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Logistic regression applied to the incidence of *Aspergillus* producer of mycotoxin in cocoa beans cultivated in the state of Rondônia, Brazil

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The presence of fungi of the genus *Aspergillus* must be investigated because it is related to the diminishment of quality and consequently to the risk to health of consumers due to the possible production of mycotoxins. In this context, this study was performed with the aim to evaluate the presence of *Aspergillus* species in cocoa beans produced in the state of Rondônia, Brazil, as well as verify the toxigenic potential of these species and the effect of the geographical origin on the incidence of toxigenic and non-toxigenic species of fungi. The technique of logistic regression was used to estimate the probability of occurrence of the genus *Aspergillus*. A total of 185 *Aspergillus* were identified. Among the tested *Aspergillus carbonarius,* 79.41% produced ochratoxin on levels from 0.08 to 44.09 µg/g in the culture medium. For the other *Aspergillus* species, the production potential was only assessed, and 3.13% of the *A. niger* presented potential to produce OTA and 83.33% of *A. flavus* which was able to produce aflatoxins. The presence of these toxigenic species indicates a potential risk of mycotoxin in cocoa beans and in their derived products in the studied region. Besides, the occurrence of *Aspergillus* species differed in function of geographical coordinates and temperature, presenting higher probability of occurrence on cocoa beans of municipalities located in Southern Rondônia, Brazil.

Key words: Cocoa beans, *Aspergillus,* mycotoxins, ochratoxin A, aflatoxins, logistic regression, thin layer chromatography, high performance liquid chromatography.

INTRODUCTION

The importance of cocoa to the industry is attributed to its beans, from which the chocolate and other by products are obtained. Brazil is the sixth largest cocoa producer of the world (International Cocoa Organization, 2013),

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highlighting the state of Rondônia between the three largest national producers (Brazilian Institute of Geography and Statistics, 2013). The state of Rondônia is located in the Western Amazon in northern Brazil and has suitable conditions for the production of this Brazilian commodity. It is located at 11°30'20'' south latitude and 63°34'20'' west longitude. The average annual temperature is 24-26°C, precipitation of 1400-2600 mm/year and has hot and humid climate.

The primary cocoa processing occurs on farms and involves the fruit opening stages, fermentation, drying and storage. The microbial succession that naturally occurs during the fermentation of cocoa was already established and observed on studies developed on Brazil. According to Schwan and Wheals (2004), the cocoa seeds are contaminated with microorganisms when the fruit is opened by the cutting tool, when they are transported on baskets and also through insects that may be present on the local. These microorganisms contribute to the process of spontaneous fermentation that occurs thereafter (Schwan and Wheals, 2004).

On the steps of fermentation, drying and storage, the contamination by filamentous fungi on cocoa beans may occur (Mounjouenpou et al., 2008; Sánchez-Hervás et al., 2008) and the presence of these fungi may cause the cellulose hydrolysis, production of acids and undesirable flavors on the beans (Schwan and Wheals, 2004). Furthermore, some species of these fungi may produce mycotoxins, which may endanger its consumption (Copetti et al., 2010, 2011a, b; Mounjouenpou et al., 2008; Sánchez-Hervás et al., 2008).

One of the mycotoxins of greatest interest on cocoa beans, which is produced by species of the genus *Aspergillus,* is the ochratoxin A (OTA), a potent nephrotoxin with teratogenic and carcinogenic action (International Agency for Research on Cancer, 1993; O'brien and Dietrich, 2005).

Therefore, the knowledge regarding the incidence of fungi of the genus *Aspergillus* and its relation with the processing practices are important for the creation of control strategies during the primary processing of cocoa beans in order to minimize the exposition of the consumer to these toxic compounds.

In this sense, the present study aimed to investigate the presence of fungi of the genus *Aspergillus* on cocoa beans cultivated on municipalities of the state of Rondônia, Brazil, besides evaluating the toxigenic potential of these species, as well as the effect of the geographical origin over the incidence of toxigenic and non-toxigenic fungi.

