated with pathogenicity in plants (Chen *et al.*, 2009) and in humans (Zhu & Williamson, 2004).

Significant progress has been made in understanding the genetics of *A. flavus*, from which the complete genome has been sequenced (Payne *et al.*, 2006). Also, great

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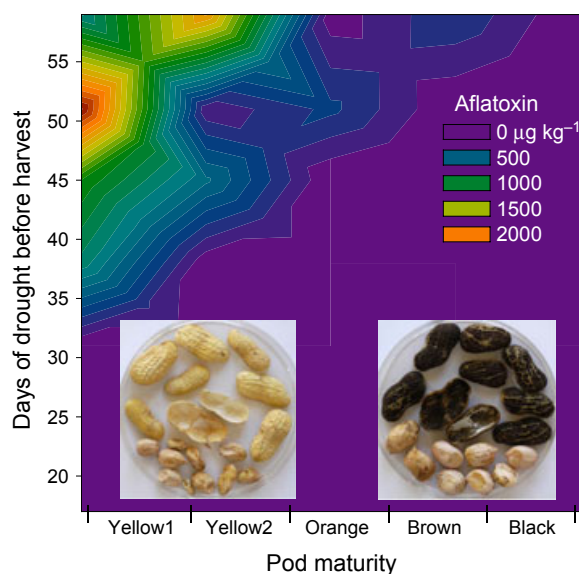


Figure 1 Aflatoxin content in peanut from soil at 29°C, influence of maturity stage of seeds and length of terminal drought (contour representation of numerical data published by Dorner *et al.*, 1989); examples of blasted peanut pods exhibiting yellow or black mesocarp.

progress has been achieved in characterizing peanut phytoalexins (Sobolev *et al.*, 2006, 2009, 2010, 2011). However, there is no information about the possible role of *A. flavus* laccases in phytoalexin detoxification, pathogenicity, or about the genetic regulation of these enzymes during peanut infection. The objectives of this work were to analyse the impact that kernels, hulls (shells without pericarp and without seeds), and low water potential have on the regulation of *A. flavus* laccases, and to analyse the possible role of these enzymes in facilitating peanut invasion, while monitoring the production of peanut phytoalexins.

Materials and methods

Aspergillus flavus strain and culture medium

The aflatoxigenic fungal pathogen *A. flavus* (NRRL 3357), from which the genome has been sequenced, was kindly provided by Dr Bruce Horn (National Peanut Research Laboratory, Dawson, GA, USA). Spores of NRRL 3357 were used to inoculate 50 mL Czapek-Dox medium (Oxoid), referred to hereafter as liquid medium, and grown for 3 days at 28°C. The culture was transferred to 400 mL fresh liquid medium, divided into two 500 mL Erlenmeyer flasks, and incubated overnight at 28°C. The 450 mL of culture were transferred to a 3 L Fernbach flask containing 2 L of liquid medium, and then 35 mL of the culture suspension were dispensed into 125 mL Erlenmeyer flasks.

Plant material

Pods of *Arachis hypogaea* cv. Georgia06 harvested in 2011 were used for the experiments. Exocarps were removed from the pods using a water-impact blaster to determine their maturity stage (Williams & Monroe, 1986). Only kernels from black-mesocarp

Pods (Fig. 1) were used. In a laminar flow, kernels were surface sterilized as follows: (i) 1 min 70% ethanol, rinsed with sterile water, (ii) 5 min incubation in 2.5% hypochlorite and two rinses with sterile deionized water (repeated once), and (iii) allowed to imbibe submerged in sterile water for 2 h. Each kernel was then cut into quarters under sterile conditions, and kept in sterile water until their distribution (<1 h) in the previously prepared 125 mL Erlenmeyer flasks.

Experimental design

In each experiment, 12 quarters of surface-sterilized kernels randomly picked were added to each 125 mL Erlenmeyer flask (12 pieces/flask) containing 35 mL liquid medium, with or without *A. flavus* culture, and incubated at $28 \pm 1^\circ\text{C}$ with agitation at 110 rpm. Various sets of experiments were prepared and each treatment repeated over time with three biological replicates/treatment/sampling date. In experiments (a) the treatments were: mycelium only, mycelium + kernels, kernels alone, and mycelium + dead kernels. 'Dead kernels' refer to those placed in a forced-air oven at 65°C for 4 days. In experiments (b) the treatments were mycelium, mycelium + 20% polyethylene glycol 3350 MW (PEG; PEG 3350, Sigma), and mycelium + kernels + 20% PEG. Water potential of the PEG solution was calculated according to the formula $y = 10.29 \ln(x) + 19.05$ (Coleman *et al.*, 1989), where y is the molarity of PEG, and x is the water potential in MPa; thus, 20% PEG corresponds to -1.1 MPa, which corresponds to a water activity (a_w) 0.982 (Markarian & Schlenoff, 2010). Water activity was also measured throughout the experiments using an AquaLab Series 3 (Decagon Devices, Inc.), although no changes were detected. Because PEG is considered a matricum and not an osmoticum (Steuter *et al.*, 1981; Coleman *et al.*, 1989) and water stress has broader use in agriculture as MPa, these units are used in the present work.

In experiments (c) 1 g of surface-sterilized yellow (immature) or black (mature) hulls were added to each flask with liquid medium and mycelium prepared as in previous experiments. The surface sterilization consisted of 70% ethanol for 5 min, followed by 30 min UV light (253.7 nm, 100 μW) on the concave side and 30 min on the convex side of the hulls in a laminar flow. To sample *A. flavus* from the hull-containing liquid medium, two sterile forceps were used to remove the mycelium that coated both sides of the hulls. Other samples were directly poured into volumetric centrifuge tubes, centrifuged at 7000 g for 5 min, and the mycelium volume quantified. Samples from experiments (a) to (c) were collected at different times between 0 and 67 h of incubation, counted from the time each experiment was set up.

