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Selenium and Aflatoxins in Brazil Nuts

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1. Introduction

Brazil nuts (*Bertholletia excelsa* H.B.K.) are considered a high nutritious food. Apart from carbohydrates, lipids, sulphur proteins and minerals, Brazil nuts are known to be rich in selenium - Se (Barclay et al., 1995; Coutinho et al., 2002, Souza et al., 2004, Pacheco and Scussel, 2007). Despite of that, when their shell are cracked either when pods fall on the ground, or during pod opening for nut extraction (done by an axe) and exposed to high moisture and temperature of the tropical forest, fungi may grow, leading to nut spoilage. If fungi are toxigenic they may produce aflatoxins (AFLs).

Se has been reported to be an antioxidant and studies have reported differences on its levels in Brazil nuts from the two Amazon regions being the Eastern nuts richer in Se than the Westerns (Chang et al., 1995; Pacheco and Scussel, 2007). Its content may vary when grown in different soils of the Amazon basin. The aflatoxigenic *Aspergillus* species of *A. flavus* and *parasiticus* are intimately related to agricultural crops, including tree nuts and their growth are influenced by environmental conditions. Although Brazil nuts have tested positive for *A. flavus*, less is known about its populations on Brazil nuts, how they grow and vary among the two Amazon regions, in the different stages of nut collection prior reaching the factory and how the processing affect them (Castrillon and Purchio, 1988; Freire et al., 2000; Candlish et al, 2001, Caldas et al., 2002). However, AFLs have been reported contaminating Brazil nuts. Their pods are harvested after they fall onto the forest soil. They stay directly in contact with the soil for several days or weeks prior to collection. It is during that time that pods may get contaminated with *Aspergillu*s sp. and so with AFLs. Post harvesting operations are expected to have major influence on further contamination of the nuts (Bayman, 2002; Campos/Pas, 2004). For fungi growth and for their normal maintenance, a number of metals are required in different amounts. Many microorganisms are known to be able to use Se (i.e., selenite, selenate of other forms) in their metabolism (Roux et al., 2001; Fleet-Stalder et al., 2000). Se has been added to the media as sodium selenite (Na_2SeO_3), to understand its effect in different concentrations on fungi behaviour. The inhibitory action of Se on the growth rate of various fungi such as *Aspergillus*, *Penicillum* and *Fusarium* was reported by several authors (Ramadan et al., 1983, Ragab, et al., 1986, Zohri et al., 1997, Li et al, 2003). When it was used in Czapek Dox agar medium to evaluate the *A. parasiticus* behavior concerning morphological growth and toxin production, it was observed that fungus growth decreased by the increasing of the Se concentration (Zohri et al., 1997). In an

experiment carried out by Li et al. (2003) utilizing Na_2SeO_3, the authors observed from stimulating to toxic effects on organisms depending on the levels applied in the media and concluded that the presence of Se in high concentration in the growth environment can lead to morphological distortion of the fungi characteristics. Se compounds have been reported also been acting as antagonists to the mutagenic and carcinogenic activities of some agents such as UV light and AFLs (Martin et al., 1984, Gregory, 1984, Overvad et al., 1985). AFLs are produced by *Aspergilllus flavus* in the Brazil nuts that have Se in their composition and the Amazon forest has optimal conditions for fungi growth such as high temperatures (> 25 ºC) and relative humidity (RH) > 80 % (Bayman et al., 2002; Freire et al, 2000; Scussel, 2004; Arrus et al., 2005a; Arrus et al, 2005b; Pacheco and Scussel, 2006). Considering that Brazil nuts are rich in Se, its concentration may vary among regions, AFL contamination has been reported on Brazil nuts and that Na_2SeO_3 has either stimulation or toxic effects on organisms depending on its levels in media, a work was carried out in order to evaluate the effect of Brazil nut Se content from different Amazon regions and Na_2SeO_3 on the aflatoxigenic *A. flavus* FC1087 in terms of (a) fungal growth behavior, (b) colonies characteristics and (c) AFL production.