MATERIALS AND METHODS

Samples

Forty samples of cocoa beans were collected on properties located in the state of Rondônia, the third largest producer of cocoa on Brazil. The samples were collected in 2012 in 12 municipalities: Ariquemes (5), Buritis (3), Cacoal (3), Cacaulândia (3), Colorado do Oeste (4), Governador Jorge Teixeira (2), Jaru (3), Machadinho d'Oeste (5), Ouro Preto do Oeste (5), São Felipe d'Oeste (3), Urupá (3) and Vilhena (1).

Identification of species of the genus *Aspergillus*

The technique of direct plating was used for the isolation of fungi from cocoa beans, according to Samson et al. (2000). One hundred randomly collected beans of each sample were submitted to surface disinfection with sodium hypochlorite (1%) during 30 s and then washed with sterile distilled water three times consecutively. Samples were put on Petri dishes containing the culture medium Dichloran Rose Bengal Chloranphenicol (MERCK). Plates were incubated at 25°C during seven days (Pitt and Hocking, 1997). After seven days, beans were evaluated in relation to the growth of fungi and representative colonies of the genus *Aspergillus* were isolated in medium Malt Agar (MA). Results were expressed as the percentage of contaminated beans according to the methodology of Pitt and Hocking (1997). After obtaining pure colonies, the isolates were transferred to two standardized media: Czapec yest agar (CYA) and malt extract agar (MEA) at 25°C during seven days for posterior identification. The species identification was made according to Klich and Pitt (1988), Klich (2002), Samson et al. (2002) and Samson et al. (2004).

Evaluation of the toxigenic through the thin layer chromatography

The ochratoxigenic and aflatoxigenic potential was evaluated by thin layer chromatography, according to Filtenborg et al. (1983). Species of the Section *Nigri* were cultivated in medium CYA (K_2HPO_4:1.0 g; Czapec Concentrate: 10.0 mL; yeast extract: 5.0 g; Agar: 15.0 g; $NaNO_3$:30.0g, KCl: 5.0 g, $MgSO_4.7H_2O$: 5.0 g, $FeSO_4.7H_2O$: 0.1 g, $ZnSO_4.7H_2O$: 0.1 g, $CuSO_4.$ $5H_2O$: 0.05 g; Distilled Water: 1000 mL) at 25°C for seven days. The *Aspergillus* Section *Flavi* were cultivated in médium – yeast extract sacarose (YES) Agar (yeast extract: 20.0g; sacarose: 150 g; Agar: 20.0 g; metalic solution $ZnSO_4.7H_2O$: 0.1 g, $CuSO_4.5H_2O$: 0.05 g; distilled water: 1000 mL), at 25°C for seven days.

Standard solution of OTA, AFB_1, AFB_2, AFG_1 E AFG_2, (SIGMA-ALDRICH), plates of thin layer chromatography (MERK-SILICA GEL 60, 20x20) and as mobile phase TEF- toluene ethyl acetate and formic acid 90% (60:30:10 v/v/v) were used. Confirmation of toxin production was performed in ultraviolet light (λ 366nm) in Cromatovisor CAMAG (UF-BETRACHTER). The isolates considered producers had a retention factor (Rf), and a fluorescent spot pattern similar to that of the toxin evaluated.

Quantification of ochratoxin A produced by *Aspergillus carbonarius* by HPLC method

The *A. carbonarius* were inoculated into a Petri dish containing the medium Czapek yeast agar (CYA) and incubated at 25°C for 10 days. After this, OTA was extracted according to the modified method of Bragulat et al. (2001). Three plugs of culture were removed from the centre, middle and edge of each colony on the tenth day of the incubation period. The plugs were weighed in test tubes, and then 1 mL of methanol was added. The tubes were homogenised vigorously for five seconds and kept at 25°C for 60 min. The extracts were filtered through polytetrafluoroethylene (PTFE) membranes (0.22 µm) (Millipore) and then analysed by high-performance liquid chromatography (HPLC). The equipment used was an HPLC Shimadzu coupled with two high-pressure