A priori induction

An experiment was conducted by separately inducing phytoalexin production in kernels, and inducing laccase expression in *A. flavus*, and then incubating kernels and *A. flavus* mycelium together. Induction of phytoalexin in kernels was done as follows: kernels were surface sterilized, imbibed and cut as indicated above. Then, 100 mL of a 3-day-old culture of *A. flavus* in liquid medium was heated to 100°C for 10 min to kill the mycelium. A sterile 5 mL aqueous suspension of 0.5 g low-molecular weight chitosan (Cat. 448869, Aldrich) and 0.5 g food-quality pectin were added to the medium containing dead mycelium. Surface-sterilized cut kernels were added to the mixture and incubated for 48 h at 27°C with agitation at 110 rpm to induce phytoalexin accumulation. Fungal laccases were

induced by replacing the liquid medium of a 3-day-old *A. flavus* culture with liquid medium containing 20% PEG, and incubating the culture for 24 h at $27 \pm 1^\circ\text{C}$ with agitation at 110 rpm. The 48-h induced kernel pieces and the 24-h induced *A. flavus* culture were used to set up an experiment with two replicates per treatment. Treatments were: kernels + liquid medium, kernels + liquid medium + 20% PEG, and kernels + liquid medium + 20% PEG + mycelium. The cultures were incubated at $27 \pm 1^\circ\text{C}$ with agitation at 110 rpm. Samples were collected at 0, 22 and 46 h incubation.

Sampling

Three flasks per treatment were randomly collected per sampling date. Kernels were placed into 50 mL Falcon tubes, frozen in liquid nitrogen and stored at -80°C for later processing. The liquid cultures containing mycelium were poured into 50 mL Falcon tubes, centrifuged at 4°C and 5200 g for 5 min, and the supernatant was saved in some cases for further analysis. The volume of mycelium in millilitres was measured using volumetric centrifuge tubes. Part of the mycelium samples (c. 0.3 mL) were directly placed into 2 mL tubes containing ceramic beads (for RNA extraction), immediately frozen in liquid nitrogen and stored at -80°C for later processing. Mycelium biomass was calculated as percentage of packed-cell volume (PCV) based on the total volume of the liquid culture (volume of mycelium in mL $\times 35 \text{ mL}^{-1} \times 100$). PCV (%) data were converted to arcsin (\sqrt{x}) for normalization before statistical analysis. Three biological replicates per treatment and per sampling date were used, and the experiments were repeated. In the experiments with a priori induction, two biological replicates were used. The converted values were subjected to analysis of variance (ANOVA), and mean comparison by Tukey's test using SIGMAPLOT v. 11 (Systat Software).

Primer design

DNA sequences of *A. flavus* genes annotated as putative laccases were downloaded from the *Aspergillus* Comparative Sequencing Project, Broad Institute of Harvard and MIT (www.broadinstitute.org). Three putative laccases, AFL2G_01113 (587 aa), AFL2G_10583 (586 aa) and AFL2G_09420 (598 aa) (hereafter referred as Lac 1, Lac 2 and Lac 3) were aligned with their corresponding transcripts using CLONE MANAGER PROFESSIONAL v. 9 (SciEd), and primers were designed for real-time PCR by spanning introns within each gene. Primer efficiency was tested using

twofold dilutions of an *A. flavus* cDNA generated from peanut-exposed mycelium. Translated transcripts of the three laccases were analysed with MOTIF (www.genome.jp/tools/motif).

Primers previously reported for the 18S rRNA gene (HQ026744, GenBank; Walsh *et al.*, 2011) were used as control. Primer efficiency was calculated using REST 2009 v. 2.0.13 (QIAGEN; Pfaffl *et al.*, 2002). Primers used in this work, and their corresponding efficiencies, are listed in Table 1.

Analysis of *A. flavus* mycelium: RNA extractions, cDNA synthesis and real-time or quantitative qPCR

Each experiment was performed using three biological replicates per treatment per sampling date (e.g. for three treatments and four sampling dates, 36 individual flasks were sampled and analysed), and each experiment was repeated. Each biological sample was analysed by RT-PCR in triplicate for each of the genes (e.g. one biological sample and three genes, required nine RT-PCR reactions, plus another three reactions of the housekeeping gene). Frozen mycelium samples were pulverized with 0.28 mm ceramic beads in 2 mL tubes using an Omni Bead Ruptor 24 (Omni International). RNA was extracted from mycelia using the FastRNA Green Kit (MP Biomedicals) and quantified using NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesized in all the treatments of all the experiments using 690 ng RNA of each biological replicate with a combination of random hexamers and oligo-dT; the SuperScript III First Strand Synthesis Super Mix (Invitrogen) was applied according to manufacturer's instructions. Real-time qPCR was performed on ABI 7500 real-time PCR, using RT² SYBR Green ROX qPCR Mastermix (QIAGEN). The qPCR was done in 25 μL reactions with 0.5 μM of each primer and 12.5 μL qPCR Mastermix. Conditions for amplification were: 2 min at 50°C , 10 min at 95°C , followed by 45 cycles of 15 s at 95°C , 1 min at 58°C , and a dissociation curve analysis of 15 s at 95°C , 1 min at 58°C , 15 s at 95°C , and 15 s at 60°C . Each biological sample was analysed for three laccase genes and for a housekeeping gene (18S rRNA) using three biological samples per treatment and three technical replicates per biological sample. Expression levels were normalized to the levels of 18S rRNA expression for each sample (Schmittgen & Zakrajsek, 2000). The expression of each tested gene in the treated samples relative to that of untreated samples was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Data were represented as relative fold change (\log_2) normalized to the level of expression at time zero, before treatment. Levels of gene expression for the first

Table 1 Primers used in RT-PCR analysis of *Aspergillus flavus* laccases

Gene	Accession ^a	Primer	Primer sequence (5'→3')	Position (nt) ^b	Amplicon (bp)	T _m °C	e
Lac 1	AFL2G_01113	Lac1-01113-Fw	GTTTCGAGGCGACCGTTCATT	222	194	63	99.6
		Lac1-01113-Rv	TGGCACCATACAGTCCATCC	465c		63	
Lac 2	AFL2G_10583	Lac2-10583-Fw	GCCAACGACCTGGTTCCTAT	892	204	63	99.0
		Lac2-10583-Rv	CGATGGAGAAGACCGTAGT	1148c		63	
Lac 3	AFL2G_09420	Lac3-09420-Fw	TCGAGTTGCGACAAGGAGAC	191	100	63	99.5
		Lac3-09420-Rv	GGAGTGCCATGTTGTTTCGAT	341c		63	
18S rRNA	HQ026744 (GenBank)	A. flav-Fw ^c	CGAGTGTAGGGTTCCTAGCGA	59	88	65	97.3
		A. flav-Rev	CCGCGCGCCATGAAT	133c		61	

nt: nucleotide; bp: base pairs; e: efficiency calculated using REST 2009 v. 2.0.13 (Pfaffl *et al.*, 2002).

^aAccession numbers are according to www.broadinstitute.org except for 18S rDNA.

^bThe letter c after nucleotide position indicates complementary strand.

