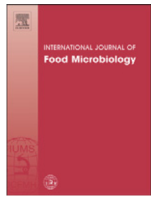
International Journal of Food Microbiology 148 (2011) 141–144

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro



Short communication

Aflatoxigenic fungi and aflatoxin in cocoa

Marina V. Copetti [a,*], Beatriz T. Iamanaka [b], José Luís Pereira [c], Maria H. Fungaro [d], Marta H. Taniwaki [b]

[a] Universidade Federal de Santa Maria (UFSM), Departamento de Tecnologia e Ciência de Alimentos, CEP 97105, 900, Santa Maria/RS, Brazil
[b] Instituto de Tecnologia de Alimentos (ITAL), C.P. 139, CEP 13070-178, Campinas/SP, Brazil
[c] Universidade Estadual de Campinas (UNICAMP), Departamento de Ciência de Alimentos, C.P. 6121, CEP 13083-862, Campinas/SP, Brazil
[d] Universidade Estadual de Londrina (UEL), Centro de Ciências Biológicas, C.P. 6001, CEP 86051-990, Londrina/PR, Brazil

ARTICLE INFO

Article history:
Received 3 March 2011
Received in revised form 4 May 2011
Accepted 23 May 2011
Available online 27 May 2011

Keywords:
Aflatoxin
HPLC
Aflatoxigenic fungi
Theobroma cacao
Aspergillus flavus
Aspergillus parasiticus
Chocolate

ABSTRACT

This paper reports the occurrence of aflatoxigenic fungi and the presence of aflatoxins in 226 cocoa samples collected on Brazilian farms. The samples were taken at various stages of fermentation, drying and storage. A total of 819 potentially aflatoxigenic fungi were isolated using Dichloran 18% Glycerol agar after surface disinfection, and identified by standard techniques. The ability of the fungi to produce aflatoxins was determined using the agar plug technique and TLC. The presence of aflatoxins in cocoa samples was determined by HPLC using post-column derivatization with bromide after immunoaffinity column clean up. The aflatoxigenic fungi isolated were *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. A considerable increase in numbers of these species was observed during drying and storage. In spite of the high prevalence of aflatoxigenic fungi, only low levels of aflatoxin were found in the cocoa samples, suggesting the existence of limiting factors to the accumulation of aflatoxins in the beans.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Aflatoxins represent the group of the most studied mycotoxins, especially due to their widespread occurrence in foods and toxicological and carcinogenic potential associated with their consumption. Since aflatoxins have hepatotoxic, teratogenic, mutagenic and carcinogenic properties, many countries have imposed regulations aiming to minimize the human exposure to aflatoxins (IARC, 1993). This legal restriction has resulted in rejection of products causing great economic losses for producers, processors and marketers of contaminated products (Cotty and Jaime-Garcia, 2007).

Aflatoxins are found as contaminants of food due to the development of fungi both pre-and post-harvest; the level of contamination depends on the plant stress, temperature, humidity, genotype and culture and storage conditions (Wilson and Payne, 1994). An inhibition of their production has also been related to the presence of some food components such as caffeine and polyphenols (Lenovich and Hurst, 1979; Hasan, 1999; Molyneux et al., 2007).

Despite the ability of aflatoxin production detected in several species of fungi: *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. toxicarius*, *A. parvisclerotigenus*, *A. bombycis*, *A. pseudotamarii*, *A. ochraceoroseus*, *A. rambelli*, *Emericella astellata* and *E. venezuelensis* (Frisvad et al., 2005); *A. flavus* and *A. parasiticus* remain as the most important and representative aflatoxin producer species naturally occurring in agricultural commodities (Cary and Ehrlich, 2006). *A. nomius*, which is also reported as a major producer of aflatoxins, has a more restricted occurrence (Kurtzman et al., 1987).

The occurrence of aflatoxigenic species is high in tropical countries. Although a wide variety of foods are susceptible to aflatoxin contamination, they have been most commonly associated with peanuts, pistachio, dried fruits, nuts, spices, figs, vegetable oils, cocoa beans, corn, rice and cotton seed (JECFA, 1998; ROC, 2003).