2 Material and methods

2.1 Material

a. *Brazil nuts:* two batches of raw, medium size nuts, with 18.4 and 43.5 mg/kg Se in their composition. Those nuts were from the Western and Eastern regions of the Brazilian Amazon basin, respectively. Nuts were previously tested for AFL. No AFL was detected up to the method limit of detection (LOQ) of 1.95 μg/kg.
b. *Toxigenic Aspergillus strain:* aflatoxigenic *A. flavus* FC1087 isolated from the Amazon forest and supplied by the Oswaldo Cruz Foundation (Fiocruz), Manaus, Amazon State, Brazil. AFLs total (AFB_1+AFB_2+AFG_1+AFG_2) and AFB_1: 90.3 and 109.2 μg/kg, respectively.
c. *Culture media*: aqueous tween 80 and potato dextrose agar (PDA) from Merck.
d. *Se standard:* Na_2SeO_3, analytical grade, Baker. *(d.1) for ICP analysis:* acidified aqueous solution with nitric acid at 10 % (certificate N° *SRM 3149)*, NIST and *(d.2) for mycological study:* acidified aqueous stock solution (100 μg/mL) with sulfuric acid at 10% - sterile.
e. *Aflatoxin standards:* AFB_1, AFB_2, FG_1 and AFG_2 from Sigma.
f. *Chemicals:* acetonitrile and methanol (HPLC grade), Baker. Ammonium acetate, ammonium sulfate, hydrochloric acid, nitric acid and anhydrous sodium sulfate (analytical grade) also from Baker. Ultrapure water (Mili-Q), Milipore.
g. *Equipment* for *(g.1) Se analysis:* atomic emission spectrophotometer with inductively coupled plasma (ICP) -optical emission spectrometry (OES), Model Otima 2000, Perkin Elmer and for *(g.2) Aflatoxins analysis:* ultra violet cabinet (365 nm), Tecnal; spectrophotometer, Hitachi; (g.3) *Mycology*: bacteriological oven and autoclave, Fanem, colony counter, Marconi and microscope stereoscope, lenses 40X, model Q714TZ-1, Quimis. Thin layer chromatographic aluminum sheets (20 x 20) with G60 silica gel from Merck and industrial Brazil nut cracker, CIEX.
h. *Other materials:* sterilized stainless steel blenders, trays (400 x 250 mm) and scissors. Sterilized polyethene bags. Round and straight platinum wires, Petri dishes - plates (90x15 mm, volume: 15 ml) and Neubauer counting chamber, Optik.