^cPrimers previously reported (Walsh *et al.*, 2011).

data point were compared to the samples collected at time 0 h of starting the experiments, and for the following data points, each treatment was compared to the control treatment of the same sampling time. ANOVA and mean comparisons by Tukey's test, or pairwise multiple comparisons among all treatments were calculated by Holm-Sidak, for the $\log_2(x)$ transformation of the levels of expression, using SIGMAPLOT v. 11 (Systat Software).

Extraction and analysis of phytoalexins from peanut kernels

Kernel pieces from each biological sample were freeze-dried in a Labconco Freezone 6 (Labconco Corp.) before processing. For analytical purposes, 0.17 to 1.43 g of each biological replicate were extracted with a triple amount of methanol (w/v) for 24 h at 23–24°C. Filtered aliquots (9 μ L) of the extracts were used for direct determination of target compounds by HPLC. All manipulations were carried out under minimal lighting conditions to avoid any possible photoisomerization of the stilbenoid phytoalexins.

Reference compounds

Pure peanut stilbenoids (*trans*-arachidin-2, *trans*-arachidin-3, *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene, chiricanine A and arahypin-1) were obtained as previously described (Sobolev *et al.*, 1995, 2009). The identities of the reference compounds were confirmed by APCI-MS/MS (MS^2) and UV spectroscopy. These data are given in parentheses as $[M + H]^+$ values followed by UV absorption maxima (in HPLC mobile phase): *trans*-resveratrol (m/z 229; 305 and 317 nm), *trans*-arachidin-2 (m/z 297; 308 and 322 nm), *trans*-arachidin-3 (m/z 297; 335 nm), *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene (m/z 295; 295 nm), chiricanine A (m/z 281; 312 nm) and arahypin-1 (m/z 281; 327 nm). The above results were in agreement with published data (Keen & Ingham, 1976; Aguamah *et al.*, 1981; Cooksey *et al.*, 1988; Sobolev *et al.*, 1995, 2009; Ioset *et al.*, 2001).

HPLC-DAD-MS analysis

For tandem HPLC-MS analysis, a Surveyor HPLC system equipped with MS Pump Plus, Autosampler Plus, a PDA Plus

Detector (Thermo Electron Corp.) and a 75×4.6 mm i.d., 2.5μ m, XTerra MS C18 analytical column (Waters) was used. The column was maintained at 40°C. H₂O (A), methanol (B), and 2% HCOOH in H₂O (C) were used in the following gradient: initial conditions, 60% A/39% B/1% C, changed linearly to 11% A/88% B/1% C in 11 min, changed linearly to 0% A/99% B/1% C in 0.01 min, held isocratic for 2 min, changed to initial conditions in 0.01 min. The flow rate was 0.8 mL min⁻¹. APCI-MSⁿ data were obtained on a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an APCI interface and operated with XCALIBUR v. 1.4 software (Thermo Electron Corp.). All data were acquired in the full-scan positive polarity mode from m/z 100 to 2000. Capillary temperature was 175°C, APCI vaporizer temperature 400°C, sheath gas flow 60 units, auxiliary/sweep gas flow 10 units, source voltage 4.5 kV, and source current 5 μ A. In MS² analyses, the $[M + H]^+$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analyses, the isolation width, relative fragmentation energy, relative activation Q , and activation time were m/z 1–6, 35%, 0.25 and 30 ms, respectively. Concentrations of compounds of interest were determined by reference to peak areas of corresponding pure compounds. Concentrations of phytoalexins from three biological replicates, expressed in μ g mL⁻¹ were analysed by ANOVA and mean comparisons by Tukey's test, or pairwise multiple comparisons by Holm-Sidak using SIGMAPLOT v. 11 (Systat Software).

Results

In the presence of peanut kernels, *A. flavus* biomass measured as packed-cell volume (PCV) of mycelium, was significantly higher than in the liquid medium (Czapek-Dox) without kernels ($P \leq 0.01$). Using initial mycelium inoculums of 1 and 2% PCV, the PCV in the presence of kernels was double that of the control after 21 h incubation, and reached 44–51% PCV at 67 h, compared to 16–20% in the control, respectively (data from one experiment shown in Figure 2a). Initial inoculums of 2% PCV in Czapek-Dox medium alone, resulted in down-regulation of 18S rRNA expression (unexpected higher number of cycles for $C_t = 1$) after 40 h incubation, thus

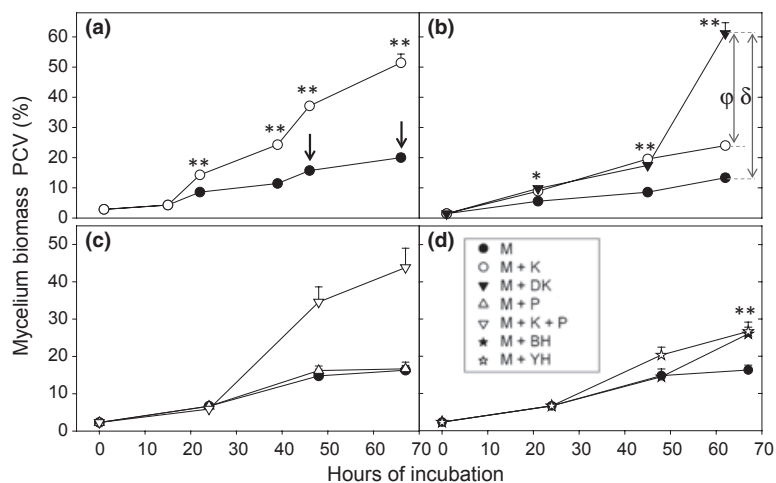


Figure 2 *Aspergillus flavus* biomass from various experiments. M: mycelium; K: kernels; DK: dead kernels; P: 20% polyethylene glycol (3350 MW); BH: black hulls; YH: yellow hulls. * $P < 0.05$; ** $P < 0.01$. ↓: biomass limit in Czapek-Dox medium at which the 18S rRNA gene was down-regulated. Error bars are the mean standard errors of three biological replicates.

lower initial inoculums were used in subsequent experiments. Down-regulation of 18S rRNA commonly occurs in fungi under stress or when there is lack of nutrients (French *et al.*, 2003; Dasgupta *et al.*, 2007). In the presence of 'dead kernels', PCV (δ) at 62 h was 61%, 4.5-fold higher than the control (13.3%), and significantly higher than in the presence of live kernels (24.0%; $P \leq 0.01$; Fig. 2b). The additional biomass δ can be attributed to nutrients released by kernels, and the difference with live kernels ϕ suggests it is due to the effect of phytoalexins and perhaps some other defence mechanism from the kernels (Fig. 2b). The ϕ effect reached 39–60% biomass reduction at 62–67 h incubation. *Aspergillus flavus* biomass in the presence of 20% PEG (–1.1 MPa) was not different from the control, and no detrimental effect was observed by the presence of 20% PEG when kernels were added (Fig. 2c). After 67 h incubation, significantly higher ($P \leq 0.01$) *A. flavus* biomass was observed in the presence of black (26% PCV) and yellow (27% PCV) hulls compared to the control (16% PCV; Fig. 2d). The weight of immature yellow hulls is only 57% the weight of mature black ones.

