Aflatoxin production by *Aspergillus flavus* in Brazil nuts

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Abstract

Experiments were conducted to evaluate the effects of relative humidity (r.h.; 75%, 80%, 85%, 97%) and temperature (10, 13, 15, 25, 30 °C) on aflatoxin production in previously dried (3.5% moisture content; m.c.) Brazil nuts. Initially *Aspergillus* spp. were isolated from the surfaces of whole in-shell (WIS) Brazil nuts imported from Peru using A. flavus and A. parasiticus agar (AFPA). Isolates were subsequently screened for aflatoxin production using yeast extract sucrose medium. Total aflatoxin ($B_1 + B_2 + G_1 + G_2 + M_1$) was analyzed using an immunoassay technique while the presence of aflatoxin was confirmed using thin-layer chromatography. The surface of shelled half-nuts (simulating damaged or trimmed nuts), shelled whole (SW) nuts, and WIS nuts following a chlorine wash and water rinse, served as sites for inoculation (10 µl; 10^5/ml) using an aflatoxigenic isolate. Maximum concentrations of total aflatoxin and B_1 were detected in nuts stored at 97% r.h. and at temperatures of 25–30 °C. Shelled half-nuts contained the highest total (6817 ng/g) and B_1 (4483 ng/g) aflatoxin. WIS nuts contained the least total and B_1 toxin with maximum concentrations of 93 and 49 ng/g, respectively. Aflatoxin was not detected (detection limit of 1.75 ng/g) in nuts maintained at either 10 °C (97% r.h.) or at 30 °C (75% r.h.) for up to 60 d. Maximal moisture contents (%) and water activity values (a_W) for nuts stored at these conditions were 4.50 and 0.78, and 9.14 and 0.92, respectively. Results of this study indicate that the limiting moisture content and a_W values required to control aflatoxin production (<4 ng/g) in SW and WIS stored at 30 °C for up to 60 d are 4.5, 0.68, 5.0, and 0.75, respectively. Overall, increasing

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the relative humidity and temperature during storage resulted in an increase in aflatoxin and these were shown to be the most significant variables influencing toxin production in Brazil nuts.

1. Introduction

Brazil nut or castaña trees (*Bertholletia excelsa* Humb. and Bonpl.) are indigenous to the Amazon forests of South America and represent some of the oldest living trees on earth, many of which date back more than 1100 years (FAO, 1995). Harvesting of Brazil nuts, a major non-timber forest product, not only helps in preserving the Amazon rainforest but also creates an economy on which thousands of local people depend.

The occurrence of aflatoxins, produced by *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare, in Brazil nuts has been confirmed in several studies (Castrillon and Purchio, 1988b; Steiner et al., 1992; Freire et al., 2000; Caldas et al., 2002). The highest concentration of aflatoxin B_1 (AFB_1) in Brazil nuts (Brazil crop of 1988), for example, was reported to be 4.0 mg/kg (Steiner et al., 1992). In many instances, the presence of the mycotoxin was detected on the surface of shelled nuts exhibiting visible mold growth and/or inside shriveled, cracked, or brown spotted nuts (Freire et al., 2000). Shelled nuts exhibiting yellow fluorescence when screened under UV light were also shown to be positive for aflatoxin (Steiner et al., 1992).

Several environmental factors are known to influence aflatoxin production, but temperature and relative humidity (r.h.) are considered to be the most critical. Studies performed on hazelnuts and pistachios suggested that optimum temperature and relative humidity for aflatoxin production is 25–30 °C and 97–99%, respectively (Diener and Davis, 1966, 1967; Schindler et al., 1967; Northolt et al., 1976; Şimşek et al., 2002). Additional factors such as water activity, moisture, substrate composition (Sakai et al., 1984), storage time, insect damage (Lynch and Wilson, 1991; Schatzki and Ong, 2001), and presence of a shell (Ayerst and Budd, 1960) also influence fungal growth and aflatoxin production. It is also important to recognize, however, that the interaction of all these factors may provide for varying results in regards to fungal growth and mycotoxin production even on identical substrates.

The presence of aflatoxin is a serious concern for exporters of Brazil nuts especially since 1998, when the European Community decreased the maximum tolerance limit of total and B_1 aflatoxins to 4 and 2 ng/g, respectively (EC, 1998). Moreover, since temperature and relative humidity are important factors for aflatoxin production, it was of interest to evaluate the effect of these parameters on aflatoxin production during storage of Brazil nuts. The objective of this study was to provide Peruvian Brazil nut harvesters and processors with some of the necessary information for the safe handling and storage of nuts in order to limit aflatoxin production and thereby help maintain a viable export trade. By maintaining this trade, forests that are managed for Brazil nut harvest can be legally protected against deforestation.

2. Materials and methods

2.1. Brazil nuts and isolation of Aspergillus flavus

Shelled whole (SW) and whole in-shell (WIS) Brazil nuts (February–March 2002 harvest) were imported from a Peruvian Brazil nut processor and 50 nuts of each type were randomly chosen, then surface treated by immersion in 0.4% sodium hypochlorite solution for 2 min (Pitt et al., 1992). Using alcohol-flamed forceps, 25 groups each consisting of two SW nuts and 25 groups each consisting of WIS nuts were transferred onto the surface of deep Petri dishes (100 × 20 mm) pre-poured with *A. flavus* and *A. parasiticus* agar (AFPA; Oxoid Inc., Nepean, ON). Following incubation (30 °C, 3 d), the dishes were examined for fungal growth. A yellow/orange colour on the reverse of the colony was used to indicate presumptive *A. flavus/parasiticus* (Hocking, 1982; Pitt et al., 1983). Presumptive cultures were subsequently purified and cultivated on both Czapek and malt agar (Difco, Detroit, USA) at 28 °C until extensive formation of conidia was observed. The isolates were then compared to known *A. flavus* and *A. parasiticus* strains grown on the same media. *Aspergillus flavus* strains were tentatively identified and several were confirmed by the National Fungal Identification Laboratory, Agriculture and Agri-Food Canada (Ottawa, ON). Cultures were maintained on AFPA slants at 5 °C following growth at 25 °C for 5 d.