pumps (model SPD-M20A), degasser DGU 20A_3, interface CBM-20A, auto injector SIL-10AF and RF-10 A_{XL} fluorescence detector. The Agilent ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5 µm) column was used and it was connected to a pre-column Agilent ZORBAX Eclipse XDB-C18 4-Pack (4.6 x 12.5 mm, 5 µm). The chromatographic conditions for wavelength were 332 nm for excitation and 476 nm for emission. The flow used throughout the analysis was equal to 0.8 mL min^{-1}, and the injected volume of the samples and standard was 20 µl. The elution was performed using an isocratic system of 35:35:29:1 (methanol : acetonitrile : water : acetic acid). The average retention time for OTA determination was 11 ± 0.1 min. The amount of OTA in the samples was determined using an analytical curve obtained by linear regression (y = 1.11756 x 10^7x – 2592.1485, where y = peak area and x = OTA concentration). The calculation defined the peak area versus the concentration of the respective standard solution obtained by the setting of coefficient (R^2 = 0.9999). The detection limit (DL) and quantification limit (QL) were estimated through parameters obtained by the analytical curve and were calculated according to the following: DL = 3 SD/m and QL = 10 SD/m (where SD = standard deviation and m = angular coefficient of the linear regression). The values obtained for the DL and QL were 0.0004 and 0.0016 µg/g, respectively. All samples were analysed in duplicate, and the standard OTA solutions were assessed in triplicates.

Statistical analysis

In order to determine the probability of occurrence of total and toxigenic species of the genus *Aspergillus* in the state of Rondônia, Brazil, the technique of logistic regression was used in function of the following independent variable: geographical coordinates (latitude and longitude) and temperature. The response variable was characterized by the presence and absence of total and toxigenic fungi of the genus *Aspergillus*.

Thereunto the models of logistic regression were fitted, adjusted using the software R Core Team (2013), considering the covariates represented by temperature (T), by geographical coordinates that identify a city given by longitude (Long) and latitude (Lat). Thus, it was assumed that the Y = 1 encoding, featuring the presence of total species and Y = 0, indicating the absence of these species (Equation 1). Similarly, considering the same covariates, but assuming Y = 1 coding related to the presence of toxigenic species and Y = 0, the absence, the adjusted logistic model is given in Equation 2.

$$P(Y=1|\text{Long, Lat, T}) = \frac{\exp(36.047 - 0.8294\text{Long} + 0.428\text{Lat} - 0.690\text{T})}{1+\exp(36.047 - 0.8294\text{Long} + 0.428\text{Lat} - 0.690\text{T})} \quad (1)$$

Where, P (.) is set equal to the probability for the occurrence of species of total fungi of the genus *Aspergillus*.

$$P(Y=1|\text{Long, Lat, T}) = \frac{\exp(-20.5807 - 0.4682\text{Long} - 0.2002\text{Lat} - 0.0805\text{T})}{1+\exp(-20.5807 - 0.4682\text{Long} - 0.2002\text{Lat} - 0.0805\text{T})} \quad (2)$$

Where, P (.) is set equal to the probability for the occurrence of species of toxigenic fungi of the genus *Aspergillus*.

After the adjustment of models through the Hosmer-Lemeshow test by means of adjusted probabilities obtained from Equations 1 and 2, the graphs of response surface and contours were built in order to ease the identification of the potential regions for the incidence of total and toxigenic fungi of the genus *Aspergillus.*

Table 1. Species of *Aspergillus* isolated from cocoa beans processed and stored on cocoa properties of Rondônia, Brazil.

Genus/species	Nº of genus/species	(%)
Aspergillus* Section *Nigri	**166**	**89.73**
A. aculeatus	12	7.23
A. carbonarius	34	20.48
A. foetidus	17	10.24
A. japonicus	5	3.02
A. niger	64	38.55
A. niger aggregate	20	12.05
A. tubingensis	14	8.43
Aspergillus* Section *Flavi	**19**	**10.27**
A. flavus	18	94.74
A. oryzae	1	5.26

RESULTS AND DISCUSSION

Identification of *Aspergillus* species

Only 2.5% of all the analyzed samples (40) did not present contamination by fungi species, 97.5% were contaminated by filamentous fungi and nine of these samples presented 100% of contamination.

