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Influence of sub-lethal antioxidant doses, water potential and temperature on growth, sclerotia, aflatoxins and *aflD* (=*nor-1*) expression by *Aspergillus flavus* RCP08108

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ABSTRACT

Effects of interacting conditions of sub-lethal levels of antioxidants, water potential (Ψ) and temperature were evaluated on growth, sclerotial characteristics, aflatoxin B_1 (AFB_1) production and *aflD* (=*nor-1*) gene expression by *Aspergillus flavus* strain RCP08108. These studies were carried out on peanut meal extract agar osmotically modified to −2.8,−7.1, −9.9 and −16.0 MPa and incubated at 28 and 20 °C. The food grade antioxidants added were butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) at (1 + 1 mM – M1) and (5 + 5 mM – M2). To relate the *aflD* expression after toxigenic *A. flavus* grew under interacting stress conditions, real-time PCR was used. Antioxidant mixtures caused a higher and significant ($p < 0.001$) reduction in growth rate. The major impact on size and volume sclerotia was produced by Ψ; followed by antioxidant mixtures. High AFB_1 levels were observed in response to the M1 applied at −7.1 MPa. Induction of the *aflD* gene was observed in response to the M1 treatment at −2.8, −7.1 and −9.9 MPa; but significant decreases of AFB_1 production and *aflD* transcripts were observed; when the fungus grew in the presence of the M2 treatment.

These results showed that it is necessary to apply food-grade antioxidants into the peanut storage system at levels higher than 5 mM. This is an important tool to avoid sub-lethal antioxidant doses that can lead to fungal growth, increase resistance structures, and stimulate *aflD* gene expression and AFB_1 accumulation in this substrate.

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1. Introduction

Among the most common toxigenic fungi affecting the food chain are *Aspergillus* spp. These fungi infect commodities such as corn, peanut, cotton, tree nuts, sorghum, and other oil seeds. In particular, *Aspergillus flavus* is of great importance due to its impact on agriculture and human health. During the life cycle of *A. flavus* in peanut, the pathogen can infect oil seeds before and after harvest (Barros et al. 2003; Passone et al. 2008a). Preharvest peanut seeds contain mycelia and spores of aflatoxigenic fungi which cause important economic losses resulting in serious damage and aflatoxin accumulation when they are stored (Passone et al. 2010). Aflatoxins are the most toxic and carcinogenic compounds among toxins. In particular aflatoxin B_1 (AFB_1), the most potent natural hepatocarcinogen that has been characterized to date, possesses the greatest risk in food for human and animal consumption (IARC 1993; Urrego Novoa and Díaz 2006). Consequently, the European Union and Food and Drug Administration U.S. instituted legislation to protect the health of consumers and set limits for total aflatoxins and AFB_1 in peanuts (European Commission (EC) Commission Regulation, No 165/2010; FDA U.S. Regulations, CPG Sec. 570.375/10).

Fungal growth and survival are markedly affected by environmental factors, especially water availability and temperature, which are limiting factors in the functioning of ecosystems (Giorni et al. 2009). If these fungi grow in the storage ecosystem under the optimum conditions for water potential (Ψ) and temperature, the life cycle may be completed with sclerotia formation (Gqaleni et al. 1996). *Aspergillus* sclerotia are resistance structures that serve primarily to produce conidia (Wicklow and Donahue 1984; Wicklow

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and Wilson 1986). Consequently, *Aspergillus* species invasion and concomitant production of aflatoxin may occur. In previous studies it was demonstrated that in the peanut storage agroecosystem, there were toxigenic *Aspergillus* section *Flavi* strains that produced sclerotia (Passone et al. 2008a).

Previous studies have identified that the effect of antioxidant treatments on germination, growth and AFB_1 accumulation are all influenced by water availability (Nesci et al. 2003; Passone et al. 2005). However, no study has tried to link this data at a fundamental level to expression of early biosynthetic genes (e.g. *aflD* = *nor-1* gene). The structural gene *aflD* encodes a 31 KDa NADPH-dependent ketoreductase which is involved in the conversion of norsolorinic acid (NA) to averantin (AVN) (Zhou and Linz 1999). Early aflatoxin pathway intermediates including NA do not possess a bisfuran ring with a double bond; the presence of this double bond is strongly associated with DNA adduct formation, mutation, and cancer (Mori et al. 1985).

A previous report has demonstrated a direct relationship between aflatoxin content and *aflD* transcripts measured by real-time PCR (Scherm et al. 2005). However, there are no studies which have examined the *aflD* gene expression in relation to eco-physiological factors and food-grade antioxidants. Previous studies showed that the antioxidant mixture BHA–BHT (10 + 10 mM) totally inhibited growth and aflatoxin production by *A. flavus* and *Aspergillus parasiticus in vitro* on irradiated and natural peanut grains, regardless of Ψ assayed (Passone et al. 2007, 2008c). However, at *in situ* level, this mixture only was effective up to ≤ -24.0 MPa (=0.84 a_W) owing to the fact that stimulation of aflatoxin production was observed in peanuts stored with −17.6 MPa initial Ψ (=0.88 a_W) (Passone et al. 2009).