Laccases

Some properties of the three *A. flavus* laccases chosen for this work, Lac 1, Lac 2 and Lac 3, are shown in Table 2. In all three laccases, the software MOTIF detected the Pfam of Cu-oxidases 2 and 3 with independent E-values in the range of 9.5×10^{-21} and 4.5×10^{-36} . In Lac 1 and Lac 3, a prosite pattern of multicopper oxidase and a ProDom of oxidoreductase laccase-precursor-signal conidiation oxidase I metal-binding urishiol repeat were found. These patterns were not found in Lac 2. Sequence alignments of these proteins showing copper-binding sites are in Figure S1.

Levels of mRNA expression of Lac 1, Lac 2 and Lac 3 monitored by qPCR for *A. flavus* grown on liquid medium, and sampled between 0 and 62 h incubation are shown in Figure S2. A rapid and significant ($P \leq 0.01$) increase in the level of expression (300-, 100- and 36-fold, for Lac 1, Lac 2 and Lac 3, respectively) was observed within the first hour of incubation, apparently due to the 3% sucrose in the medium, and was followed by a steady decrease in expression, mainly for Lac 2 (Fig. S2).

Table 2 Some properties of the laccases used in this work

	Accession	MW	pI	% match DNA	% match AA	Most abundant AA (%)
Lac 2	AFL2T_10583	64611	4.61	39	27	Ser (10.4); Thr (9.0)
Lac 1	AFL2T_01113	65418	5.62	64	58	Leu (8.5)
Lac 3	AFL2T_09420	66138	5.53	67	60	Leu (8.7)

MW: molecular weight; pI: isoelectric point; AA: amino acid.

Presence of live or dead kernels in the medium, sampled between 2 and 66 h, showed an initial increase in mRNA expression of the three laccases. A 10–18-fold increase was observed at 2 h for mycelium alone on liquid medium. In the presence of live kernels, the increased expression was only two- to threefold at 2 h, and in the presence of dead kernels all three laccases were down-regulated (0.46–0.96-fold; Fig. 3). In general, reduced expression of laccases was observed in the presence of kernels, and even higher reduction in the presence of dead kernels during the first hours of incubation, e.g. expression of Lac 2 and Lac 3 at 2 h incubation were significantly lower for M + DK than for M + K ($P \leq 0.01$; Fig. 3).

In the presence of black or yellow hulls (M + BH, M + YH) at 24 h incubation, the expression level of the three *A. flavus* laccases was significantly lower ($P \leq 0.05$) compared to the control at time 0 (before incubation). At 24 h, expression of the three laccases in the presence of black or yellow hulls was about one-sixth to one half (0.16–0.48) of the expression in the control at time 0. Although in general peanut hulls down-regulated *A. fla-*

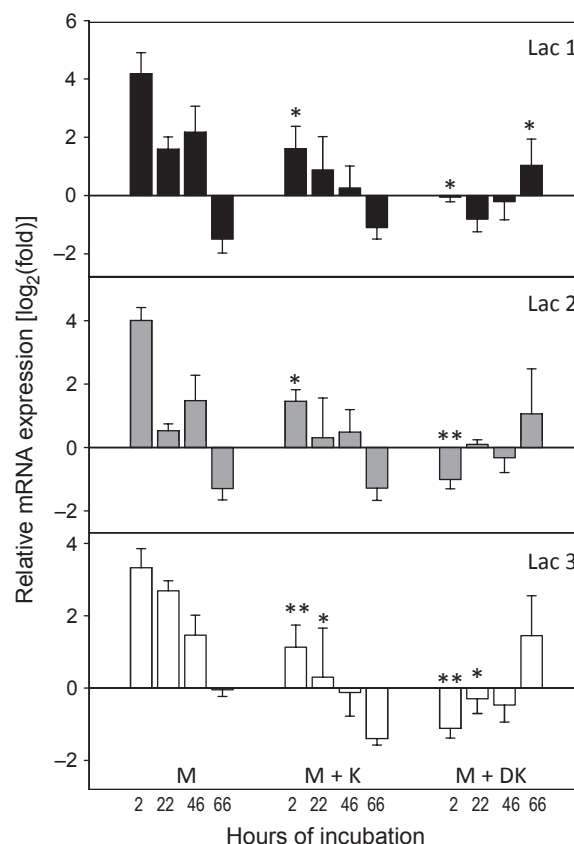


Figure 3 Relative mRNA expression of three laccases of *Aspergillus flavus* quantified by RT-PCR in the presence of live kernels (K), dead kernels (DK), and on mycelium (M) only. * $P < 0.05$; ** $P < 0.01$, compared to M at each sampling time. Error bars are the mean standard errors of three biological replicates.

flavus laccases, highly variable results were observed after 24 h, and this will need further investigation (Fig. 4). At 24 h, the laccases in the control (M) were up-regulated (3.8-, 6.15- and 5-fold for Lac 1, Lac 2 and Lac 3, respectively), followed by the typical steady down-regulation over time (Fig. 4). Water stress of -1.1 MPa ($0.982 a_w$) generated using PEG 3350 (Hutton *et al.*, 1996) in liquid medium resulted in a significant ($P \leq 0.001$) up-regulation of the three laccases. At 24 h, mRNA expression for M + P was up by 157-, 248- and 85-fold, for Lac 1, Lac 2 and Lac 3, respectively, whereas for M + P + K, it was 139-, 241- and 57-fold for Lac 1, Lac 2 and Lac 3, respectively. In the presence or absence of kernels, the up-