2.2 Methods

a. *Brazil nut preparation:* 1 kg of in-shell Brazil nuts, of each Se content, after de-shelling was finely grounded (particle size <100 μm), homogenized and three portions of 250 g were separated for Se, AFLs and the mycology tests.
b. *Se analysis*: the method used was that reported by US EPA (1996) using atomic emission spectrometry-ICP-OES. The limit of detection (LOD) was 2.00 mg/kg and the limit of quantification (LOQ) was 3.50 mg/kg. LOQ was defined as the lowest point of the calibration curve with high repeatability-axial view. Three replicates each (n=3).
c. *AFLs analysis:* by AOAC (2005) with a LOD and LOQ for total AFLs of 0.97 and 1.95 μg/kg, respectively. Two replicates each (n=3).
d. *Se culture media preparation:* Media were divided into three groups containing different Se origin and concentrations. They were prepared by adding Se in PDA as follows. **Group I**: ground Brazil nuts from Western (18.4 mg/kg) and Eastern (43.5 mg/kg) Amazon basin (portions of each Brazil nut batch were added to PDA to obtain final Se concentration of 0.018 and 0.044 mg/kg in the plates, respectively). **Group II**: Se as Na_2SeO_3 into six different Se concentrations (volumes of the Na_2SeO_3 solution was added to PDA accordingly to get the following increasing concentrations: 0.01; 0.02; 0.1; 0.2; 0.4 and 0.6 mg/kg in the plates). Poured plates were gently shaken to allow proper homogenization of the metal into the medium. **Group III:** no Se was added to the media - as a Control - to evaluate the strain normal behaviour. The media were sterilized by autoclavation for 15 min at 121°C. See details of the media on the Groups and Se concentration in Table 1.
e. *Spore suspension preparation:* the suspension concentration of the *A. flavus* spores was set using a Neubauer counting chamber and diluting the reference strain original suspension in 0.2% aqueous tween 80 to get a final concentration of 3.0 x 10 spores/mL.
f. *Fungal total count:* the suspension was spread on the media plates (Group I; Group II and Group III) previously prepared and incubated for 5 days at 28 °C. After that period the total colonies were counted utilizing the colony counter. Note: to observe further colony behavior and development, the plates were kept incubated up to 14 days at the same temperature. Four replicates each (n=4).
g. *The effect of Se on A. flavus* FC1087*:(g.1) growth and colony features*: the fungi spore suspension was also inoculated on another set of the three Groups media, by means of a straight wire to give a single point inoculum on the plate centre, to evaluate the colony radius growth (by a fine measuring scale) and other features behaviour such as shape and colour. After fungi inoculation, dishes were incubated for 14 days at 28 °C. Fungi growth rate related to Se content was checked every day by measuring their radius, examining their morphological changes and their verse and reverse colour. At the end of the incubation period, the morphological colonies features (size, colour, shape, reverse colour) were registered (Table 1). Colony colour was defined according to the Methuen Handbook of Colour (Kornerup and Wanscher, 1989).
g. *(2) AFL production*: The strain AFL production related to Se concentration was evaluated in each media by examining the AFL characteristic fluorescence (white/bluish) development. Each media fluorescence positive had their AFLs extracted (Moss and Badii, 1982) and analyzed as in (c). Four replicates each (n=4).
h. *Statistical analysis:* the data were statistically analyzed using analysis of variance (ANOVA).

3 Results and discussion

The influence of Se on fungi growth, colony diameter, verse/reverse colour, fluorescence development and AFL production are shown in Table 1. The data obtained shows that Se affected the *A. flavus* F1087 strain proliferation and AFL production when inoculated in media added either of ground Brazil nuts or Na_2SeO_3 at different concentrations.

3.1 Effect of Se concentration on the total fungi growth

A. flavus FC1087 was able to grow in presence of both Brazil nut Se concentrations (18.4 and 43.5 mg/kg: Western and Eastern region nuts, respectively) – Group I. It was observed that the highest total *A. flavus* colonies count and growth rate was obtained in the media containing the Western Brazil nuts (Se: 0.02 mg/kg in the plate with 2.3 x 10 cfu) and so for the Control (2.8 x 10 cfu) - Group III. The Eastern nuts media with higher Se content (0.04 mg/kg) presented lower number of colonies than the Western's (1.2 x 10 cfu). Similar to the Eastern nut total colonies count, was observed in the tested Na_2SeO_3 media concentrations (0.01 to 0.20 mg/kg) – Group II, with total counts of 1.5, 1.3, 1.3 and 1.1 x 10 cfu, respectively. Growth decreased from Na_2SeO_3 level of 0.20 to 0.4 mg/kg reaching 0.9 x 10 cfu. At the highest Na_2SeO_3 content (0.60 mg/kg) fungi failed completely to grow, probably because of its toxic level to the strain. These findings are corroborated by previous authors (Badii et al, 1986; Aboul-Dahad 1991; Zohri et al, 1997) that have also found reduction on fungi growth as the concentration of Na_2SeO_3 increase in the media. The toxic Se effect at higher doses reduces microorganism growth due to the detoxification process that takes place. That involves fungi transformation of the inorganic Se (more toxic) to organics (less toxic). It also involves the reduction of the Se oxyanions, Na_2SeO_3 and selenate to inert elemental Se within the mycelium. The toxic action of Se in fungi is believed to be due to its incorporation into the protein amino acids instead of sulfur which can lead to alteration of the tertiary structure thus to dysfunction of the fungi proteins and enzymes (Gharieb and Gadd, 1998). Figure 1 shows the *A. flavus* FC1087 growth from Day 5 to Day 14 of incubation in the media containing Se Western and Eastern Brazil nuts Se.