Cocoa beans, the principal raw material of chocolate, have an astringent, unpleasant taste and flavor and have to be fermented, dried and roasted to obtain the characteristic cocoa flavor. During the processing at farm, cocoa beans are exposed to bacteria, yeasts and filamentous fungi present in the environment. Bacteria and yeasts have an essential role in production of enzymes, acids and other metabolites that take part in biochemical transformations of the beans, forming the precursors of chocolate flavor (Schwan and Wheals, 2004). On the other hand, despite the frequent reports of filamentous fungi (Ribeiro et al., 1986; Ardhana and Fleet, 2003; Mounjouenpou et al., 2008; Sanchez-Hervas et al., 2008; Copetti et al., submitted for publication), their function in the process is not clear. Fungal presence in cocoa is generally regarded as undesirable and often related to the formation of off flavors, spoilage and mycotoxin accumulation (Schwan and Wheals, 2004; Gilmour and Lindblom, 2008; Copetti et al., 2010).

In this study, we evaluated the occurrence and distribution of aflatoxigenic species through the stages of cocoa processing at the

* Corresponding author. Tel.: +55 55 3220 8822.
E-mail address: mvc@smail.ufsm.br (M.V. Copetti).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.05.020

farm and their capacity to produce aflatoxins in culture medium, as well as the occurrence of aflatoxins in these products, aiming to correlate them in order to establish the critical steps in aflatoxin production on cocoa beans.

2. Materials and methods

2.1. Samples

Two hundred and twenty six samples of cocoa beans corresponding to varieties of the groups Forastero and Trinitario were collected from farms in Bahia, the main cocoa producing region in Brazil. These samples were made up of 25 cocoa beans at the opening of the pods, 51 from different times of fermentation in wooden boxes (1 to 6 days), 85 from different phases of sun drying on wooden floor platforms with movable roofs (1 to 12 days) and 64 of dried beans in storage.

2.2. Water activity determination

The water activity (a_w) of cocoa bean samples was determined in triplicate in an Aqualab Series 3TE instrument (Decagon, USA) at 25 ± 0.1 °C.

2.3. Identification of fungal species with the potential to produce aflatoxins

Samples (about 200 g) were subsampled (50 g) and surface disinfected in a sodium hypochlorite solution (0.4%) for 2 min. A total of 33 beans (eleven particles per plate) was placed aseptically on Dichloran 18% Glycerol agar (DG18) (Pitt and Hocking, 2009). The plates were incubated for 7 days at 25 °C. After incubation, beans were inspected for fungal growth and all colonies isolated on Czapek Yeast Extract agar (CYA) (Pitt and Hocking, 2009) for subsequent identification of potentially aflatoxigenic fungi based both on macroscopic (colony diameter, color, exudate and soluble pigment production) and microscopic characters, following appropriate keys (Klich and Pitt, 1988a; Samson et al., 2002). The isolation frequency of each species was expressed as the percentage of particles infected by that species.

Fungi identified as potential producers of aflatoxins were inoculated onto Yeast Extract Sucrose agar (Samson et al., 2002) for 7 days at 25 °C and then the agar plug technique (Filtenborg et al., 1983) was used to evaluate the capability of isolates to produce aflatoxins. Fungal extracts taken as plugs with a cork borer were placed on TLC plates, developed in a toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v) mobile phase, and visualized under UV light at 365 nm. A mixture of aflatoxins B_1, B_2, G_1 and G_2 standards (Sigma, St. Louis, USA) was used for comparison.

2.4. Analyses of aflatoxins on cocoa beans

2.4.1. Clean-up

Two g of NaCl were added to 20 g of finely ground cocoa and extracted with 120 mL of methanol: water solution (8:2, v/v). Suspensions were blended (3 min) at high speed (10,000 rpm) using an Ultra-Turrax homogenizer (Polytron, Switzerland). The homogenized solution was filtered through Whatman No. 2 filter paper and Whatman A-H glass microfiber filter (Whatman, England). The filtrate (4 mL) was diluted in phosphate buffered saline (24 mL) and applied to an Aflatest WB immunoafinity column (Vicam, USA) at a flow rate of 2–3 mL/min. The column was then washed with distilled water (30 mL), and aflatoxins eluted with methanol (4 mL) into an amber vial. After evaporation to dryness at 40°C under a stream of N_2, the dry residue was redissolved in methanol:water (2:3, v/v; 1 mL) and filtered through Millex PTFE 0.45 μm (Millipore, USA).