The quantification of *aflD* transcripts by real-time PCR permits a sensitive and specific approach to evaluate the effects of sub-lethal antioxidant treatments on aflatoxin biosynthesis. This allows a much more sensitive method for examining the influence of eco-physiological conditions than conventional analyses of phenotypic mycotoxin production.

The objectives of this study were to evaluate the effects of interacting conditions of sub-lethal antioxidant mixtures, Ψ and temperature on growth, sclerotial characteristics, AFB_1 production and *aflD* gene expression.

2. Materials and methods

2.1. Fungal isolate

A. flavus isolate RCP08108 was used. This strain was originally isolated from stored peanut in Córdoba, Argentina, in the August–December, 2007 period (Passone et al. 2008a), and it was previously demonstrated to produce aflatoxin in peanut meal extract agar (PMEA; 0.99 a_W; 71.6 ng g^{-1} AFB_1). Sclerotial production with a mean sclerotium diameter of 1122.1 ± 82.6 μm was observed on Czapek Dox medium according to the methodology of Horn et al. (1996), thus *A. flavus* RCP08108 was classified as an L strain. This isolate was deposited in the Microbiology and Immunology Department of the National University of Río Cuarto culture collection. The fungus was maintained on slants of malt extract agar (MEA) at 4 °C and stored as spore suspensions in 15% glycerol at −80 °C.

2.2. Growth medium and water activity modification

The medium used in this study was 3% PMEA, made by boiling 30 g of dried peanut meal in 1 L water for 60 min and filtering the resultant mixture through a double layer of muslin. The volume was made up to 1 L with distilled water (Passone et al. 2005). This medium was specifically used since the *A. flavus* isolated proceeded from stored peanuts.

The Ψ was modified osmotically with the nonionic solute glycerol to −2.8, −7.1, −9.9 and −16.0 MPa of Ψ = water activities (a_W) of 0.98, 0.95, 0.93 and 0.89, respectively. The water activity of all media was determined with an AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.).

2.3. Antioxidants

The antioxidant chemicals used were 2(3)-*tert*-butyl-4 hydroxyanisole (BHA) and 2,6-di (*tert*-butyl)-*p*-cresol (BHT) (Eastman Chemical Company, Kingsport, USA). BHA and BHT were dissolved in ethyl alcohol-distilled water (95:5, v/v) and the appropriate volume of each antioxidant was added in combination to PMEA to produce a mixture with a final concentration of 1 and 5 mM. In the control cultures only ethyl alcohol-distilled water (95:5, v/v) was added to the medium.

2.4. Inoculation, incubation, and growth assessment

The fungus was grown on PMEA for 5 days at 28 °C to obtain heavily sporulating cultures. PMEA plates at different a_W conditions were amended with the appropriate concentration of each antioxidant and sterile cellophane overlays (P400, Cannings Ltd, Bristol, U.K.) were used to enable removal of the mycelial biomass for *aflD* expression analyses. *A. flavus* strain RCP08108 was centrally inoculated using a semi-solid agar (0.2% agar in water) spore suspension (10^5 spores mL^{-1}), into different treatments (Pitt 1979). Petri dishes of the same a_W values were sealed in polyethylene bags and incubated at 28 ± 2 and 20 ± 2 °C. Growth assessment was made every day during the incubation period, and two radii of the growing colonies were measured at right angles in two directions until the control colony reached the edge of the plates. All colony radii were determined in six replicates for each test. The radii of the colonies were plotted against time, and a linear regression was applied, in order to obtain the growth rate (mm h^{-1}) as the slope of the line. Lag phase for growth was defined as the time (hours) needed by each colony to reach 10 mm in diameter, for each treatment (Marín et al. 1995). Mycelial biomass was extracted and frozen at −20 °C until RNA extraction and aflatoxin quantification by HPLC. Three replicates were used for aflatoxin and three for *aflD* expression analyses.

2.5. Sclerotial characterization

Following the methodology proposed by Nesci and Etcheverry (2009), spores of *A. parasiticus* RCP08300, obtained from a 7-day-old culture on MEA, were used. For sclerotia production, *A. flavus* was inoculated at the plate centre, using a semisolid agar spore suspension (10^5 spores mL^{-1}). The Petri dishes were incubated at 20 ± 3 °C and 28 ± 3 °C for 30 days. Plates of the same a_W were incubated in polyethylene bags. Sclerotia were obtained by scraping the surface of the culture plates in three replicates per treatment, over a Whatman No 4 sieve during irrigation with water containing Triton X-100 (100 μL L^{-1}). Sclerotia were further cleaned in a beaker with repeated rinses and decanting, then air dried and stored in a desiccator. Dried sclerotia were used to estimate their volume (from average length and width), number and weight. Length (a) and width (b) of 100 sclerotia (if available) from each replicate plate were measured by light microscopy at 10×. Sclerotium shape was approximated as a prolate spheroid, and the volume (V) was determined from the equation $V = 4/3\pi \times a \times b^2$ (Horn et al. 1996). Sclerotia were counted on each whole plate for each replicate.