2.2. Aflatoxin screening of fungal cultures

Aspergillus flavus isolates (designated as AF strains 1–7) were screened for aflatoxin production using a procedure described by Davis et al. (1966). Flasks (500 ml) containing 100 ml sterile YES medium (2% yeast extract and 20% sucrose) were inoculated with a single conidium obtained from each isolate and incubated statically for 7–10 d in the dark at room temperature (21 °C). Following incubation, the flask contents were filtered (Whatman No. 1), dried at 70 °C for 24 h in a convection air oven and weighed (Davis et al., 1966). Individual filtrates (50 µl) were freeze-dried, dissolved in chloroform (100 ml), mixed vigorously, and then quantified for total aflatoxin using an ELISA kit (Ridascreen®, R-Biopharm AG, Darmstadt, Germany). Aflatoxin was confirmed by spotting extracts (10 µl) onto pre-coated TLC plates (20 × 20 cm) of silica gel (SIL G25-HR, Machery-Nagel, Duren, Germany) and allowed to develop in a solvent consisting of acetone: chloroform (9:1) at room temperature (AOAC, 1996). To detect fluorescence, plates were illuminated in the dark using UV light (365 nm). A standard aflatoxin solution (R-Biopharm AG) prepared in chloroform was loaded concurrently onto the plate.

2.3. Aflatoxin production on Brazil nuts

Aspergillus flavus strain AF-3 was grown on malt extract agar slants at 25 °C until extensive formation of conidia was observed (10 d). Conidia were harvested by washing the slants with peptone (0.1%) containing Tween 80 (0.1%; Andrews, 1996) and inocula were standardized to 10^7 conidia per ml using a Neubauer counting chamber (VWR Scientific, West Chester, PA). Spore concentrations were confirmed using serial dilution and direct plating (AFPA; 30 °C, 3 d).

Nuts simulating three stages in processing were selected for mold inoculation: shelled half nuts (SH; simulating damaged or trimmed nuts), SW, and WIS. All nuts used for inoculation were

carefully examined for visible signs of damage and mold growth. SW or WIS nuts exhibiting any discoloration or blemishes were discarded. SH nuts were prepared by slicing through the longitudinal axis of WS nuts using a utility knife. All nuts were surface disinfected by immersion in a 0.4% solution of sodium hypochlorite for 2 min, rinsed in sterile water, and dried overnight on paper towels in a laminar-flow hood (Pitt et al., 1992).

For SH and SW nuts, 5 μl (10^4 conidia) inocula were pipetted onto each of their ends following the creation of small bore holes to help contain the inoculum. WIS nuts were inoculated (5 μl; 10^4 conidia) at each stem ending (locule). All samples were transferred aseptically to deep Petri dishes (10×20 mm; 4 nuts per dish) and placed on perforated porcelain plates in grease-sealed glass desiccators adjusted to specific relative humidity values using saturated salt solutions: $97 \pm 1\%$, potassium sulfate; $84 \pm 1\%$, potassium chloride; $79 \pm 1\%$, ammonium sulphate; and $75 \pm 1\%$, sodium chloride (Meites, 1963). Temperature was maintained by placing the desiccators in thermostatically controlled incubators.

Two experimental protocols were used in this study. In the first protocol, WIS nuts were maintained at 30 °C under 75, 80, 85, and 98% r.h. For each temperature–relative humidity combination, eight replicates consisting of four nuts per replicate were used. At 30 and 60 d, four replicates (16 nuts) were assessed for aflatoxin (total and/or B_1). A similar procedure was used for SH and SW nuts. In the second protocol, WIS nuts were maintained under 98% r.h. and at temperatures of 10, 13, 15, 25, and 30 °C. For each temperature–relative humidity combination, eight replicates consisting of four nuts per replicate were used. At 30 and 60 d, four replicates were assessed for total and B_1 aflatoxin. A similar procedure was used for SW and SH nuts. Temperature and relative humidity were monitored on a daily basis using a hygrometer ($\pm 5\%$ accuracy) and thermometer (± 1 °C accuracy). The moisture content of the nuts was determined following oven drying (Beuchat, 1973) whereas available moisture was assessed using a water activity meter (Novasina A_W Sprint Th-500, ± 0.01) after 30 and 60 d of storage. For both the analyses, shell material was not included in WIS measurements. The analyses were performed in duplicate.