3.2 Effect of Se concentration on *A. flavus* FC1087 colony features

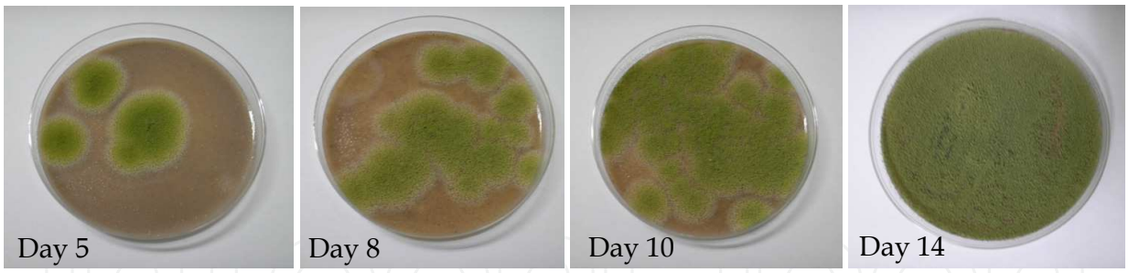
When *A. flavus* strain was inoculated in a single point on the media that contained either the two Brazil nut Se concentrations (Group I) or the six increasing Na_2SeO_3 concentrations (Group II), it was possible to observe their diameter, colours (verse/reverse) and fluorescence variations. Their diameters increased (5±2 to 19±4 mm) with the reduction of Na_2SeO_3 concentration (0.4 to 0.01 mg/kg). The highest colony diameter was obtained in the Western Brazil nut media with 31±5 mm. Eastern nuts colonies diameter was smaller with only 18±2 mm. Most of the colonies grown in Na_2SeO_3 media presented a characteristic green colour with the exception of the ones grown in the lowest Na_2SeO_3 concentration (0.01 mg/kg) that had only the centre green with a white, broad, non sporulating margin. That non sporulating margin was also observed in the Brazil nut both regions media colonies. On the other hand, the colonies reverse colour changed as the selenite concentration increased in the media from brown (lower Na_2SeO_3 concentration = 0.01 and 0.05 mg/kg) to orange-red (higher Na_2SeO_3 concentration = 0.2 and 0.4 mg/kg). Some authors have studied and reported these colour changes. McCready et al. (1966) reported that orange intracellular granules in bacteria (*Salmonella Heidelberg*), when grown in the presence of Na_2SeO_3, as amorphous red elemental Se. Badii et al in 1986, also observed a deep orange pigment on the

undersurface of the *A. parasiticus* colonies in presence of Na_2SeO_3. Aboul-Dahab (1991) found that these colours were due to the reduction of Na_2SeO_3 with deposition of elemental Se within the fungal cells, as well as, in the growth media. Presumably, the fungus metabolizes Se to produce strong reducing and oxidizing agents into the media environment. Moss et al. (1987) reported that *A. parasiticus* is able to reduce also sodium bis selenite with the deposition of granules of elemental Se within the mycelium, being that red-orange colour a biological reduction of Se compounds into elemental colloidal Se. As far as the fluorescence of possible AFL presence is concerned, all media, containing Se despite of its origin or concentration, presented the characteristic white/bluish fluorescence and so the Control plates (except at the selenite highest concentration). That was an indication of the AFL presence, thus they were submitted to AFL analysis. However, it was observed that the fluorescence intensity reduced as the Na_2SeO_3 level increased in the media, reaching the highest concentration (0.60 mg/kg) with no fluorescence at all. In fact, *A. flavus* FC1087 failed to grow at that concentration caused probably by the toxic effect of the Na_2SeO_3 (Zohri, et al., 1997, Li et al., 2003). Figure 2 shows the *A. flavus* FC1087 growth in selenite at the 0.01 and 0.4 mg/kg with the development of orange to red pigment.