2.6. Aflatoxin analysis

Aflatoxin quantities were determined by high-performance liquid chromatography (HPLC), following the detection methodology proposed by Trucksess et al. (1994). After 11 days of incubation a piece of PMEA (1 cm × 1 cm) from the centre of each colony was taken, weighed and transferred to an Eppendorf tube, to which 500 μL of chloroform were then added. The mixture was shaken for 20 min. The piece of agar was then removed and the chloroform extract allowed to evaporate to dryness in N_2 flow. The residue was redissolved in 200 μL of acetonitrile and derivatized with 700 μL of trifluoroacetic acid/acetic acid/water (2:1:7, v/v/v). The derivatized aflatoxins (50 μL solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of an HP 1100 pump (Hewlett Packard, Palo Alto, CA, USA) connected to an HP 1046A programmable fluorescence detector, and quantification was performed by an HP workstation. Chromatographic separations were performed on a stainless steel, C_{18} reversed-phase column (150 mm × 4.6 mm i.d., 5 μm particle size, Luna-Phenomenex, Torrance, CA, USA). Water/methanol/acetonitrile (4:1:1, v/v/v) was used as the mobile phase, at a flow rate of 1.5 ml min^{-1}. Aflatoxin derivative fluorescence was recorded at excitation and emission wavelengths of 360 and 440 nm, respectively. Aflatoxin standards were quantified on the basis of HPLC fluorometric response compared with aflatoxins standards (Sigma Chemical St Louis, MO, USA). The mean recovery of the method used was calculated by spiking peanut seeds at different levels ranging from 5 to 100 ng g^{-1} and was estimated at 94.5%. The lowest detection limit was 1 ng g^{-1}.

2.7. RNA isolation and reverse transcription

After 72 h of incubation mycelial biomass was extracted for *aflD* expression analyses (Gallo et al. 2010). An aliquot of 100 mg of mycelium corresponding to each treatment was used for isolation of total RNA with the Absolutely RNA® Miniprep Kit (Stratagene) following the manufacturer's instruction. For tissue disruption, the samples were transferred into microtubes containing acid washed glass beads and then shaken in a vortex for 5 min at maximum speed, in presence of the disruption buffer. The integrity of the total RNA was checked on agarose gel and concentration was calculated by spectrometry (DU 800, Beckman Coulter, USA). DNAse I treatment was applied to remove genomic DNA contamination from the samples, and first strand cDNA synthesis was performed using QuantiTect reverse Transcription Kit (Qiagen). Each 20 μL reaction contained 500 ng of total RNA, 1 μL of RT primer Mix, 4 μL of 5× Quantiscript RT buffer, 1 μL of Quantiscript Reverse Transcriptase and RNase-free water up to the final volume. Synthesis of cDNA was performed in a Thermocycler (Cycler, BioRad, out for 15 min at 42 °C, followed by 3 min at 95 °C to inactivate Quantiscript Reverse Transcriptase. The cDNA samples were kept at −20 °C.

2.8. RT-PCR assay

The expression of structural gene *aflD* involved in the aflatoxin biosynthetic pathway was assayed. Real-time PCR was performed in a Thermocycler (Mx3000P, Stratagene, USA). The primers and the internal probe used in the reaction were those proposed by Mayer et al. (2003). The primer/probe set had the following nucleotide sequence: nortaq-1,5′-GTCCAAGCAACAGGCCAAGT-3′; nortaq-2,5′-TCGTGCATGTTGGTGATGGT-3′; norprobe, 5′-TGTCTTGATCGGCGCCCG-3′ enclosing an amplicon of 66 bp of *aflD* (=*nor-1*) for isolate *A. flavus* (AY510455) (Geisen 1996). For PCR reaction 1 μL of the cDNA sample solution was mixed with 24 μL of the PCR stock solution containing 2.5 μL of 10X PCR buffer (5 Prime, GmbH Hamburg Deutschland), 2 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM dNTP mixture, 1 μL of each primer (10 μM), 0.5 μL probe (10 μM), 0.2 μL of 5 U/μL enzyme (5 Prime, GmbH Hamburg Deutschland) and 16.3 μL sterile deionized H_2O. The amplification thermal profile was: 4 min at 95 °C followed by 40 amplification cycles at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 20 s.

Template plasmids containing a larger PCR fragment of the *aflD* gene, generated with the primer nortaq1 and nortaq3 (5′-AGTGTGGCAGGCATCTGTG-3′) were used to generate a standard curve as an external standard. Plasmids were quantified by spectrometry with the DU 800 (Beckman Coulter, USA) and copy numbers were estimated based upon the molecular weight of the template. A range of 2×10^4 to 2×10^9 copies of the serially diluted cloned target DNA were included in each run. The number of target DNA sequences present in each PCR mixture was calculated by comparing the crossing points of the samples with those of the standard curve (slope and R^2 value were −3.281 and 0.997) using the Mx3000P software (Stratagene, USA). The constitutively expressed rRNA fragment amplified with ITS1 and ITS4 (White et al. 1990) was used as a control.

2.9. Statistical analysis

All experiments were carried out with three replicates and all variables were analyzed based on completely randomized design. Statistical tests were performed using SigmaStat program Version 3.10. (Systat Software Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was performed on growth rate, sclerotium size, AFB_1 production and *aflD* gene expression. Duncan's New Multiple Range Test ($p = 0.05$) was applied to establish significant differences in growth rate and sclerotium size between treated and control samples. To compare differences in AFB_1 production levels and *aflD* gene expression data between treated and control samples, Fisher's LSD test ($p < 0.001$) was applied.