2.4. Determination of aflatoxin

Total and aflatoxin B_1 were determined using an ELISA kit (Ridascreen®). The detection limits for the total and B_1 test kits were 1.75 and 0.625 ng/g, respectively. Preparation of Brazil nut samples including extraction and aflatoxin determination was performed according to the manufacturer's instructions. Aflatoxin B_1 standard (R-Biopharm AG) dissolved in methanol was used to prepare calibration curves. The cross reactivity of the antibodies in the kit used to measure total aflatoxin was as follows: 100% aflatoxin B_1; 200% aflatoxin B_2, 15% aflatoxin G_1, 16% aflatoxin G_2, and 63% aflatoxin M_1. The cross-reactivity of the antibodies used to measure aflatoxin B_1 was as follows: 100% aflatoxin B_1, 0.2% aflatoxin B_2, 1.1% aflatoxin G_1, and $< 0.1\%$ aflatoxin G_2, M_1, M_2.

Brazil nuts were macerated into a paste using a mortar and pestle. A portion (2 g) was subsequently mixed with methanol (70%, 10 ml) for 10 min on a shaker at room temperature. Following filtration (Whatman No.1) the filtrate (100 μl) was diluted with PBS buffer (900 μl); 50 μl was used per test. The absorbance of the samples at 450 nm (Multiskan MCC/340 MK 11) was integrated into the software (provided by R-Biopharm) to obtain aflatoxin concentrations.

Aflatoxin was confirmed by the Romer minicolumn method following AOAC Official method 975.36 (AOAC, 1996). Efficiency of aflatoxin recovery was assessed using the Ridascreen® Total aflatoxin kit. Aflatoxin B_1 (1 mg) standard was dissolved in methanol (70%) and diluted to 5, 50, and 100 ng/g. Known quantities of the toxin were then added to macerated Brazil nut meal, extracted, and quantified. Aflatoxin-free SW nuts from Peru, certified by an accredited laboratory (Inassa International Analytical Services S.A., Lima, Peru), were also evaluated for aflatoxin using the test kit.

2.5. Data analysis

The data obtained in the study were analyzed using the analysis of variance (ANOVA) procedure of SAS (SAS Institute, Cary, NC). Differences among means were compared using Tukey's test. Aflatoxin levels were log-transformed before analysis.

3. Results

Aspergillus parasiticus was not recovered from any of the nuts examined. The majority of *Aspergillus* spp. isolated on AFPA were identified as *A. flavus* of which seven were subsequently screened for total aflatoxin production (Table 1). Aflatoxin concentrations at 10 d ranged from 10 to 778 ng/g and biomass in terms of mycelial growth did not appear to correlate with toxin production. Based on total aflatoxin production, isolate AF-3 was chosen for future challenge studies.

During the first 2 weeks of storage, mold growth was not observed on any of the inoculated nuts. By the third week, however, mold was observed on nuts stored at both 85 and 97% r.h. particularly at temperatures of 25 and 30 °C. By 4 weeks, mold growth was also observed on nuts maintained at 80% r.h. (30 °C) and at 97% r.h. (15 °C). However, mold was not observed at 97% (10 °C) and only minimal growth occurred at 75% (30 °C) even after 60 d. The most luxuriant growth was observed at 30 °C on SH nuts which, regardless of relative humidity, appeared to yield the highest fungal growth.

Table 1
Growth and total aflatoxin production in YES medium[a] by *A. flavus* strains isolated from Brazil nuts

Isolates	Total aflatoxin[b] (ng/ml)	Mycelia[b] (g/100 ml YES)
AF-1	13.12 ± 5.23[c]	2.43
AF-2	627.60 ± 64.19	2.61
AF-3	778.09 ± 25.98	2.42
AF-4	12.58 ± 1.74	2.90
AF-5	12.88 ± 2.35	1.64
AF-6	10.02 ± 2.43	2.68
AF-7	612.32 ± 147.62	2.33

[a]Incubated at 21 ± 1 °C for 10 d.
[b]Mean of three values.
[c]Mean value $\pm$ S.D.

Aflatoxin was not detected in any nuts stored at either 75% r.h. (30 °C) or 10 °C (97% r.h.) even after 60 d of storage (data not presented). As shown in Fig. 1, total aflatoxin production at 30 °C was significantly ($P \leqslant 0.05$) affected by relative humidity (especially when it increased
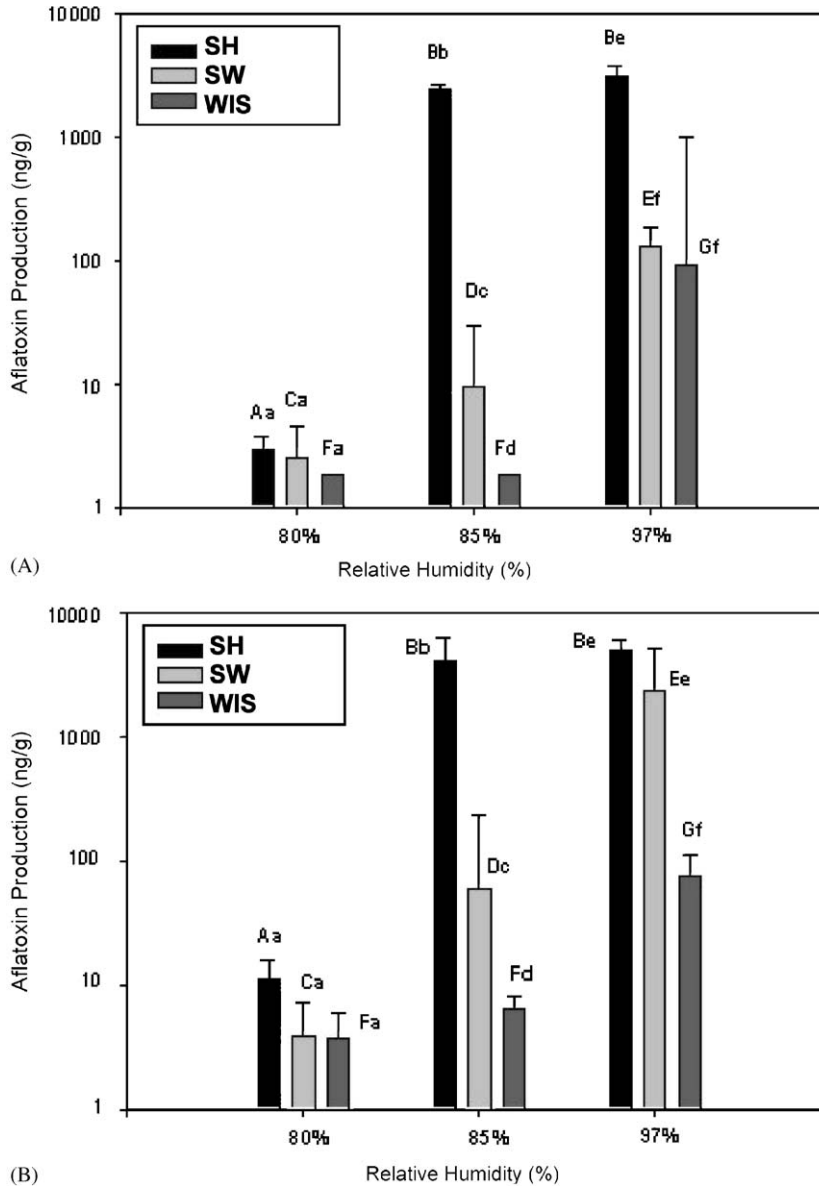