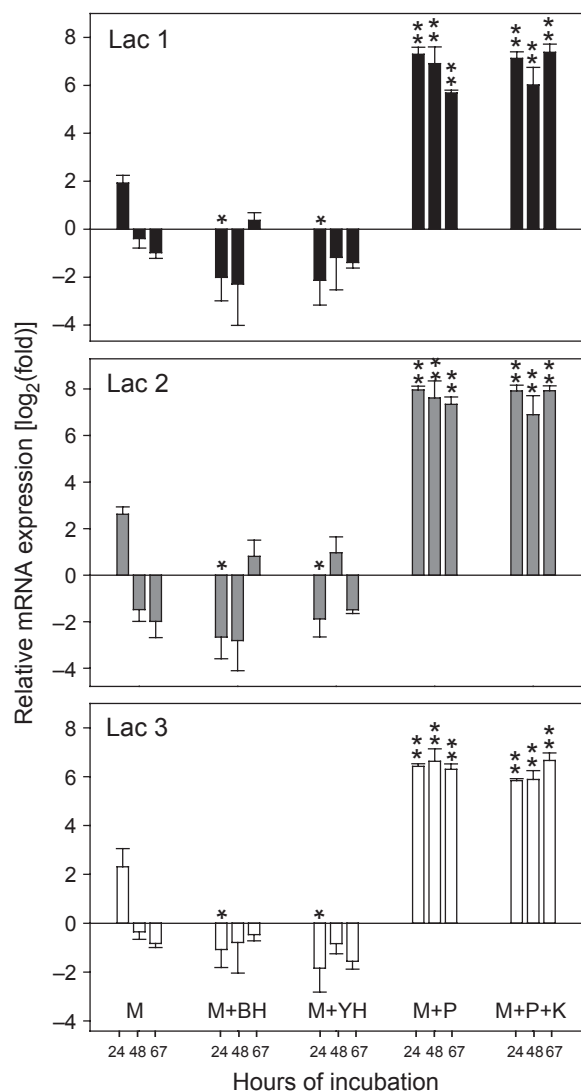


Figure 4 Relative mRNA expression of three laccases of *Aspergillus flavus* quantified by RT-PCR on mycelium (M), in the presence of: black hulls (BH), yellow hulls (YH), 20% polyethylene glycol (P) and live kernels (K). * $P < 0.05$; ** $P < 0.01$, compared to M at each sampling time. Error bars are the mean standard errors of three biological replicates.

regulation stayed at high levels over the 67 h incubation (Fig. 4).

Phytoalexins

No phytoalexins were detected in peanut kernels immediately after being cut in quarters, or after 1 h incubation in any of the treatments (Figs 5a,b & S3f). Kernels incubated in liquid medium without mycelium, accumulated the stilbenoid IPD, and very low amounts of arachidin-2 ($1.6\text{--}4.1 \mu\text{g g}^{-1}$) and arachidin-3 ($1.2\text{--}4.7 \mu\text{g g}^{-1}$; Fig. 5a). IPD reached $64 \mu\text{g g}^{-1}$ dry weight in kernels after 67 h incubation (Fig. 5a). Live kernels incubated in liquid medium in the presence of *A. flavus* accumulated increasing amounts of stilbenoid phytoalexins (at 67 h): IPD ($62 \mu\text{g g}^{-1}$), chiricanine-A ($54 \mu\text{g g}^{-1}$), arachidin-2 ($41 \mu\text{g g}^{-1}$), arachidin-3 ($19 \mu\text{g g}^{-1}$) and arahypin-1 ($7 \mu\text{g g}^{-1}$; Figs 5b & S3e). Chiricanine-A and arahypin-1 were not detected at any given time in kernels not exposed to mycelium. IPD, arachidin-2 and arachidin-3 were the first ones detected in all the samples after 15 h incubation; however, arachidin-2 and arachidin-3 were in general at least 85% higher in kernels exposed to *A. flavus* mycelium. Chiricanine-A, arachidin-2, arachidin-3 and arahypin-1 showed slower rates of increase after 45 h incubation (Fig. 5b). The heat-treated kernels were kept in liquid medium for several days after

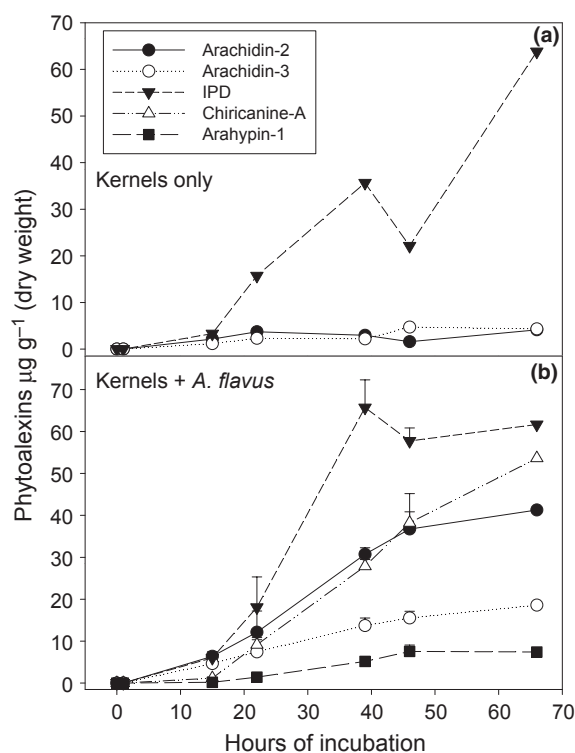


Figure 5 Phytoalexin quantification by HPLC-DAD-MS in peanut kernels after cultivation in liquid Czapek-Dox medium in the absence (a) or presence (b) of *Aspergillus flavus*. Error bars are the mean standard errors of three biological replicates.

the experiments ended, and in only one instance out of dozens of kernels, two embryos had survived the heat treatment, resulting in detectable IPD ($60.7 \pm 7.2 \mu\text{g g}^{-1}$) and minimal amounts of arachidin-3 ($3.6 \pm 0.4 \mu\text{g g}^{-1}$) at 67 h.

Phytoalexins and PEG

Kernels incubated at -1.1 MPa in the presence of *A. flavus* showed significant amounts of chiricanine-A ($20.0 \mu\text{g g}^{-1}$) and arachidin-3 ($22.8 \mu\text{g g}^{-1}$) at 48 h incubation, whereas at 67 h only significant amounts of chiricanine-A ($37.2 \mu\text{g g}^{-1}$) were detected. At 67 h, IPD and arachidin-3 were below the detection level, and arachidin-2 and arahypin-1 were at 3.64 and $1.96 \mu\text{g g}^{-1}$, respectively. The possibility that phytoalexins may have been released to the medium was analysed in a few samples of medium after 67 h of mycelium, PEG and kernels. The result was additional presence of phytoalexins in the medium as follows: resveratrol ($5.58 \mu\text{g g}^{-1}$), arachidin-2 ($9.18 \mu\text{g g}^{-1}$), arachidin-3 ($2.95 \mu\text{g g}^{-1}$), IPD ($0.99 \mu\text{g g}^{-1}$), chiricanine-A ($6.47 \mu\text{g g}^{-1}$) and arahypin-1 ($7.82 \mu\text{g g}^{-1}$).