3. Results

3.1. Effects of antioxidants, Ψ and temperature on growth

Table 1 shows the effects of antioxidant mixtures on the lag phase of *A. flavus* RCP08108 on peanut meal extract agar (PMEA) at four Ψ levels (−2.8, −7.1, −9.9, −16.0 MPa) and two temperatures (28 and 20 °C). The lag phase of untreated controls increased when Ψ and temperature decreased. At −2.8 MPa and 28 °C the lag phase of the control was 64.3 h, but at 20 °C an increase was observed (9.8 h). Same effects in the control samples occurred when the two incubation temperatures (28 and 20 °C) were compared at the other Ψ tested; increases in lag phase were estimated in 35.0, 72.9 and >101.5 h at −7.1, −9.9 and −16.0 MPa, respectively. When the fungus grew at −2.8 MPa and 28 °C in presence of BHA–BHT mixtures (1 + 1 mM/M1) and (5 + 5 mM/M2), the lag phase increased 13.8 and 99.5 h, respectively. Similar behaviours were observed at 28 °C and at the other Ψ assayed, showing increasing times between 2.3 and 57.4 h and 206.0 and >300 h for M1 and M2, respectively. At 20 °C, both M1 and M2 treatments increased the lag phase between 22.4 and 86 h and >300 h, respectively.

Fig. 1 shows the relative growth rate of *A. flavus* RCP08108 on PMEA in response to antioxidant treatments and water stress at 28 and 20 °C. The growth rate was markedly reduced at 20 °C. At this temperature, practically no growth occurred with the M2 treatment at all Ψ stress tested. An ANOVA was performed to analyze the effect of the single factors considered in the study (antioxidant treatments, water stress and incubation temperature) on growth rate, as well as two- and three-way interactions. All of these factors and their interactions showed significant effects; the major effects were produced by antioxidant treatments and substrate

Table 1
Comparison of lag phase of *A. flavus* RCP08108 in response to antioxidant mixtures and Ψ at 28 °C and 20 °C.

	Lag phase (h)[a]					
	28 °C			20 °C		
Ψ (MPa)	C	M1	M2	C	M1	M2
−2.8	64.3 ± 1.3	78.1 ± 2.4	163.8 ± 1.6	74.1 ± 4.4	96.5 ± 6.4	>
−7.1	72.8 ± 1.3	80.5 ± 1.1	>	107.8 ± 3.0	157.7 ± 6.4	>
−9.9	81.1 ± 5.0	83.4 ± 2.5	287.8 ± 86.8	154.0 ± 6.5	206.5 ± 4.7	>
−16.0	198.5 ± 2.0	255.9 ± 8.5	>	>	>	>

Key: (C) control; (M1) BHA–BHT 1 + 1 mM; (M2) BHA–BHT 5 + 5 mM; $\geq$300 h.
[a] Mean of six replicates.

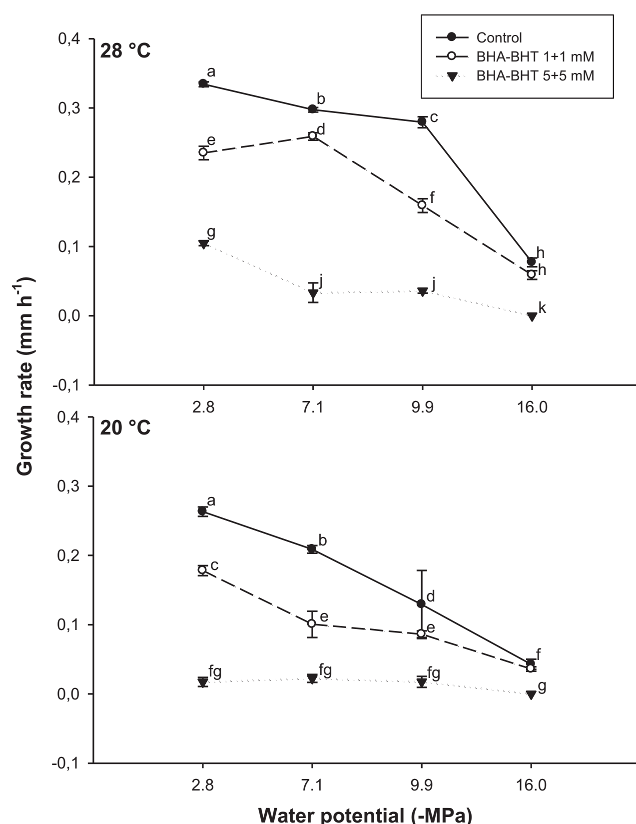

Fig. 1. Comparison of growth rate of *A. flavus* RCP08108 in response to antioxidant treatments and Ψ at 28 °C and 20 °C. Data with the same latter for each Ψ are not significantly different according to Duncan's New Multiple Range Test (p = 0.05).

water availability (Table 2). At 28 °C control growth rates were comparable or slightly lower in response to Ψ stress, except for −16.0 MPa. At 20 °C control growth rates decreased with the substrate Ψ in the order of 41.9%. Chemical treatments application caused a higher and significant (p < 0.001) reduction in growth rate estimated at 30.3 and 89.2% for M1 and M2 treatments, respectively regardless of Ψ stress imposition or incubation temperature.