Fig. 1. Total aflatoxin production in Brazil nuts after (A) 30 d and (B) 60 d of storage at 30 °C: influence of relative humidity and inoculation site. Results are the means of four trials analyzed in duplicate. The bars represent the standard deviation. Results accompanied by the same upper-case letter for similar inoculation sites are not significantly different ($P \leqslant 0.05$) according to Tukey's test. Results accompanied by the same lower-case letter for each relative humidity are not significantly different ($P \leqslant 0.05$) according to Tukey's test.

from 80% to 85%) and inoculation site. Although maximum toxin concentrations were observed in SH nuts stored at 97% r.h. (3103 and 5046 ng/g at 30 and 60 d, respectively), they were not significantly different from those concentrations in SH nuts maintained at 85% either for 30 or 60 d. In contrast, WIS nuts contained the least amount of toxin. For example, at 60 d and 97% r.h., the total aflatoxin concentration in these nuts was approximately 85.5 ng/g.

Temperature was also found to significantly affect mycotoxin production. As shown in Fig. 2 when nuts were maintained at 97% r.h., concentrations of aflatoxin generally increased with increasing temperature. Highest levels at 60 d, 6817, and 5046 ng/g were obtained at 25 and 30 °C, respectively in SH nuts. Overall, the least amount of toxin occurred in WIS nuts; at 25 and 30 °C levels of aflatoxin accumulation at 30 d in SH nuts were approximately seven and 23 times greater than in SW nuts, respectively. It is interesting to note, however, that by 60 d, total aflatoxin concentrations in SH nuts at 25 and 30 °C were only 1.3 and 1.7 times greater, respectively, than SW nuts.

AFB_1 production in Brazil nuts at 30 °C is shown in Fig. 3. Similar to total aflatoxin production, synthesis of AFB_1 in SH nuts significantly increased with an increase in relative humidity especially from 80% to 85%. In this regard, the AFB_1 concentrations increased by approximately 400 and 1860 ng/g at 30 and 60 d, respectively. AFB_1 concentrations in SW and WIS nuts, however, did not exhibit any significant increase either at 30 or 60 d when the relative humidity was increased from 80% to 85%. Production of AFB_1 in nuts maintained at various temperatures under 97% r.h. is shown in Fig. 4. Toxin concentrations were below 1 ng/g at 30 and 60 d for SW and WIS nuts maintained at 13–15 °C. At 30 d, the highest concentration of AFB_1 (1934 ng/g) occurred in SH nuts kept at 30 °C. By 60 d, the concentration had increased marginally to 2202 ng/g. At 25 °C, toxin concentrations increased by approximately 4400 ng/g. AFB_1 concentrations on average comprised about 76–79% of the total aflatoxin produced.

The moisture content and water activity of Brazil nuts maintained under various relative humidity environments is shown in Tables 2 and 3. Initial moisture (3.5%) and a_W levels (0.705) in all nuts (with the exception of SH nuts and SW nuts at 75% r.h.) increased during storage; nuts stored at 97% r.h. exhibited the highest moisture content gain. Interestingly, by 60 d, WIS nuts gained the highest amount of moisture regardless of storage relative humidity.