2.4.2. HPLC parameters

A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a fluorescence detector set at 362 nm excitation and 455 nm emission for aflatoxins G_1 and G_2 and 425 nm emission for aflatoxins B_1 and B_2. A Shimadzu CLC G-ODS (4×10 mm) guard column and Shimadzu Shimpack (4.6×250 mm) column were employed. The mobile phase used was water: acetonitrile: methanol (6:2:3, v/v/v) containing KBr (119 mg/L) and nitric acid (4 M, 350 μL/L). The flow rate was 1 mL/min. A mix of aflatoxin B_1, B_2 G_1 and G_2 standard was used to construct a five point calibration curve of peak areas versus concentration (μg/mL). The injection volume was 100 μL for both standard solution and sample extracts. The post-column derivatization of aflatoxins B_1 and G_1 was performed with bromine using a KobraCell (R-Biopharm Rhône Ltd, Scotland).

2.4.1. Optimization of method performance

Two different levels of contamination were tested to determine the recovery of aflatoxins from cocoa using the above methodology, in triplicate. Cocoa was spiked with a mix of aflatoxins containing 0.4, 0.2, 0.15 and 0.18 μg /kg; and 4.0, 2.4, 1.5 and 1.8 μg/kg respectively of aflatoxin B_1, B_2, G_1 and G_2. For limit of detection (LOD) determination, a concentration of aflatoxins close to an estimated detection limit was spiked in 8 cocoa samples and the extractions were performed in parallel. After quantification, the standard deviation was calculated and the limit of detection was determined according to recommendations of Eurachem Guides (1998).

2.5. Statistical analyses

Statistical analyses (correlation analyses) were carried out with the software The Unscrambler® 9.2 (Camo Process AS, Norway). Correlation coefficients (r) were calculated to identify possible associations between the incidence of fungi, the potential aflatoxin producers and aflatoxin B_1 levels (the most prevalent aflatoxin) in the cocoa in all samples evaluated. Interpretation of values was performed according to Pearson's coefficient (r) which are: very weak $0.000 \leq r \geq 0.200$; weak $0.201 \leq r \geq 0.400$; moderate $0.401 \leq r \geq 0.600$; strong $0.601 \leq r \geq 0.800$ and very strong $0.801 \leq r \geq 1.000$ (Christmann and Badgett, 2009).

3. Results and discussion

Cocoa processing on the farm resulted in a large reduction in a_w levels (Table 1). Most cocoa farms visited during this work used empirical methods to decide when beans were dry enough to be stored. Our results indicate that the critical point for aflatoxigenic fungi to infect cocoa beans is the sun drying stage, when the beans start to lose water. The decrease of a_w reduces the number of competitors due to the high sensitivity of bacteria and yeasts to low water availability (Beuchat, 1987). All the farms in this study used a wooden drying floor, where it is difficult to maintain good hygienic conditions and this can contribute to a high fungal load.

Out of 604 isolates of *A. flavus*, 386 (63.9%) were producers of aflatoxins B_1 and B_2. The species *A. parasiticus* and *A. nomius* occurred at a lower percentage, but all 212 isolates of *A. parasiticus* and the 3 *A. nomius* tested were able to synthesize aflatoxins B_1, B_2, G_1 and G_2.

There is a big difference in the distribution of these species in foods as well their capacity to produce aflatoxins. *A. flavus* has a wider distribution and higher frequency of occurrence in foods when compared to *A. parasiticus*. However, according to literature, about half of the isolates of *A. flavus* are aflatoxigenic and this species produces only aflatoxins from the class B, while about 100% of *A. parasiticus* have such ability, and synthesize aflatoxins from groups B and G (Klich and Pitt, 1988b; Vaamonde et al., 2003).

A. flavus was present in samples from all stages after the beginning of cocoa fermentation. About 40% of the samples analyzed from sun

Table 1
Water activity and aflatoxin contamination in cocoa beans at different processing stages at farm.[a]

Stage (n)	a_w	Aflatoxins (μg/kg)				
		B_1	B_2	G_1	G_2	Total
Before fermentation (25)						
Mean	0.99	<LOD	<LOD	<LOD	<LOD	<LOD
Range	0.98–0.99	<LOD	<LOD	<LOD	<LOD	<LOD
Positive (>LOD)	–	0	0	0	0	0
Fermentation (51)						
Mean	0.99	<LOD	<LOD	<LOD	<LOD	<LOD
Range	0.98–0.99	<LOD–0.1	<LOD–0.04	<LOD–0.06	<LOD	<LOD–0.2
Positive (>LOD)	–	2 (4%)	1 (2%)	1 (2%)	0	2 (4%)
Sun drying (85)						
Mean	0.81	0.11	0.02	<LOD	<LOD	0.13
Range	0.49–0.99	<LOD–6.66	<LOD–0.37	<LOD	<LOD	<LOD–7.03
Positive (>LOD)	–	11 (13%)	4 (4%)	0	0	11 (13%)
Positive (>1 μg/kg)	–	2 (2.4%)	0	0	0	2 (2.4%)
Storage (65)						
Mean	0.67	<LOD	<LOD	<LOD	<LOD	<LOD
Range	0.40–0.85	<LOD–0.14	<LOD	<LOD	<LOD	<LOD–0.14
Positive (>LOD)	–	3 (5%)	0	0	0	3 (5%)
LOD	–	0.001	0.002	0.003	0.02	