Table 2
ANOVA of the effects of antioxidant mixtures (A), water potential of substrate (Ψ), temperature (T), and their interactions on growth rate of *A. flavus* RCP08108.

Source of variation	*df*	Mean square	*F* value[a]
A	2	0.19	1244.14*
Ψ	3	0.14	867.36*
T	1	0.06	399.07*
T vs Ψ	3	0.01	53.90*
Ψ vs A	6	0.01	70.11*
T vs A	2	0.02	148.49*
T vs Ψ vs A	6	0.003	20.96*
Error	48	0.0002	

[a] Snedecor's *F* test.
* Highly significant at p < 0.001.

3.2. Effects of antioxidants, Ψ and temperature on sclerotial characteristics

Sclerotia production by *A. flavus* RCP08108 took place on PMEA at −2.8 MPa and at both incubation temperatures, but at −7.1 MPa this strain was only able to form these resistance structures at 28 °C (Table 3). The effect of Ψ stress imposed on sclerotia production was higher than that of incubation temperature; that is, the sclerotia number produced by control cultures at −2.8 MPa/28 °C was near 308 and 14 times greater than at −7.1 MPa/28 °C and −2.8 MPa/20 °C, respectively. At −7.1 MPa/20 °C the isolate was not able to produce sclerotia. *A. flavus* strain RCP08108 produced an increased number of sclerotia (16.1%) when grown in the presence of the M1 treatment. Moreover, the dry weight of these resistance structures increased 51.1% by the application of this antioxidant mixture.

Fig. 2 shows the sclerotium size of *A. flavus* strain cultured on PMEA for 30 days at 28 and 20 °C in response to antioxidant treatments and Ψ stress. The ANOVA showed statistically significant effects for all of the factors considered, except when considering the temperature and interaction between temperature and antioxidant treatments (Table 4). The major effect was produced

Table 3
Influence of antioxidants on sclerotial production by *Aspergillus flavus* RCP08108 in relation to different Ψ and temperatures.

Temperature (°C)	Ψ	Antioxidant concentrations (mM)	Sclerotium numbers[a]	Dry weight (g)[a]
28	−2.8	0	616 ± 42	$1.7 \times 10^{-1} \pm 3.8 \times 10^{-2}$
		1	734 ± 130	$1.8 \times 10^{-1} \pm 5.3 \times 10^{-2}$
	−7.1	0	2 ± 1	$4.0 \times 10^{-4} \pm 2.8 \times 10^{-4}$
		1	8 ± 2	$7.7 \times 10^{-3} \pm 6.3 \times 10^{-3}$
20	−2.8	0	45 ± 29	$1.6 \times 10^{-2} \pm 7.0 \times 10^{-3}$
		1	66 ± 42	$3.4 \times 10^{-2} \pm 1.5 \times 10^{-2}$
	−7.1	0	0 ± 0	
		1	0 ± 0	

0 = fungal growth without sclerotial production.
[a] Mean value of three replicate and standard error.

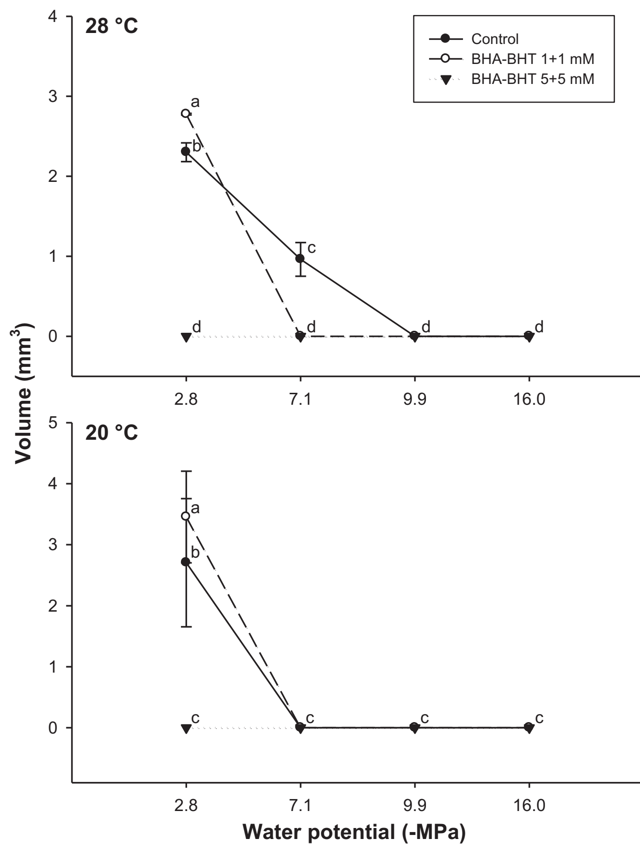

Fig. 2. Effect of antioxidants on *A. flavus* RCP08108 sclerotium size under different Ψ conditions (−2.8, −7.1, −9.9, −16.0 MPa) and temperatures (28 and 20 °C). Data with the same letter for each Ψ are not significantly different according to Duncan's New Multiple Range Test (p = 0.05).