4. Discussion

The failure to detect *A. parasiticus* from any of the nuts evaluated in this study is consistent with the majority of investigations dealing with Brazil nuts (Holubova-Jechova, 1970; Freire et al, 2000; Bayman et al., 2002). Only one study (Castrillon and Purchio, 1988a) reported the presence of *A. parasiticus.* In all these studies, *Aspergillus* spp. were the dominant fungi recovered and as such the major toxigenic species. Although all of the isolates we recovered were aflatoxigenic, a number of authors have reported that not all *A. flavus* are capable of producing aflatoxin (Diener and Davis, 1966; Varma and Verma, 1987). During the first 2 weeks of storage, mold growth was not observed on any of the inoculated nuts and it is presumed that the low initial moisture content (3.5%) and water activity (0.705) either prevented or inhibited growth as the minimum a_W for
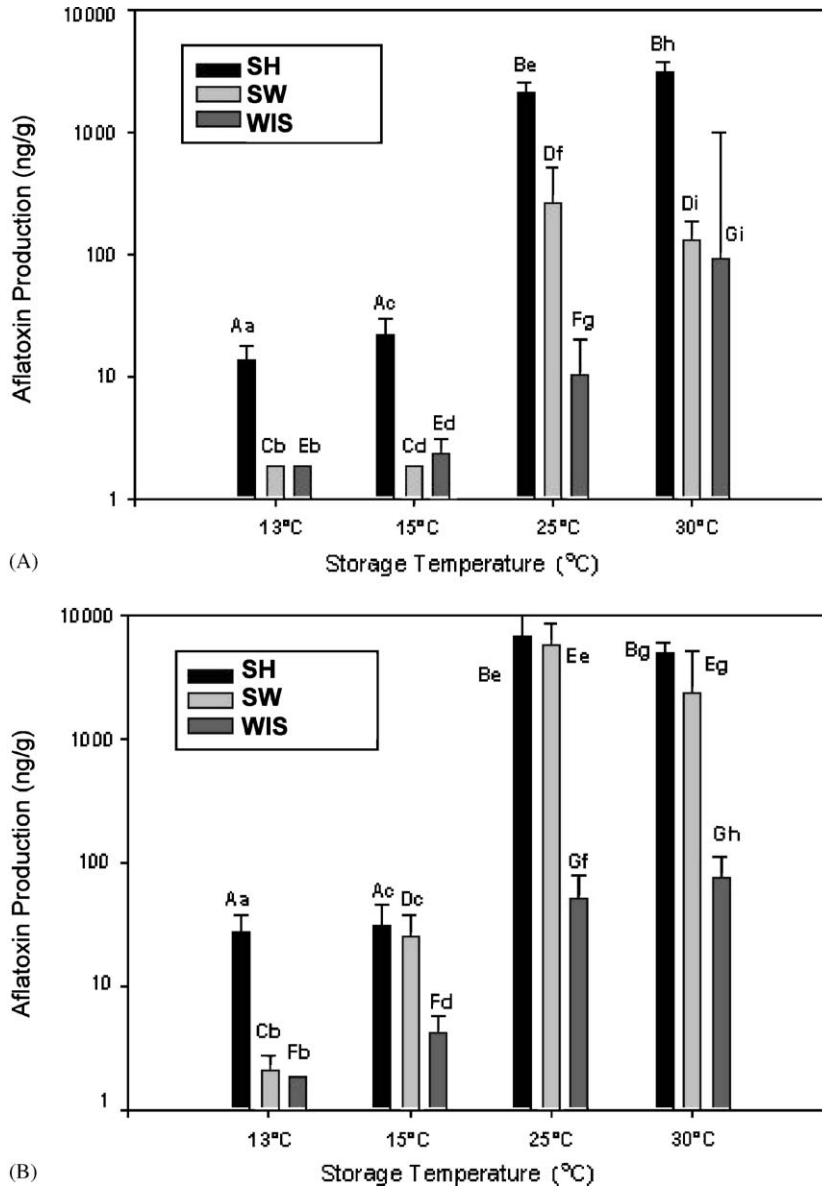
Fig. 2. Total aflatoxin production in Brazil nuts after (A) 30 d and (B) 60 d of storage at 97% r.h.: influence of temperature and inoculation site. Results are means of four trials analyzed in duplicate. Bars represent the standard deviation. Results accompanied by the same upper-case letter for similar inoculation sites are not significantly different ($P \leqslant 0.05$) according to Tukey's test. Results accompanied by the same lower-case letter for each temperature are not significantly different ($P \leqslant 0.05$) according to Tukey's test.