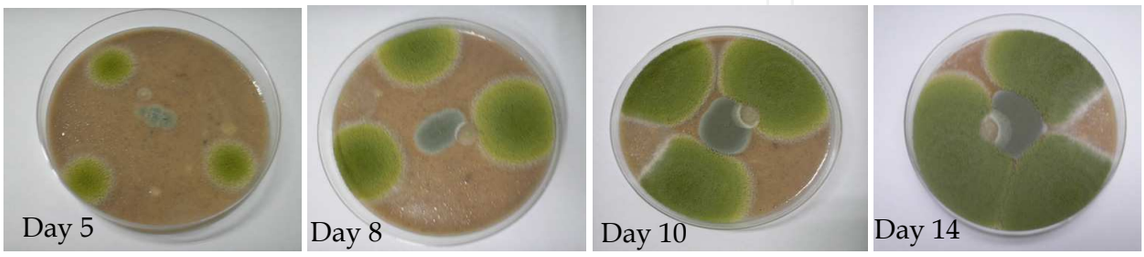
PDA[a]		Total fungi count (cfu/ml)	Colony features				Aflatoxin (µg/kg)	
Group	Se content[a] (mg/kg)		Diameter[b] (mm)	Colour		Fluorescence[c]		
				Verse	Reverse		Total[d]	AFB_1[e]
I	**Brazil nuts**							
	Western[f] 0.02	2.3 x 10	31±5	Green[g]	Colourless	+	ND	ND
	Eastern[h] 0.04	1.2 x 10	18±2	Green[g]	Orange Red	+	50.2±0.6	28.5±0.6
II	**Na_2SeO_3[i]**							
	0.01	1.5 x 10	19±4[j]	Green[g]	Brown	+	98.7±0.3	75.0±0.5
	0.05	1.3 x 10	13±4	Green	Brown	+	102.6±0.3	70.4±0.2
	0.10	1.3 x 10	10±3	Green	Light brown	+	90.3±0.5	67.9±0.3
	0.20	1.1 x 10	9±2	Gray	Orange Red	+	70.3± 0.5	49.2±0.5
	0.40	0.9 x 10	5±2	Gray	Orange Red	+	30.6±0.5	19.2±0.5
	0.60	None	NG[j]	NA[k]	NA	-	NA	NA
III	**Control**							
	NS[l]	2.8 x 10	30.5±5	Green	Colourless	+	109.2 ±0.2	90.3±0.5

[a] Potato dextrose Agar and Se concentration in each Petri dish
[b] The values of colony diameters are means based on four replicates (n = 4)
[c] Under ultra violet light at 365 nm
[d] ∑ total AFL LOD: 0.390 µg/kg
[e] AFB_1 LOD: 0.04 µg/kg
[f] PDA+ Brazil nuts from the Western Amazon region with Se content of 18.4 mg/kg
[g] With white broad nonsporulating margins
[h] PDA+ Brazil nuts from the Eastern Amazon region with Se content of 43.5 mg/kg
[i] In Se standard acidic solution (sodium selenite)
[j] No fungi growth
[k] Not applicable
[l] No Se added

Table 1. Effect of Brazil nuts natural Se content and Se as Na_2SeO_3 on the behaviour aflatoxigenic *A. flavus* FC1167