[a] Method mean recovery: 94.79%.

drying were contaminated, reaching 100% of infected beans in some samples. During storage the contamination by *A. flavus* remained high (32% of infected samples) and so did the number of infected beans in each sample, which also reached 100% in some cases (Table 2).

Compared to *A. flavus*, the isolates of *A. parasiticus* appeared to be more sensitive to a_w reduction. This species was isolated from about 25% of the samples at sun drying and only 14% during storage. A reduction in the number of infected beans was also observed, which reached 78% infection during drying and no more than 48% in storage (Table 2). In this regard, these important aflatoxigenic species may show different physiological patterns.

There are differences between the minimum a_w of a substrate to allow fungal growth and that which is necessary for mycotoxin production; the latter is generally more restrictive. The growth of *A. flavus* and *A. parasiticus* has been verified with a minimum a_w of 0.78 but a minimum a_w of 0.82 and 0.86 is required for *A. flavus* and *A. parasiticus* to produce aflatoxins, respectively (Pitt and Hocking, 2009). These a_w values were still found at the sun drying stage (Table 1).

Among the samples collected before the beginning of fermentation and during the fermentation, none showed any contamination by aflatoxins. Table 1 shows the results of aflatoxin analyses in samples collected during the primary processing of cocoa beans on the farm.

At the stage of sun drying on platforms, only 11 (13%) samples were contaminated with aflatoxins (Table 1). From these, two samples presented total aflatoxin levels of 0.1 and 0.5 μg/kg, 3 samples exceeded 0.5 μg/kg, one of these reaching 6.66 μg/kg aflatoxin B_1. *A. flavus* was isolated in the 3 samples with higher levels of aflatoxins and *A. parasiticus* was co-occurring in one sample. This low level of aflatoxins in the samples was unexpected given the high frequency of occurrence of aflatoxigenic fungi in the samples, and it can be visualized through Pearson's correlation coefficient values analyses (Table 3).

There was a weak correlation between contamination by aflatoxin B_1 and presence of aflatoxigenic fungi (both *A. flavus* or *A. parasiticus*), suggesting the existence of anti-toxigenic properties in cocoa, limiting the accumulation of aflatoxins in this product (Table 3).

The action of polyphenols on the synthesis and accumulation of aflatoxins in food has been observed by Molyneux et al. (2007). They evaluated the effect of different phenolic constituents commonly present in oilseeds (walnuts, almonds and pistachios) on mycotoxin production and found inhibition between 59.5 and 99.8% of the synthesis of aflatoxin. It is known that cocoa is a product rich in polyphenols (Wollgast and Anklam, 2000; Lee et al., 2003), which could act as inhibitors of aflatoxins in this product.

A study conducted by Hasan (1999) evaluated the role of caffeine and tannin (a polyphenol) in anti-toxigenic properties of coffee and tea, since *A. flavus* is a common contaminant of these products, and the amount of aflatoxin produced in these products was very low. The researcher found a 5-fold increase in aflatoxin production in detannin-caffeinated coffee and tea compared with normal, when inoculated with *A. parasiticus*. The addition of tea extract to a culture

Table 2
Isolation frequency of aflatoxigenic species and incidence of infected cocoa beans at different processing stages.[a]

Stage	Fermentation		Drying		Storage	
(n)	(51 samples)		(85 samples)		(65 samples)	
	IF (%)	RI (%)	IF (%)	RI (%)	IF (%)	RI (%)
Aspergillus flavus	3.92	ND-6	39.51	ND-100	32.31	ND-100
Aspergillus nomius	0	ND	3.70	ND-3	0	ND
Aspergillus parasiticus	1.96	ND-6	25.92	ND-78	13.85	ND-48

RI = Range of Infection % (Range of infected beans in a sample, %).
[a] IF = Isolation frequency % (number of samples containing a fungal species/ total of samples evaluated, %).

Table 3
Correlation coefficients (*r*) of aflatoxigenic fungi, water activity and aflatoxin B_1.