growth of *A. flavus* is between 0.80 and 0.83 (Pitt and Miscamble, 1995). Interestingly, in this study growth as evidenced by the presence of toxin (Diener and Davis, 1967; Yokoya et al., 1970), was detected on nuts having a_W below this range.
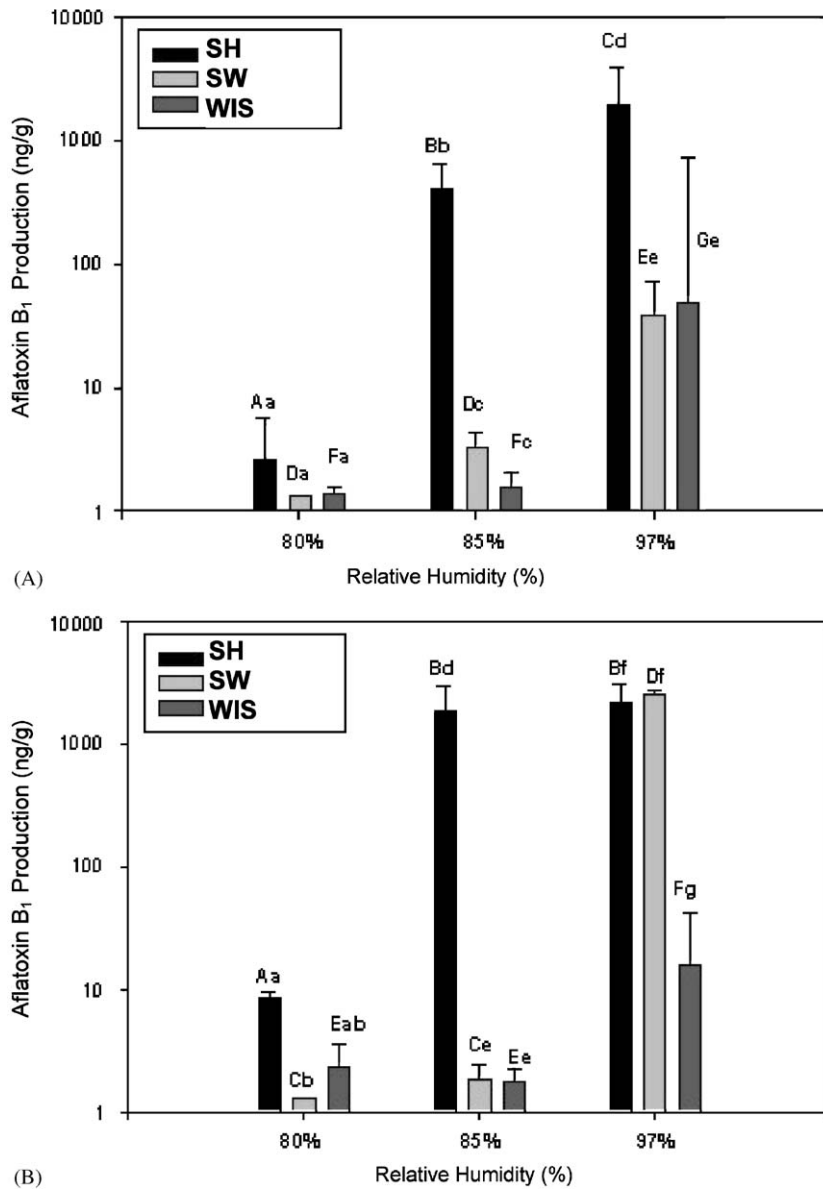
Fig. 3. Aflatoxin B_1 production in Brazil nuts after (A) 30 d and (B) 60 d of storage at 30 °C: influence of relative humidity and inoculation site. Results are means of four trials analyzed in duplicate. Bars represent the standard deviation. Results accompanied by the same upper-case letter for similar inoculation sites are not significantly different ($P \leqslant 0.05$) according to Tukey's test. Results accompanied by the same lower-case letter for each relative humidity are not significantly different ($P \leqslant 0.05$) according to Tukey's test.

When maintained at 75% r.h. and 30 °C, growth of *A. flavus* was not observed on any of the nuts even at 60 d. It was interesting to observe, however, that inoculated and especially control nuts had growth of *A. chevalieri* Mangin, a tropical and subtropical species that proliferates under
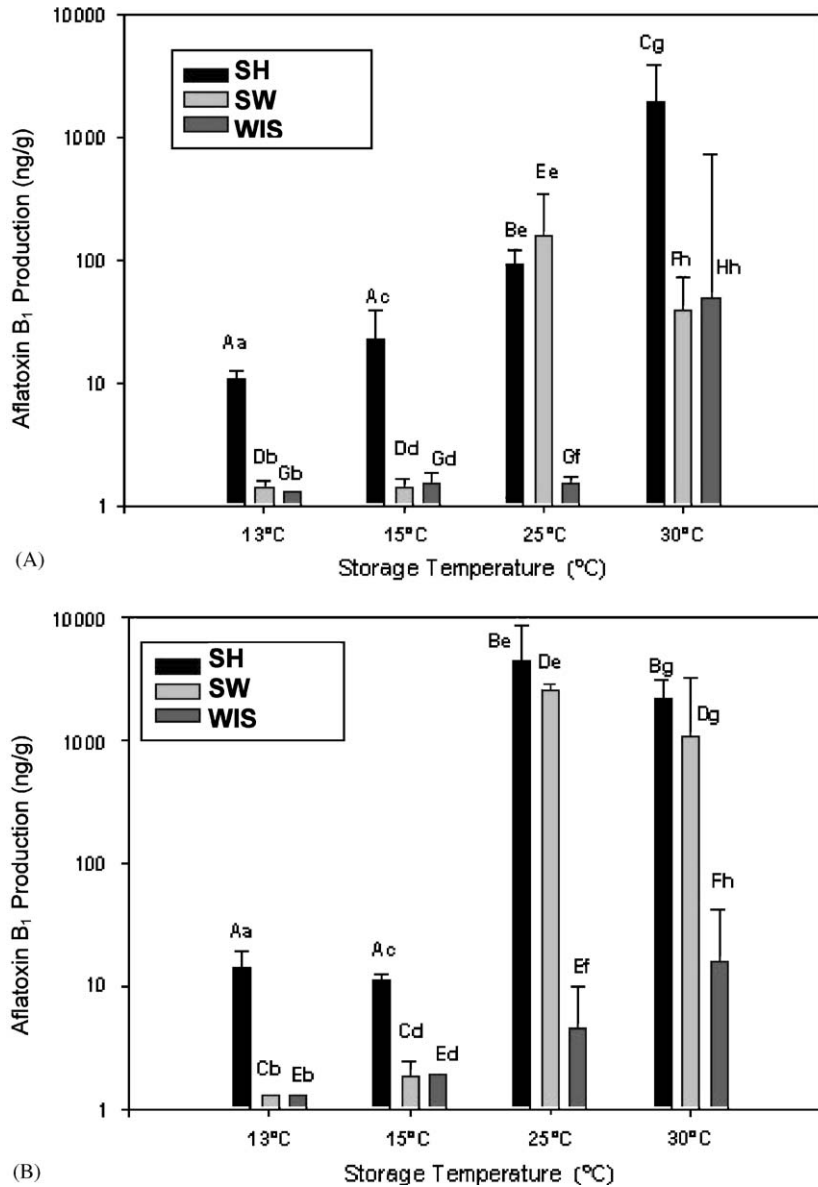
Fig. 4. Aflatoxin B_1 production in Brazil nuts after (A) 30 d and (B) 60 d of storage at 97% r.h.: influence of temperature and inoculation site. Results are means of four trials analyzed in duplicate. Bars represent the standard deviation. Results accompanied by the same upper-case letter for similar inoculation sites are not significantly different ($P \leqslant 0.05$) according to Tukey's test. Results accompanied by the same lower-case letter for each temperature are not significantly different ($P \leqslant 0.05$) according to Tukey's test.