The fungi were identified to the species level due to the high incidence in cocoa beans and the risk for the production of ochratoxin A in tropical regions. The species identified in this study are presented on Table 1.

The species were isolated and identified as members of the Sections *Nigri* (*Aspergillus niger*, *Aspergillus carbonarius*, *Aspergillus niger* aggregate, *Aspergillus foetidus*, *Aspergillus japonicus, Aspergillus tubingensis* and *Aspergillus aculeatus*) and *Flavi* (*Aspergillus flavus* and *Aspergillus oryzae*), and the species of Section *Nigri* were the most frequent (89.73%).

The following species were isolated and identified: *A. niger*, *A. carbonarius*, *A. niger* aggregate, *A. foetidus, A. japonicus*, *A. tubingensis* and *A. aculeatus*, belonging to Section *Nigri*, and *A. flavus* and *A. oryzae* of the Section *Flavi*. The species of Section *Nigri* were the most frequent (89.73%). A similar result was found by Sánchez-Hervás et al. (2008) on cocoa beans produced on Sierra Leone, Equatorial Guinea and Ecuador. It is known that some species of these two Sections of Aspergillus are considered the most important contaminating and toxin producer fungi, which has increased the interest on the isolation and identification of these species (Moss, 1996; Samson et al., 2004).

Among the species of Section *Nigri*, *A. niger* predominated both in this study and other studies regarding cocoa (Copetti et al., 2011b; Mounjouenpou et al., 2008). *A. niger* is a species of frequent occurrence of the Section *Nigri* and it is known by its wide distribution,

both in the tropics and in temperate regions (Pitt and Hocking, 1997).

Mounjouenpou et al. (2008) isolated the species *A. carbonarius, A. flavus, A. fumigatus, A. niger, A. tamari* and *A. versicolor* from cocoa beans cultivated on Cameroon. Sánchez-Hervás et al. (2008) obtained *A. niger* aggregate as main species of the Section *Nigri* and also related the presence of *A. carbonarius* in lower amounts, but did not find any uniseriate species (*A. aculeatus* and *A. japonicus*). Copetti et al. (2011b) made a similar study on Brazil and also isolated some species mentioned above, like *A. versicolor* and *A. fumigatus*, as well as *A. clavatus, A. niger, A. ochraceus* and *A. parasiticus*, confirming the existence of a toxigenic microbiota associated with the culture of cocoa, as well as the risk of producing ochratoxin A and aflatoxins on cocoa beans.

In relation to Section *Flavi*, *A. flavus* predominated in this section. In the study performed with cocoa beans on Sierra Leone, Equatorial Guinea and Ecuador by Sánchez-Hervás et al. (2008), this species also predominated on the Section *Flavi.* Other main species member of the Section *Flavi* responsible for the production of aflatoxins, *A. parasiticus* (Horn, 2007), was not isolated in this study.

No species of the Section *Circumdati* considered ochratoxigenic, like *A. ochraceus* (Pitt and Hocking, 1997) was identified in this study. Sánchez-Hervás et al. (2008) related a low incidence of species of the Section *Circumdati.* The results shows that probably *A. ochraceus* is not an important source of OTA on cocoa cultivated in these regions.

Potential toxigenic species of genera *Aspergillus*

A total of 166 species of Section *Nigri* were registered in relation to the capacity to produce ochratoxin A by the method of thin layer chromatography. From all the isolates, 29 (17.47%) were capable of producing toxins, it means, they presented retention factor and fluorescence spot similar to the standard solution of OTA. From the 29 isolates with toxigenic potential, 27 were identified as *A. carbonarius* and 2 as *A. niger*.

However, 7 *A. carbonarius* did not present potential to produce OTA. This means that from the total of *A. carbonarius* isolates only 79.41% produced OTA. Some studies showed that the percentage of OTA production by this species may range between 70 and 100% *A. carbonarius.* Only two isolates identified as *A. niger* were able to produce OTA. Differently from the observed in the present work, Mounjouenpou et al. (2008) found 70% of the species of *A. niger* producing OTA in cocoa beans. However, the same percentage (3%) of *A. niger* was recorded as OTA producer in coffee (Taniwaki et al., 2003) and grape (Lasram et al., 2007). The high percentage found by Mounjouenpou et al. (2008) was not confirmed by other studies conducted with this species in different producer regions (Bellí et al., 2004a; Iamanaka et al., 2005; Leong et al., 2007; Magnoli et al., 2007; Taniwaki et al., 2003).