Phytoalexins and laccases, a priori induction

A priori incubation of *A. flavus* mycelium at -1.1 MPa highly induced ($P < 0.001$) mRNA expression of the three laccases (119-, 168- and 90-fold, for Lac 1, Lac 2 and Lac 3, respectively), shown as 0 h in Figure 6. A further increase in mRNA expression was observed after adding a

priori induced kernels to the medium (146-, 403- and 93-fold, for Lac 1, Lac 2 and Lac 3, respectively) as shown at 22 h, and was significantly higher for Lac 2 (from 168- to 403-fold; $P < 0.01$). At 46 h incubation, laccases were still up-regulated but at a much lower level (2-, 2.5- and 5-fold, for Lac 1, Lac 2 and Lac 3, respectively; Fig. 6).

Kernels induced for 48 h to produce phytoalexins were then placed at -1.1 MPa in the presence of induced mycelium, and samples were collected at 0, 22 and 46 h incubation (Fig. 6).

A priori incubation of peanut kernels in the presence of chitosan, pectin and dead mycelium resulted in: chiricanine-A ($32.5 \mu\text{g g}^{-1}$), arachidin-2 ($22.2 \mu\text{g g}^{-1}$), IPD ($4.5 \mu\text{g g}^{-1}$), arachidin-3 ($2.5 \mu\text{g g}^{-1}$) and arahypin-1 ($0.95 \mu\text{g g}^{-1}$; Figs 6 & S3a). After removing the inducing factors, the concentration of four phytoalexins significantly increased: chiricanine-A, IPD, arachidin-3 and arahypin-1; and the concentration of one of them significantly decreased: arachidin-2 ($P < 0.01$). Phytoalexin concentrations at 46 h after removing the induction factors were: arachidin-2 ($2.4 \mu\text{g g}^{-1}$), arachidin-3 ($19 \mu\text{g g}^{-1}$), arahypin-1 ($16 \mu\text{g g}^{-1}$), IPD ($37 \mu\text{g g}^{-1}$) and chiricanine-A ($57 \mu\text{g g}^{-1}$) in kernels (Figs 6 & S3b). When a priori induced kernels were placed at -1.1 MPa , both in the presence or absence of induced mycelium, their phytoalexin concentration decreased (Figs 6 & S3c,d). A comparison of induced kernels placed at -1.1 MPa in the presence or absence of induced mycelium showed no significant difference in accumulation of arachidin-2 or arahypin-1. However, in the same samples, significantly

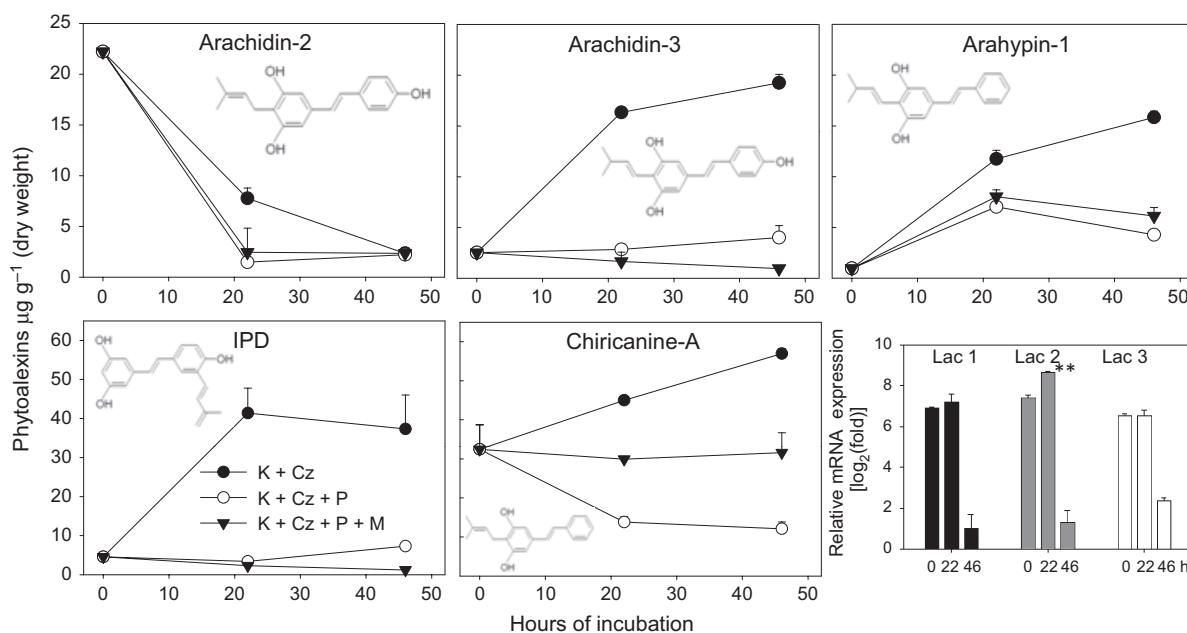


Figure 6 Phytoalexin quantification by HPLC-DAD-MS in peanut kernels after 48 h of artificial induction followed by incubation in liquid Czapek-Dox medium; presence of 20% polyethyleneglycol (P); and presence of P plus *Aspergillus flavus* mycelium (P + M). K: kernels; Cz: Czapek-Dox medium; M: mycelium. In the same experiment: relative mRNA expression of three laccases of *Aspergillus flavus* quantified by RT-PCR on laccase-induced mycelium in the presence of P and phytoalexin-induced kernels. ** $P < 0.01$, compared to the initial level of expression on the induced mycelium. Error bars are the mean standard errors of three biological replicates.

lower concentration of arachidin-3 ($0.96 \mu\text{g g}^{-1}$) was observed in the presence of mycelium compared to the absence of mycelium ($4.0 \mu\text{g g}^{-1}$; $P \leq 0.05$). Also, a significantly lower accumulation of IPD ($1.2 \mu\text{g g}^{-1}$) occurred in the presence of mycelium compared to -1.1 MPa without mycelium ($7.3 \mu\text{g g}^{-1}$; $P \leq 0.01$); and a significantly higher accumulation of chiricanine-A ($31.6 \mu\text{g g}^{-1}$) in the presence of mycelium compared to -1.1 MPa in the absence of mycelium ($12.1 \mu\text{g g}^{-1}$; Fig. 6). The low level of phytoalexins in kernels incubated at -1.1 MPa with or without mycelium indicated that either phytoalexins were not being produced at low water potential, were being released to the medium or were being metabolized in the presence of mycelium. Thus, the supernatants of the cultures were examined, and adding the amount of phytoalexins in supernatant and in kernels (7.9 g, 12 kernel pieces/flask), the total amounts were only 6, 23, and 32% for IPD, arachidin-3, and arachidin-3, respectively (Figs 6 & S3d). This indicates that in the presence of induced mycelium, these compounds may have been degraded. Figure S3 shows examples of phytoalexin accumulation in peanut kernels in the presence of *A. flavus* mycelium at 0 h (Fig. S3f) and at 67 h incubation (Fig. S3e).