conditions of low moisture (Samson et al., 1995). It is assumed that the difference in the amount of *A. chevalieri* growth between control and inoculated nuts may have been due to greater competition. Regardless of inoculation site, incubation at 30 compared to 25 °C resulted in more visible mold growth; however, total aflatoxin concentrations particularly by 60 d were consistently

Table 2
Moisture content and water activity of Brazil nuts after 30 and 60 d storage at 30 °C and at various relative humidities

Relative humidity (%)	Shelled half nuts (SH)			Shelled whole nuts (SW)			Whole in-shell nuts (WIS)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
75	3.50[a]	3.55	3.50	3.50	3.55	3.50	3.50	4.50	4.12
	(0.705)[b]	(0.695)	(0.682)	(0.700)	(0.695)	(0.680)	(0.711)	(0.780)	(0.720)
80	3.50	3.57	4.57	3.50	4.35	4.50	3.50	4.75	5.05
	(0.705)	(0.717)	(0.705)	(0.700)	(0.735)	(0.685)	(0.711)	(0.794)	(0.751)
85	3.50	6.43	6.60	3.50	5.34	5.35	3.50	6.73	6.90
	(0.705)	(0.895)	(0.823)	(0.700)	(0.775)	(0.815)	(0.711)	(0.880)	(0.886)
97	3.50	8.57	11.14	3.50	7.19	10.22	3.50	8.20	11.26
	(0.705)	(0.897)	(0.953)	(0.700)	(0.859)	(0.936)	(0.711)	(0.915)	(0.952)

[a] Moisture content (%). Mean of two values.
[b] Water activity. Mean of two values.

Table 3
Moisture content and water activity of Brazil nuts after 30 and 60 d storage at various temperatures and 97% r.h.

Temperature (°C)	Shelled half nuts (SH)			Shelled whole nuts (SW)			Whole in-shell nuts (WIS)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
10	3.50[a]	5.40	9.14	3.50	6.32	6.45	3.50	4.15	5.50
	(0.705)[b]	(0.870)	(0.922)	(0.700)	(0.858)	(0.891)	(0.711)	(0.725)	(0.830)
13	3.50	5.71	9.43	3.50	6.38	7.26	3.50	4.52	7.86
	(0.705)	(0.890)	(0.933)	(0.700)	(0.864)	(0.900)	(0.711)	(0.745)	(0.913)
15	3.50	5.00	8.14	3.50	4.91	6.09	3.50	4.27	5.92
	(0.705)	(0.816)	(0.919)	(0.700)	(0.778)	(0.841)	(0.711)	(0.729)	(0.835)
25	3.50	8.57	11.00	3.50	7.38	8.57	3.50	7.88	8.96
	(0.705)	(0.904)	(0.917)	(0.700)	(0.898)	(0.904)	(0.711)	(0.894)	(0.933)

[a] Moisture content (%). Mean of two values.
[b] Water activity. Mean of two values.

higher in nuts stored at 25 °C. Although growth of *A. flavus* is most favored between 29 and 35 °C, (Schindler et al., 1967; Gqaleni et al., 1996) it is recognized that the biomass is not closely associated with toxin production (Priyadarshini and Tulpule, 1978). Maximum visible mold growth always occurred on the SH nuts, regardless of temperature and relative humidity. Similar observations have been reported by others when comparing damage in peanuts (Diener and Davis, 1967; Freire et al., 2000). SH nuts, which were used to simulate either trimming or damage during shelling or handling and transportation, would be expected to have enhanced fungal colonization perhaps by providing for an increase in surface area, nutrient availability and removal of protective barriers.

Overall, aflatoxin production in Brazil nuts was significantly affected by relative humidity, temperature, and inoculation site and storage time. Of the variables investigated, relative humidity and temperature were the most significant relative to toxin production (*F* ratios of 247

and 426 for relative humidity and temperature, respectively; Tables 4 and 5) and are in agreement with previous investigations (Diener and Davis, 1967; Schindler et al., 1967; Atalla et al., 2003). Maximum concentrations of aflatoxin were produced on SH nuts stored under 97% r.h. and at 25–30 °C. Although aflatoxin production in challenge studies with Brazil nuts has not been reported, several researchers concur that maximum toxin production in nuts including hazelnuts and peanuts occurs with increasing relative humidity to 99–100% (Diener and Davis, 1967; Chiou and Tsao, 1997). The failure to detect aflatoxins in nuts stored below 75% r.h., and the low amounts of toxins in SW and WIS nuts stored at 80% r.h. indicates that these may be threshold values for safe storage up to 60 d. In contrast, the relative humidity level for the safe storage of peanuts and hazelnuts was reported to be 85% (Diener and Davis, 1967; Şimşek et al., 2002).