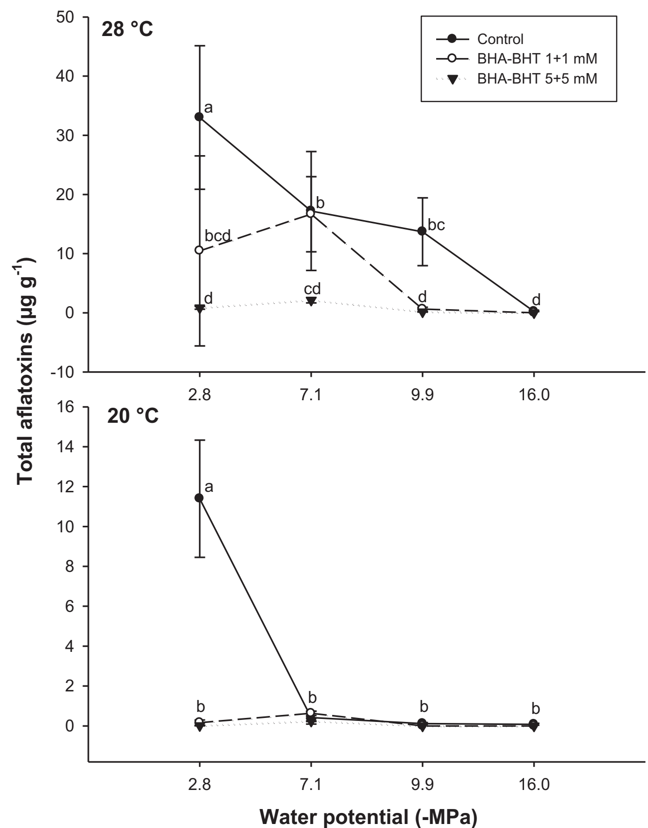

Fig. 3. Effect of antioxidant mixtures and Ψ on AFB_1 accumulation by *A. flavus* RCP08108 on PMEA medium at 28 °C and 20 °C. Data with the same letter for each Ψ are not significantly different according to Fisher's LSD Test ($p < 0.001$).

by Ψ stress followed by antioxidant mixtures. The highest effect of Ψ stress on volume decrease was observed at 20 °C, however at −7.1 MPa/28 °C the sclerotia size of control cultures were 58.2% smaller than at −2.8 MPa/28 °C. Increases in sclerotia volume of 19.4% were observed when *A. flavus* strain RCP08108 grew in the presence of M1 treatment at −2.8 MPa, regardless of incubation temperature.

3.3. Effects of antioxidants, Ψ and temperature on AFB_1 accumulation

Fig. 3 shows the effect of antioxidant mixtures and Ψ stresses on AFB_1 accumulation by *A. flavus* RCP08108 at 28 and 20 °C. The ANOVA did not show a significant interaction between all three variables (antioxidant × temperature × water potential of substrate), while all of these factors and two-way interactions significantly affected AFB_1 accumulation. The major effects were produced by incubation temperatures and antioxidant treatments (Table 5). Therefore, when the AFB_1 levels accumulated by control cultures at −2.8 MPa at temperatures were compared, a 67.6% reduction was observed at 20 °C. AFB_1 levels were undetectable at −7.1 and −9.9 MPa/20 °C. When Ψ stress was imposed, the reduction of AFB_1 accumulation were estimated at 47.9 and 58.5% at −7.1 and −9.9 MPa/28 °C, but this metabolite was not detected at −16 MPa, regardless of incubation temperature. AFB_1 levels similar to the control were observed in response to the lowest dose of antioxidant treatment at −7.1 MPa, regardless of incubation temperature, but the accumulation of this mycotoxin was practically not detected when *A. flavus* grew in presence of M2 treatment (Fig. 3).

Table 4
ANOVA of the effects of antioxidant mixtures (A), water potential of substrate (Ψ), temperature (T) and their interactions on sclerotium volume of *A. flavus* RCP08108.

Source of variation	*df*	Mean square	*F* value[a]
A	2	399.12	14.42**
Ψ	3	906.40	32.75**
T	1	16.95	0.61
T vs Ψ	3	281.29	10.16**
Ψ vs A	6	227.09	8.21**
T vs A	2	11.51	0.42
T vs Ψ vs A	6	77.57	2.80*
Error	48	27.68	

[a] Snedecor's F test.
* Significant at $p < 0.05$.
** Highly significant at $p < 0.001$.

Table 5
ANOVA of the effects of antioxidant mixtures (A), water potential of substrate (Ψ), temperature (T) and their interactions on AFB_1 accumulation by *A. flavus* RCP08108.

Source of variation	*df*	Mean square	*F* value[a]
A	2	502.12	16.94**
Ψ	3	294.79	9.95**
T	1	852.48	28.77**
T vs Ψ	3	136.87	4.62*
Ψ vs A	6	153.18	5.17**
T vs A	2	233.45	7.88*
T vs Ψ vs A	6	44.20	1.49
Error	48	29.63	

[a] Snedecor's F test.
* Significant at $p < 0.05$.
** Highly significant at $p < 0.001$.