Sánchez-Hervás et al. (2008) did not identify any *A. niger* producing OTA in cocoa, however 44.7% of the fungi identified as *A. niger* produced OTA. In the study developed with cocoa beans in Brazil by Copetti et al. (2010), *A. niger* was most commonly isolated species, but only 2.5% were able to produce the toxin.

A total of 19 species of the Section *Flavi* (18 *A. flavus* and 1 *A. oryzae*) isolated in this study were tested in relation to their capacity to produce aflatoxins. From the 18 isolates identifies as *A. flavus,* 15 (83.33%) were able to produce aflatoxin B_1 and B_2. The isolate of *A. oryzae* tested in the present study did not produce aflatoxins, thus confirming the non-toxigenicity of this species as already related by Magan and Olsen (2004). This indicates that the contamination of cocoa beans by aflatoxins does not seem to be worrying in cocoa beans cultivated in Brazil.

Production of ochratoxin A by *A. carbonarius* isolated from cocoa beans

All isolates identified as *A. carbonarius* were tested in relation to the production of OTA in medium CYA by HPLC (Table 2). Results obtained show that these isolates produced amounts of OTA ranging from 0.08 to 44.09 µg/g of culture medium.

Sánchez-Hervás et al. (2008) related the OTA production by *A. carbonarius* isolated from cocoa ranging from 0.2 to 8.0 µg/g of culture medium. In other studies with their substrates, like grape and coffee, the amount of OTA produced ranged from 1 to 10 µg/g (Bellí et al., 2004a; Lasram et al., 2007; Martínez-Culebras and Ramón, 2007). This difference of production may be related to the interference of the cultivation conditions and the origin of substrate, besides the region from where these fungi were isolated. Few studies were published on non-ochratoxigenic *A. carbonarius.*

Cabañes et al. (2013) identified 3 wild isolated of non-ochratoxigenic *A. carbonarius* from grape of different vineyards of Southern Spain through genetic sequencing. The three isolates did not produce OTA in any medium culture in all tested temperatures and in the different time of incubation, thus proving the existence of non-ochratoxigenic *A. carbonarius.*

Probability of occurrence of total and toxigenic fungi of the genus *Aspergillus* in function of geographic coordinates and temperature

Geographic coordinates and temperatures of the state of Rondônia, Brazil, were analyzed in order to relate it to the

Table 2. Effect of OCHRATOXIN A (OTA) production by *A. carbonarius*[1] isolate from cocoa beans processed and stored on cocoa properties of Rondônia, Brazil.

Isolate	Mean of OTA (µg/g)	Isolate	Mean of OTA (µg/g)
A. carbonarius 1	44.09	*A. carbonarius* 18	0.18
A. carbonarius 2	0.25	*A. carbonarius* 19	30.21
A. carbonarius 3	0.09	*A. carbonarius* 20	0.18
A. carbonarius 4	0.11	*A. carbonarius* 21	20.98
A. carbonarius 5	ND	*A. carbonarius* 22	0.19
A. carbonarius 6	0.13	*A. carbonarius* 23	0.20
A. carbonarius 7	ND	*A. carbonarius* 24	0.12
A. carbonarius 8	0.12	*A. carbonarius* 25	ND
A. carbonarius 9	ND	*A. carbonarius* 26	0.29
A. carbonarius 10	0.08	*A. carbonarius* 27	0.17
A. carbonarius 11	4.82	*A. carbonarius* 28	0.22
A. carbonarius 12	ND	*A. carbonarius* 29	0.37
A. carbonarius 13	0.39	*A. carbonarius* 30	0.29
A. carbonarius 14	ND	*A. carbonarius* 31	14.07
A. carbonarius 15	14.10	*A. carbonarius* 32	17.32
A. carbonarius 16	30.68	*A. carbonarius* 33	11.82
A. carbonarius 17	0.33	*A. carbonarius* 34	ND

[1]Analysis made by HPLC in CYA medium /10days; [ND]Non detected; LOD = 0.0004 µg/g; LOQ = 0.0016 µg/g.

levels of contamination by species of total and toxigenic fungi of the genus *Aspergillus* in cocoa beans, according to what is described in Equations 1 and 2.