Discussion

A flow diagram with the interpretation of the possible role of *Aspergillus flavus* laccases in pathogenicity, and a tentative explanation for the aflatoxin accumulation in immature peanuts, is shown in Figure 7. At high water

potential, low expression of laccases meets with high accumulation of phytoalexins in mature and immature peanut pods, resulting in no infection or aflatoxin accumulation. At low water potential, high laccase expression meets with low phytoalexin accumulation in peanut pods, but in mature pods infection is prevented by their chemical composition. However, in immature pods the low tannins/phenols/lignin are not enough to act as a barrier for infection, and additionally, the high sugar content of immature kernels increases aflatoxin production by *A. flavus* (Figs 1 & 7).

Aspergillus flavus biomass increased in the presence of peanut hulls and kernels. On average, after 65 h incubation *A. flavus* biomass increased 1.6-fold in the presence of hulls, 2.5-fold in the presence of live kernels, and 4.8-fold in the presence of dead kernels, compared to medium-only control. Such increases in biomass would be expected given that kernels are rich in nutrients: 35–37% protein content, 31–44% oil, 1.0–1.2% cellulose (Ozcan & Seven, 2003); as are peanut hulls: 6–8% protein, 29% lignin, 37% cellulose, 2.5% carbohydrates, 1.3% fat (Kerr *et al.*, 1986; Hegazy *et al.*, 1991). However, the biomass of *A. flavus* grown in the presence of live kernels was 39–60% lower (φ) than the biomass in the presence of dead kernels (δ ; Fig. 2b); assuming that both released equal nutrients to the medium, the difference φ can be mainly attributed to the presence of phytoalexins accumulated in live kernels. As φ was only detectable after 45 h incubation, it is possible that the toxic threshold of one or more phytoalexins must have been reached between 45 and 65 h (Fig. 5b). Conse-

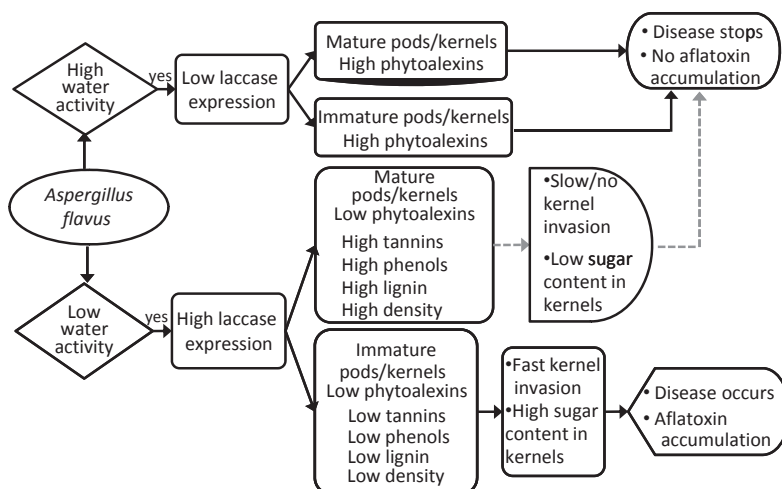


Figure 7 Potential role of *Aspergillus flavus* laccases in peanut invasion. At high water activity both mature and immature pods and kernels accumulate phytoalexins, and both kernels and hulls down-regulate *A. flavus* laccases, so the disease stops. Low water activity up-regulates *A. flavus* laccases. Mature and immature kernels cease accumulating phytoalexins at a_w 0.95, whereas *A. flavus* can still grow at a_w 0.78. Mature hulls are high in tannins, phenols and lignin, and have twice the density of immature hulls, and seed skins of mature kernels are high in tannins; all of this can slow or prevent disease even at low water activity. Also, if *A. flavus* accesses mature kernels, their low sugar content does not promote aflatoxin synthesis. In contrast, immature pods at low water activity accumulate less or no phytoalexins, and they also have less tannins, phenols and lignin. As all these compounds can be degraded by laccases, and immature hulls have half the density of mature ones, they could be more easily penetrated by the fungal pathogen. In addition, if *A. flavus* accesses the immature kernels, their high sugar content would stimulate aflatoxin production.

quently, as IPD was present at higher concentrations at 40 h than at 65 h, this might not be the main toxic compound. Then, the possible toxic threshold levels could have been $\geq 38 \mu\text{g g}^{-1}$ arachidin-2, $\geq 16 \mu\text{g g}^{-1}$ arachidin-3, $\geq 38 \mu\text{g g}^{-1}$ chiricanine-A, and/or $\geq 8 \mu\text{g g}^{-1}$ arahypin-1 (Fig. 5b). Two of these compounds, chiricanine-A and arahypin-1, have activity against fungal pathogens comparable to the fungicide captan (Sobolev *et al.*, 2011), so it would be important to study the potential role of these two phytoalexins in peanut. In doing so, the possibility of these two phytoalexins not being the products of synthesis by the plant, but products of detoxification by the pathogen should also be considered (Pedras *et al.*, 2011).