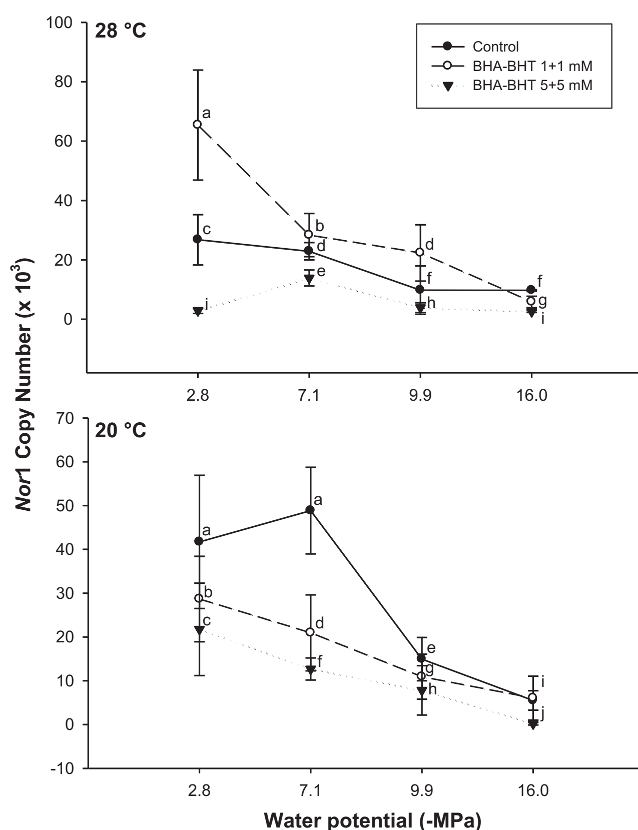

Fig. 4. Expression of the *aflD* gene of *A. flavus* RCP08108 in relation to different Ψ conditions, and antioxidant concentrations as indicated at 28 °C and 20 °C. Data with the same latter for each Ψ are not significantly different according to Fisher's LSD Test ($p < 0.001$).

3.4. Effects of antioxidants, Ψ and temperature on aflD gene expression

Fig. 4 shows the *aflD* gene expression of *A. flavus* strain cultured on PMEA for 7 days at 28 and 20 °C in response to antioxidant mixtures and Ψ stress treatments. The ANOVA showed statistically significant effects for all of the factors considered, except when considering the temperature and interactions between temperature and Ψ (Table 6). Therefore, at both temperatures the strain showed similar patterns of responses to the treatments, but the effects of Ψ stress treatments showed remarkable differences in *aflD* gene expression. A decrease of *aflD* gene expression between 58.6 and 86.5% was observed in response to the two higher Ψ stresses imposed (−9.9 and −16.0 MPa), regardless of incubation temperature. When antioxidant treatments were applied at 28 °C a statistically significant increase of *aflD* transcripts was observed in response to the lowest chemical dose (M1) at −2.8, −7.1 and −9.9 MPa, but *aflD* transcript levels were markedly decreased (67.8%) at the highest antioxidant levels (M2) (Fig. 4).

Table 6
ANOVA of the effects of antioxidant mixtures (*A*), water potential of substrate (Ψ), temperature (*T*), and their interactions on *aflD* expression by *A. flavus* RCP08108.

Source of variation	*df*	Mean square	*F* value[a]
T	1	3.21	0.29
Ψ	3	2482.71	223.20**
A	2	1837.09	165.16**
T vs Ψ	3	22.68	2.04
Ψ vs *A*	6	309.13	27.79**
T vs *A*	2	911.95	81.99**
T vs Ψ vs *A*	6	328.42	29.53**
Error	48	11.12	

[a] Snedecor's *F* test.
** Highly significant at $p < 0.001$.

4. Discussion

This is the first study that relates the growth, sclerotial characteristics, AFB_1 accumulation and *aflD* gene expression by the toxigenic *A. flavus* strain RCP08108 after sub-lethal treatment with BHA–BHT mixtures under environmental stress conditions.

Based on the results presented here, abiotic factors such as temperature and Ψ, interrelated with sub-lethal antioxidant doses, have a strong influence on the growth of *A. flavus* in PMEA. Both antioxidant treatments involved BHA–BHT increased the lag phase and reduced the growth rate of *A. flavus*, the major inhibitory effects were observed with the application of the higher concentration treatment (M2; BHA–BHT 5 + 5 mM). This treatment significantly reduced ($p = 0.05$) the growth rate of *A. flavus* between 68.6 and 93.4% and 100% at −2.8 and −16 MPa, respectively, regardless of temperature tested. However, higher antioxidant doses (BHA–BHT 10 + 10 mM) were needed to reduce the *Aspergillus* section *Flavi* population by 36.5, 46.3 and 77.4% in stored peanuts conditioned at −8.5, −24.0 and −37.8 MPa, respectively (Passone et al. 2009). Similarly, different mixtures of ferulic acid (FA) and cinnamic acid (CA) (20 + 5 mM, 25 + 5 mM, 1 + 10 mM, 10 + 10 mM and 20 + 10 mM) completely inhibited the growth of *A. flavus* and *A. parasiticus* strains on maize meal extract agar at −0.75, −3.5, −7.0 and −10.0 MPa (Nesci and Etcheverry 2006), although only CA–FA (25 + 30 mM) mixture effectively reduced *Aspergillus* section *Flavi* population on maize grains (Nesci et al. 2009).