With regards to temperature, storage of nuts at 13 °C yielded the lowest concentrations of aflatoxin which were also well below EC requirements. Considering that *A. flavus* is a mesophile, the results were not unexpected. In this study, storing Brazil nuts at 25–30 °C yielded the highest concentration of toxin. In particular, total and AFB_1 toxin concentrations in WIS nuts gradually increased with increases in temperature. With SW and HS nuts, these concentrations also increased but only up to 25 °C, thereafter decreasing.

Table 4
Analysis of Variance (ANOVA): influence of relative humidity and inoculation site on total and B_1 aflatoxin production in Brazil nuts after 30 and 60 d of storage at 30 °C

Source	F	Type III SS	Mean square	F	P
Inoculation site	2	33.96602231	16.98301115	144.12	<0.0001
Relative humidity	2	58.44310301	29.22155150	**247.97**	<0.0001
Storage time	1	3.485117853	3.48517853	29.58	<0.0001
Relative humidity*inoculation site	4	15.07412885	3.76853221	31.98	<0.0001
Inoculation site*storage time	2	0.84567909	0.42283955	3.59	0.0344
Relative humidity*storage time	2	0.07486056	0.03743028	0.32	0.7992
Inoculation site*relative humidity*storage time	4	1.62710734	0.40677683	3.45	0.0139

Table 5
Analysis of Variance (ANOVA): influence of temperature and inoculation site on total and B_1 aflatoxin production in Brazil nuts after 30 and 60 d of storage at 97% r.h.

Source	DF	Type I SS	Mean square	F	P
Inoculation site	2	33.78099853	16.89049926	233.69	<0.0001
Temperature	3	92.41273272	30.80424424	**426.20**	<0.0001
Storage time	1	5.69306779	5.69306779	78.77	<0.0001
Inoculation site*temperature	6	7.93119912	1.32186652	18.29	<0.0001
Inoculation site*storage time	2	2.56575586	1.28287793	17.75	<0.0001
Temperature*storage time	3	1.63453031	0.54484344	7.54	0.0002
Inoculation site*temperature*storage time	6	1.45915207	0.24319201	3.36	0.0056

The Brazil nuts used in this study were previously dried to a moisture content of approximately 3.5% in a processing facility using a gas-fired oven. It was imperative that the nuts be dried prior to shipment in order to avoid deterioration during transit. During storage at 30 °C, moisture and water activity levels in the WIS nuts, regardless of relative humidity, were higher compared to SW nuts. In part, this may be due to hygroscopic properties of the shell and/or the ability of the shell to retain moisture, a phenomenon also observed by Chiou et al. (1984) with peanuts. Also in some cases, especially at the lower relative humidity (75% and 80%), the moisture content of the nuts did not appear to agree with the a_W. Discounting the accuracy of the water activity meter ($\pm 0.01\%$), the variation between these values could have resulted from slight differences in moisture content among individual nuts maintained at a controlled relative humidity since different nuts were used for moisture content and a_W determinations. Despite the higher moisture content toxin levels were lower for in-shell product, indicating that the shell can only partially protect the nut from fungal invasion. For example, Frank et al. (1980) indicated that aflatoxin producers are able to penetrate the shells of Brazil nuts at relative humidity greater than 75%. It should be noted that toxin production was detected in sound WIS nuts and therefore shell porosity (Spencer, 1921), cracks (Freire et al., 2000), and the presence of a locule (narrow open channel that extends through the entire length of the shell but does not appear to contact the nut) may influence nut stability.

Based on the results of this study, it would appear that minimum moisture or a_W levels in order to control aflatoxin production (<4 ppb) in Brazil nuts is dependent on whether or not the product is in-shell and/or the extent of damage. In this respect, it is recommended that at 30 °C (typical ambient temperature in many nut gathering/storage and processing areas of Amazonia), SW and WIS nuts be maintained at moisture levels of 4.5% ($a_W = 0.68$) and 5.0% ($a_W = 0.75$), respectively, as quickly as possible following harvesting. Damaged nuts or nuts which have been trimmed to remove blemishes should be segregated and maintained at 3.5% m.c. It should be pointed out that storage of these nuts in this study was only maintained for 60 d. According to Yokoya et al. (1970), SW Brazil nuts reach equilibrium after 8 months when they are stored under high relative humidity (88–97%) but only 2 months at 70% r.h. It can be expected therefore that the moisture content levels in the nuts stored under 80–97% r.h. would increase as would mold growth.

5. Conclusions

This study confirms earlier work demonstrating the importance of relative humidity and temperature on aflatoxin production in nuts and establishes limits for both these parameters. A relative humidity of 97% accompanied by temperatures in the range of 25–30 °C were shown to promote aflatoxin production in infected Brazil nuts. Unfortunately these conditions are representative of the harvesting season. Reduction of relative humidity and or temperature may not be an economic option. Air and or mechanical drying of the nuts prior to storage could be used and would limit mold growth and toxin formation. In this respect whole shelled and in-shell nuts should be dried to a moisture content of approximately 4.5% and 5.0%, respectively.

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