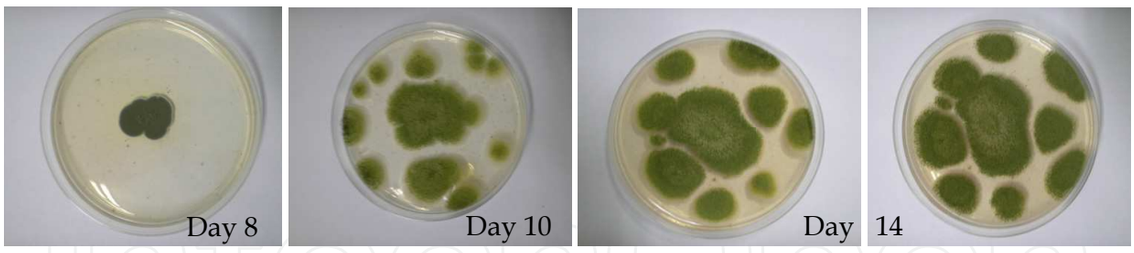


(a) PDA media with Western region Brazil nuts: 0.02 mg/kg - green colour with non-sporulating margin

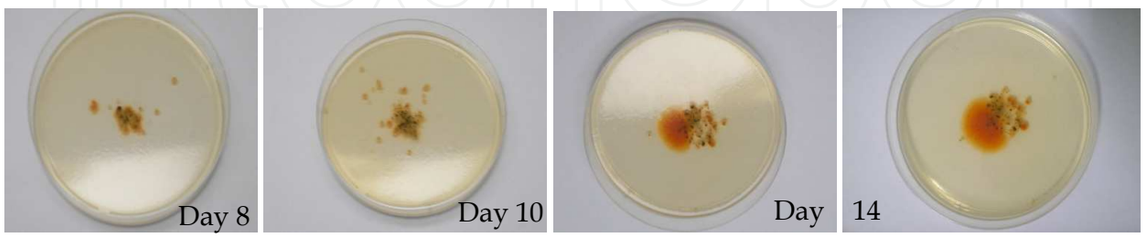


(b) PDA media with Eastern region Brazil nuts: 0.04 mg/kg- green colour with non -sporulating margin

Fig. 1. Toxigenic *Aspergillus flavus* FC1087 strain growth during 14 days of incubation at 28°C in PDA media containing natural Se content of ground Brazil Nut from (a) Western and (b) Eastern Amazon regions (*spreading technique*); Se content in the nuts: 18.4 and 43.5 mg/kg, respectively.



(a) PDA media with Se as Na_2SeO_3: 0.01 mg/kg - green colour



(b) PDA media with Se as Na_2SeO_3: 0.40 mg/kg – orange to red colour

Fig. 2. Toxigenic *Aspergillus flavus* FC1087 strain growth during 14 days of incubation at 28°C in PDA media containing Se as Na_2SeO_3 solution (*single point technique*); Se content (a) 0.01 and (b) 0.40 mg/kg.