Parameter	Aflatoxin B_1	a_w
Aflatoxin B_1	1.000	0.004
Water activity	0.004	1.000
Total fungi contamination (all fungi isolated)	0.085	−0.292
Aspergillus flavus	0.383	−0.270
Aspergillus parasiticus	−0.270	−0.003

of *A. parasiticus* was more effective than the coffee extract, reaching 95% of inhibition on aflatoxin production. According to the author, this effect could be primarily attributed to tannin, followed by caffeine.

Lenovich and Hurst (1979) carried out a study on aflatoxin production in cocoa beans. Two isolates of highly aflatoxigenic *A. parasiticus* were tested and the results showed that the higher the caffeine content in the three cocoa varieties tested, the lower was the production of aflatoxins. This correlation was not observed when considered the theobromine content in the beans. In another study analyzing 13 varieties of cocoa, Lenovich (1981) found that only very low levels of aflatoxins were produced in varieties of cocoa with caffeine content exceeding 1.8 mg/g. The author suggests that the caffeine content could explain the usual absence of aflatoxin in cocoa beans.

The aflatoxin contamination in stored samples was quite low, with only three contaminated samples and a maximum level of 0.14 µg/kg of aflatoxin B_1. Campbell (1969) reported the presence of aflatoxins at levels up to 17 µg/kg in 2 of 9 cocoa beans samples analyzed. An extensive project evaluating the presence of aflatoxins in cocoa beans and products of cocoa processing was conducted in Germany. The results found showed that 73.5% of the 334 products examined contained traces of aflatoxins (LOD = 0.01 µg/Kg). Contamination exceeding 1 µg/kg was found in 8% and greater than 2 µg/kg in 3% of the samples. A maximum level of 31.5 µg/kg was found in cocoa beans (Raters and Matissek, 2000).

There was also a very weak correlation observed between the level of beans contaminated by total fungi and the levels of aflatoxin B_1 present. It indicates that presence of moldy cocoa beans do not necessarily indicate an aflatoxin contaminated sample. These data are consistent with observations made by Raters and Matissek (2000). The authors conducted a study on the effect of storage of cocoa beans under abusive conditions of humidity on production of aflatoxins. Regardless of the strong fungal growth, no change in the aflatoxin content was observed. These observations suggest that the storage period should not be critical for the production of aflatoxin in cocoa.

Despite the high number of aflatoxigenic fungi isolated during processing of cocoa at the farm, the levels of aflatoxins found in the samples evaluated in this study were low, suggesting the existence of factors limiting the synthesis of aflatoxins in cocoa beans.

Acknowledgements

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Proc. 2005/60236-1, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Ardhana, M.M., Fleet, G.H., 2003. The microbial ecology of cocoa bean fermentations in Indonesia. International Journal of Food Microbiology 86, 87–99.

Beuchat, L.R., 1987. Influence of water activity on sporulation, germination, outgrowth, and toxin production. In: Rockland, L.D., Beuchat, L.R. (Eds.), Water Activity: Theory and Applications to Food. Marcel Dekker, New York, pp. 153–172.

Campbell, T.C., 1969. 83rd Annual meeting of the AOAC, Report presented to the Joint AOAC-AOCS Committee on aflatoxins, October, 13–16, Washington DC.

Cary, J.W., Ehrlich, K.C., 2006. Aflatoxigenicity in *Aspergillus*: molecular genetics, phylogenetic relationships and evolutionary implications. Mycopathologia 162, 167–177.

Christmann, E.P., Badgett, J.L., 2009. Interpreting assessment data: statistical techniques you can use. NSTA Press. Chapter 5.

Copetti, M.V., Pereira, J.L., Iamanaka, B.T., Pitt, J.I., Taniwaki, M.H., 2010. Ochratoxigenic fungi and ochratoxin A in cocoa during farm processing. International Journal of Food Microbiology 143, 67–70.

Copetti, M. V., Pereira, J. L., Iamanaka, B. T., Frisvad, J. C., Taniwaki, M. H., 2011. Mycobiota of cocoa: from farm to chocolate. (submitted).

Cotty, P.J., Jaime-Garcia, R., 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. International Journal of Food Microbiology 119, 109–115.

Eurachem Guides, 1998. The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics. LGC, Teddington.

Filtenborg, O., Frisvad, J.C., Svendsen, J.A., 1983. Simple screening method for molds producing intracellular mycotoxins in pure cultures. Applied and Environmental Microbiology 45, 581–585.