At high water potential, *A. flavus* grew abundantly on peanut hulls and kernels (Fig. 2a,b,d). At the same time, the presence of hulls and kernels in the medium resulted in overall down-regulation of the fungal laccases, four- to sixfold lower than the control on the same sampling date (Figs 3 & 4). At high water potential, mature and immature peanut kernels are known to accumulate phytoalexins (Wotton & Strange, 1987; Dörner *et al.*, 1989). Thus, at high water potential, phytoalexins could protect peanuts from invasion, and fungal laccases, even if they were involved in pathogenicity, would be down-regulated in the presence of hulls and kernels, reducing the risk of *A. flavus* invasion. At low water potential, -1.1 MPa , *A. flavus* biomass in the presence or absence of kernels (Fig. 2c) followed the same trend as at high water potential (Fig. 2a,b), but expression of laccases was highly up-regulated (85- to 248-fold), and in this case, the presence of kernels did not reduce the levels of expression (up-regulation by 57- to 241-fold in the presence of kernels; Fig. 4). Laccases are one of the major lignin-degrading enzymes (Baldrian, 2006; Pant & Adholeya, 2007), and can degrade a variety of phenolic compounds (Thurston, 1994) including stilbenoid phytoalexins (Chen *et al.*, 2009) and tannins (Desai & Nityanand, 2011). In peanut hulls, tannins account for 0.4% dry weight (Sanders, 1977), and most phenolic antioxidants in peanut skin are condensed tannins which can reach up to 25% dry weight (Van Ha *et al.*, 2007). Indeed, the mesocarp colour of yellow to black at different maturity stages (Williams & Drexler, 1981) is a result of increased content of tannins and phenolic compounds with maturity (Yen *et al.*, 1993). Previous work has shown that peanut cultivars with higher drought tolerance and lower aflatoxin contamination, had the ability to maintain high water potential and hence higher phytoalexin accumulation (Sobolev *et al.*, 2007; Girdthai *et al.*, 2010). The higher vulnerability of immature peanut pods to fungal invasion could be related to the low density of immature hulls; in the variety Georgia06, yellow hulls weighed 57% the weight of black hulls.

Aspergillus flavus laccases had a 36- to 300-fold increase in mRNA expression within 1 h of being placed in liquid medium containing 3% sucrose (Fig. S1). As sucrose content in peanut kernels can reach 3.6 to 7.8% (Uppala, 2011), then a similar up-regulation of laccase

expression could occur in nature. Sucrose content in immature kernels is higher than in mature ones (Manda *et al.*, 2004; Uppala, 2011), e.g. 2.4% sucrose in young kernels and 1.9% in mature kernels under the same field conditions (Uppala, 2011). Also, the presence of aflatoxin is positively correlated with sugars at harvest, with glucose/fructose/sucrose correlating with aflatoxin G2, fructose correlating with aflatoxin G1 and sucrose correlating with B1 (Manda *et al.*, 2004). In addition, in pure culture, *A. flavus* production of aflatoxin increases with increasing sucrose concentrations from 0 to 4% (Manda *et al.*, 2004). Thus, higher sucrose in immature peanut kernels could up-regulate laccase expression and also increase aflatoxin upon *A. flavus* invasion. Because sugar accumulation is one mechanism of drought resistance in plants, its effect needs to be considered when breeding peanuts for drought tolerance.

In general, the three laccases studied here had similar trends, although Lac 2 showed a higher elasticity in mRNA expression than Lac 1 and Lac 3, e.g. at 62 h incubation in liquid medium when presumably all the nutrients have been consumed, the level of expression of Lac 2 was the most down-regulated (Fig. S2). Also, when *A. flavus* mycelium was transferred to -1.1 MPa liquid medium, Lac 2 showed a higher increase in expression (248-fold), compared to Lac 1 and Lac 3 (157- and 85-fold, respectively; Fig. 4). And when laccase-induced mycelium was placed in contact with phytoalexin-induced kernels, Lac 2 showed a significant ($P < 0.01$) increase in mRNA expression at 22 h, going from 168- to 403-fold (Fig. 6). Because serine/threonine-rich proteins are involved, among many other functions, in nutritionally regulated signal transduction in fungi (Wei & Zheng, 2011), it would be interesting to investigate if the apparently higher elasticity of Lac 2 is related to its Ser/Thr-rich N-terminal (Table 2).

Using a simplistic model of liquid cultures, this study was able to analyse the regulation of *A. flavus* laccases in the presence of peanut kernels and hulls while measuring fungal biomass as PCV, and phytoalexins in kernels. Although the use of a chemical to simulate water stress is not ideal, other groups have shown that PEG 4000 was not toxic in fungal cultures even at high concentrations, not metabolized as carbon source, and its level of absorption by mycelium was less than 5% (Mexal & Reid, 1973). In the experiments here, regulation of water potential using PEG provided important information about the possible role of laccases in peanut invasion under drought conditions. Levels of chiricanine-A, arachidin-3, arahypin-1 and IPD continued increasing after removal of inducers, whereas arachidin-2 showed a fast decline. It was surprising that some of these phytoalexins were found in the liquid medium despite their low solubility. For example, resveratrol has a partition ratio of 1.35 (log P) and a solubility in water of $3 \text{ mg } 100 \text{ mL}^{-1}$ (Sobolev *et al.*, 2011); the phytoalexins reported here are more lipophilic than resveratrol and have partition ratios between 2.56 and 3.71 (Sobolev *et al.*, 2011). This study showed that total IPD, arahypin-1 and arachidin-3 in

supernatant plus kernels exposed to *A. flavus* was much less than in the control without *A. flavus*, indicating that these phytoalexins are potentially being degraded by *A. flavus* laccases. These results are taken with caution; however, they can be used to generate new hypotheses. Plant pathogens can degrade plant defence compounds and use them to suppress additional plant defences, e.g. saponin detoxification by *Septoria licopersici* suppresses induced defence response from the plant by interfering with signal transduction required for disease resistance (Bouarab *et al.*, 2002). Also in *Fusarium oxysporum* the α -tomatine detoxification suppresses the induced defence response of the plant host (Ito *et al.*, 2004). The observations seen here in a priori induction of phytoalexins in kernels and of laccases in *A. flavus* indicate that either degradation of IPD and arachidin-3 occurred, or that their accumulation ceased by presence of the laccase-induced mycelium. Apparently, arachidin-2 only accumulates in the presence of a biotic/abiotic stress, whereas arachidin-3, arachidin-1, IPD and chircanin-A showed a significant increase during the 48 h after removing the induction agents. The understanding of the mechanisms of detoxification of phytoalexins could lead to the design of effective inhibitors of phytoalexin detoxification (Pedras *et al.*, 2004), as was shown against the brassinin detoxification by *Leptosphaeria maculans* in crucifers (Pedras & Jha, 2006).

Acknowledgements

This work was supported by USDA-ARS project numbers 6604-42000-008-00D and 6604-21000-003-00D. The authors thank Dr Bruce Horn for providing the strain *Aspergillus flavus* NRRL 3357. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Protein sequence alignment of three *Aspergillus flavus* laccases.

Figure S2. Relative mRNA expression of three *Aspergillus flavus* laccases evaluated by RT-PCR in mycelium grown on fresh Czapek-Dox medium and sampled at various incubation times.

Figure S3. Examples of phytoalexin quantifications by HPLC-DAD-MS during this study.