3.3 Effect of Se concentration on *AFIs* production

AFls were detected in the media, containing Eastern Brazil nuts and Na_2SeO_3 media, with the exception of the 0.60 mg/kg Na_2SeO_3 media, as no *A. flavus* was able to grow. Only in the Brazil nut medium with 0.04 mg/kg of Se, corresponding to nuts from the Eastern Amazon region, the strain produced AFL at a level of 50.2±0.6 μg/kg for total AFL and 28.5±0.6 μg/kg for AFB_1. No AFLs were detected in the nuts from Western region, however fungi growth were much abundant in that media, and larger the colonies diameter (31±5 mm). *A. flavus* FC1087 was able to produce in the Control media 109.2 ±0.2 μg/kg of AFL. It was observed that, the strain was Na_2SeO_3 doses-dependent either for its AFL synthesis and growth, as an AFL decrease with the increased of Na_2SeO_3 concentration occurred, concomitant to the fungi growth (Table 1). Both, the total AFLs (AFB_1+AFB_2+AFG_1+AFG_2) and AFB_1 production decreased as the Na_2SeO_3 levels increased. Similar to the Control plate, tested fungus was able to produce high amounts of AFls total, reaching at the two lowest Se concentrations (0.01 and 0.05 mg/Kg) a total AFLs of 98.7±0.3 and 102.6±0.3 μg/kg, respectively. In contrary, at the highest concentrations of 0.2 and 0.4 mg/kg, to where fungi were still able to grow, toxin production reduced from 70.3±0.5 to 30.6±0.5 μg/kg. The higher Se content in the nuts, the less fungi proliferation and rate in the plate. That could be caused by the Se toxic effect on the strain leading to fungi stress thus activating second metabolism of AFL formation. That could be explained for the toxicity of high amount of Se in organism, maybe causing oxidative stress in the strain (Letavayova et al., 2006, Valko et al., 2006). The oxidative stress is a prerequisite for AFL production by *A. parasiticus* (Jayashree and Subramanyam, 2000). Thus, a Se amount could activate the mechanisms of AFL production and contaminating Brazil nuts. Bronzetti *et al* (2001) demonstrated Se compounds in yeasts exerted both mutagenic and anti-mutagenic effect at different concentrations. On the other hand, other factors, such as the interaction or the competition with different strains of *Aspergillus* seem to affect the increasing of AFL production in some substrates (Martins, Martins, Bernardo, 2000).

3.4 Brazil nut composition, Se content versus fungi and AFIs production

As far as Brazil nuts Se concentration is concerned, it is important to emphasize that there are two approached to take into account related to its benefits: First is the Se content in the nuts for (a) human consumption against diseases and the other is for (b) fungi proliferation and AFLs production in the nuts substrate. (a) Se is important to health as it has been reported being an excellent antioxidant for reducing toxic effects such as the carcinogenicity of some compounds. Its antioxidant protective effect is primarily associated with the presence of glutathione peroxides that protect DNA and other cellular components from damage by oxygen radicals. Se is an essential component of glutathione peroxides (Agar and Alpsoy, 2005). It inclusive, in some concentrations (such as 8 mg/kg), can inhibit the AFB_1 and AFG_1 mutagenic and carcinogenic effects in human blood cell culture (Geyikoglu and Turkez, 2006). (b) Regarding fungi and AFLs, in our study, it was observed that the *A. flavus* FC1087 on the Western Brazil nuts media presented similar behavior as the Control with 2.3 x 10 total count / 31±0.5 diameter / green colour / colourless reverse colonies however without AFLs production. On the other hand, the Eastern's media presented lower total *A. Flavus* FC1087 count, growth rate, and diameter, however with AFL production. Data suggests that: the higher Se content in the nuts, the less fungi proliferation and rate in the plate and high AFLs. That could be caused by the Se toxic effect on the strain leading to fungi stress thus activating the second metabolism of AFL formation.

4. Conclusion

The higher Se content present in the Brazil nuts lead to less fungi proliferation, growth rate in the plate and AFL production. That could be caused by its toxic effect to the fungus provoking stress and activating AFL production. Despite of data obtained, further studies need to be carried out utilizing Brazil nuts media with a more wide Se concentration range to find out the Se role on fungi and AFL production. It would be also necessary to investigated possible interactions and/or competition of different strains of *Aspergillus* on AFL production in Brazil nuts.

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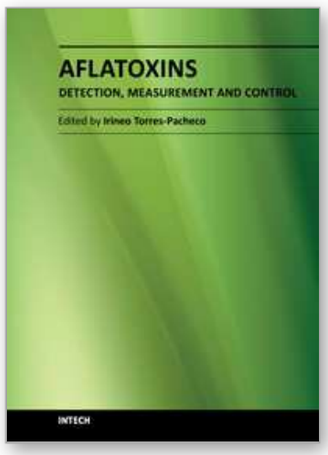

Aflatoxins - Detection, Measurement and Control

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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical prespective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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