Frisvad, J.C., Skouboe, P., Samson, R.A., 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B_1, sterigmatocystin, and 3-*O*-methylsterygmatocystin, *Aspergillus rambelly* sp. nov. Systematic and Applied Microbiology 28, 442–453.

Gilmour, M., Lindblom, M., 2008. Management of ochratoxin A in the cocoa supply chain: A summary of work by the CAOBISCO/ECA/FCC working group on ochratoxin A. In: Leslie, R., Bandyopadhyay, R., Visconti, A. (Eds.), Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade. CABI, Wallinford, pp. 231–243.

Hasan, H.A.H., 1999. Role of caffeine and tannin in anti-toxigenic properties of coffee and tea. Cryptogamie Mycologie 20 (1), 17–21.

IARC (International Agency for Research on Cancer), 1993. IARC monographs on the evaluation of the carcinogenic risks to humans, Vol. 56, some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Press, Lyon.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1998. Safety evaluation of certain food additives and contaminants – Aflatoxins. World Health Organization, Geneva.

Klich, M.A., Pitt, J.I., 1988a. A laboratory guide to common *Aspergillus* species and their teleomorphs. Commonwealth Scientific and Industrial Research Organization, Sydney. 115 pp.

Klich, M.A., Pitt, J.I., 1988b. Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus* and other closely related species. Transactions of the British Mycology Society 91, 99–108.

Kurtzman, C.P., Horn, B.W., Hesseltine, C.W., 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie van Leeuwenhoek 53, 147–158.

Lee, K.W., Kim, Y.J., Lee, H.J., Lee, C.Y., 2003. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. Journal of Agricultural and Food Chemistry 51, 7292–7295.

Lenovich, L.M., 1981. Effect of caffeine on aflatoxin production on cocoa beans. Journal of Food Science 46, 655–657.

Lenovich, L., Hurst, J.W., 1979. Production of aflatoxin in cocoa beans. Journal of the Association of Official Analytical Chemists 62 (5), 1076–1079.

Molyneux, R.J., Mahoney, N., Kim, J.H., Campbell, B.C., 2007. Mycotoxins in edible tree nuts. International Journal of Food Microbiology 119, 72–78.

Mounjouenpou, P., Gueule, D., Fontana-Tachon, A., Guyot, B., Tondje, P.R., Guiraud, J.P., 2008. Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon. International Journal of Food Microbiology 128, 234–241.

Pitt, J.I., Hocking, A.D., 2009. Fungi and food spoilage, third ed. Springer, New York.

Raters, M., Matissek, R., 2000. Vorkommen der mykotoxine aflatoxin B_1, B_2, G_1, G_2 und ochratoxin A in kakao und kakaoprodukten. Available at Projekt Nr. 9 der Stiftung der Deutschen Kakao- und Schokoladenwirtschaft, Hamburg http://www.kakao-stiftung.de/menu4_frameset_e.html. Accessed 28/08/10.

Ribeiro, N.C.A., Bezerra, J.L., Lopez, A., 1986. Micobiota na fermentação do cacau no estado da Bahia, Brasil. Revista Theobroma 16, 47–55.

ROC (Reports on Carcinogens), 2003. Aflatoxins. Available at eleventh ed. U.S. Department of Health and Human Services http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s006afla.pdf. Accessed 10/02/11.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O., 2002. Introduction to food- and airborne fungi. CBS, The Netherlands.

Sanchez-Hervas, M., Gil, J.V., Bisbal, F., Ramon, D., Martinez-Culebras, P.V., 2008. Mycobiota and mycotoxin producing fungi from cocoa beans. International Journal of Food Microbiology 125 (3), 336–340.

Schwan, R.F., Wheals, A.E., 2004. The microbiology of cocoa fermentation and its role in chocolate quality. Critical Reviews in Food Science and Nutrition 44, 1–17.

Vaamonde, G., Patriarca, A., Pinto, V.F., Comeria, R., Degrossi, C., 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* Section *Flavi* from different substrates in Argentina. International Journal of Food Microbiology 88, 79–84.

Wilson, D.M., Payne, G.A., 1994. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In: Eaton, D.L., Groopman, J.D. (Eds.), The toxicology of aflatoxins. Human Health, Veterinary, and Agricultural Significance. Academic Press, San Diego, pp. 383–406.

Wollgast, J., Anklam, E., 2000. Review on polyphenols in *Theobroma cacao*: changes in composition during manufacture of chocolate and methodology for identification and quantification. Food Research International 33, 423–447.