Another important aspect analyzed in the present study was the effect of antioxidant mixtures and abiotic factors on sclerotial characteristics. Water potential has previously been demonstrated to influence sclerotial production of different *Aspergillus* species (Ramos et al. 1999; Nesci and Etcheverry 2009). In our study, sclerotial production of *A. flavus* was significantly affected ($p = 0.05$) by medium water potential, with total inhibition of sclerotial production at −9.9 and −16.0 MPa. Reduction of sclerotia number and size at −7.0 MPa suggests that an increased sclerotium development may occur at the highest water potentials. In the present study, the low antioxidant mixture applied (BHA–BHT 1 + 1 mM/M1) increased sclerotia number, dry weight and size.

The present study demonstrated that aflatoxin production is directly related to the impact that different stresses impose on such mycotoxigenic spoilage mould. A clear inhibitory effect of antioxidant mixtures on AFB_1 production by *A. flavus* strain RCP08108 was observed, but this strain increased *aflD* expression when grown in the presence of M1 treatment at 28 °C, when compared with the untreated control. Similarly, Schmidt-Heydt et al. (2007) determined that both calcium propionate and potassium sorbate over a range of low concentration (150 and 300 ppm) at 0.98 a_W caused an up-regulation of the *otapks* gene but a reduction of OTA production by *P. verrucosum*. Similar observations were made in another study, in which reductions of aflatoxin accumulation >95% were observed when the fungus was grown in the presence of caffeic acid, despite the fact that the *aflD* gene was actively transcribed; however, expression of almost all of the genes in the aflatoxin biosynthetic gene cluster was repressed by this treatment (Kim et al. 2008). Boutigny et al. (2009) clearly showed that in ferulic acid (0.5 mM) supplemented cultures of *Fusarium culmorum*, the decrease of trichothecene production could result from a general lower expression of the *Tri* genes studied. The mode of action of the anti-aflatoxigenic activity of the antioxidants used in this work appears to be associated with attenuation of the oxidative stress response of the fungus to organic peroxides. A link between

increased aflatoxin production and oxidative stress based on chemical induction has been documented (Jayashree and Subramanyam 2000; Reverberi et al. 2005) Interestingly, it was previously shown that activities of several antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase, etc.) and the content of reduced glutathione increased as cells of aflatoxigenic aspergilla proceeded from early to late logarithmic growth phases, indicating cellular responses to oxidative stress (Jayashree and Subramanyam 2000). The involvement of environmental factors in the activation of mycotoxin biosynthetic pathways has been described in several studies (Jurado et al. 2008; Kohut et al. 2009). The only other recent study was that by Abdel-Hadi et al. (2010) who examined *A. flavus* populations and AFB_1 and the *aflD* expression in peanuts stored at three Ψ (−7.0, −14.5 and −22.4 MPa) for up to 6 weeks. They showed similar results at both −7.0 and −14.5 MPa, with high *aflD* expression especially during the first 3 weeks, but at −22.4 MPa it was not transcribed until after 3 weeks. In the same way, we observed that the accumulation of *aflD* transcripts were similar at −2.8 and −7.1 MPa, with high reduction at the driest conditions tested (−9.9 and −16.0 MPa). However, these results not accurately reflect the phenotypic production of aflatoxin, because this toxin's accumulation was more sensitive to medium water availability changes. A similar behaviour was observed with the incubation temperature modification from 28 to 20 °C. OBrian et al. (2007) demonstrated that the aflatoxin production is regulated by temperature because decreasing amounts of aflatoxin were produced as temperature increased from 34 to 37 °C and minimal amounts were produced at 37 °C. These authors also used real-time PCR to determine expression of *aflR*, *aflR* antisense, *aflS* and *aflP* and found that the levels of all these genes were relatively constant across each temperature tested. Similar behaviour was recently observed by Schmidt-Heydt et al. (2009). These authors showed that mild stress imposed by abiotic factors induced aflatoxin production, but under increased stress conditions (0.90 a_W and >37 °C), although the whole aflatoxin biosynthetic cluster was induced, with *aflS* expression prominent at certain parameter combinations (>37 °C), aflatoxin production was inhibited.

In conclusion, in this work doses of food-grade antioxidant higher than those allowed by Codex Alimentarius or FDA for oil nut content were added to the conducive medium. However, previous studies revealed that although 10 mM of BHA and BHT were sprayed on in-pod peanuts, the residual of these chemicals in seeds ranged from 0.026 mM to 0.001 mM during the storage period (Passone et al. 2008b), and therefore did not exceed the maximum residue levels allowed.

In conclusion, our *in vitro* results showed that when environmental conditions are favorable for growth, the sub-lethal antioxidant mixture assayed (M1) enhanced the production of resistance structures and stimulated the *aflD* expression by *A. flavus* strain RCP08108. Theses *in vitro* studies are critical to enable a better understanding of the ecophysiological and functional importance of specific genes to develop effective control approaches to prevent aflatoxin contamination of stored peanut.

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