The validation of the logistic model (Equations 1 and 2) was made by the probability of significance obtained in relation to the Hosmer-Lemeshow test. When considering the total and toxigenic species of fungi, the estimated probabilities were respectively, 0.907 and 0.992. Thus, confronting with the significance level fixed at 5%, since they presented a non-significant result, both models are adequate to identify the municipalities with higher probability of fungi incidence.

The logistic regression model adjusted for the identification of the occurrence of total fungi (Equation 1) allows interpreting that, considering fixed values for the geographic coordinates and temperature, the resulting probability of the model refers to the probability of occurrence of total fungi of the genus *Aspergillus*, characteristic of a determined region of the state of Rondônia (Figures 1 and 2).

In relation to the presence of toxigenic species, the surface of response with probabilities of occurrence of species in function of geographic coordinates (Equation 2), as well as the contours plot to help in interpretation are presented in Figures 3 and 4, respectively.

In relation to the geographical position of the cultivation areas, several studies with grapes had already demonstrated that the geographic area and meteorological conditions may contribute to the incidence of fungi and production of OTA (Battilani et al., 2006a, b; Bellí et al., 2006; Chiotta et al., 2009; Serra et al., 2006), however no study was done with cocoa regarding this aspect until the present one. In this context, when considering latitude, longitude and temperature, a higher probability of occurrence of total fungi of the genus *Aspergillus* was estimated for municipalities of the state of Rondônia, Brazil, located between the parallels of 11° and 13° of South latitude and meridians of 60° and 62° of West longitude, which correspond to Vilhena, Colorado do Oeste, Cacoal, São Felipe d'Oeste and Urupá.

However, we may verify that for toxigenic species of fungi, the probabilities of occurrence were similar in all the studied regions in the state of Rondônia, Brazil.

The effect of geographical area on the fungi was confirmed by Battilani et al. (2006b), showing a significant effect of localization when the incidence of species belonging to the genus *Aspergillus* Section *Nigri* in grapes was analyzed. The incidence was significantly correlated with geographical coordinates showing the positive correlation with longitude, indicating that the incidence increased from West to East, while the correlation with latitude was negative, showing a positive gradient for Southern Europe. The incidence of *A. carbonarius* also was positively correlated with latitude. These authors also verified that meteorological conditions play an important role in the fungi colonization and on the presence of OTA in grapes in Italy.

Bellí et al. (2006) studied the correlation between meteorological parameters and fungi of the Section *Nigri* in grapes of wine regions of Spain and revealed a positive correlation between the colonization by *Aspergillus* Section *Nigri* and temperature. These authors attributed the higher percentage of infection of grapes by *Aspergillus* Section *Nigri* in the year of 2003, where meteorological conditions are extremely hot during the whole year. Studies with species of the Section *Nigri* suggest that the higher frequency in grapes of warmer climates may be associated with the survival capacity of these species in the soil and the capacity of adaptation to high temperatures and low water activity (Battilani et al., 2006c; Leong et al., 2004), as well as to the fact that the black spores of Section *Nigri* species offer protection to sunlight providing a competitive advantage on warmer climates (Hocking, 2006; Leong et al., 2006; Pitt and Hocking, 1997).

A positive correlation of *A. carbonarius* on grapes with temperature was already related, and the higher occurrence of this species occurred on hot areas (Battilani et al., 2006a; Chiotta et al., 2009). *A. carbonarius* grows on temperatures between 10 and 40°C (Pitt, 2000), with optimum between 25-35°C (Bellí et al., 2004b; Mitchell et al., 2004). The mean temperatures of the municipalities which samples were collected range between 24.33 and 26.13°C, which is propitious to the growth of *A. carbonarius* in the whole state. In relation to the

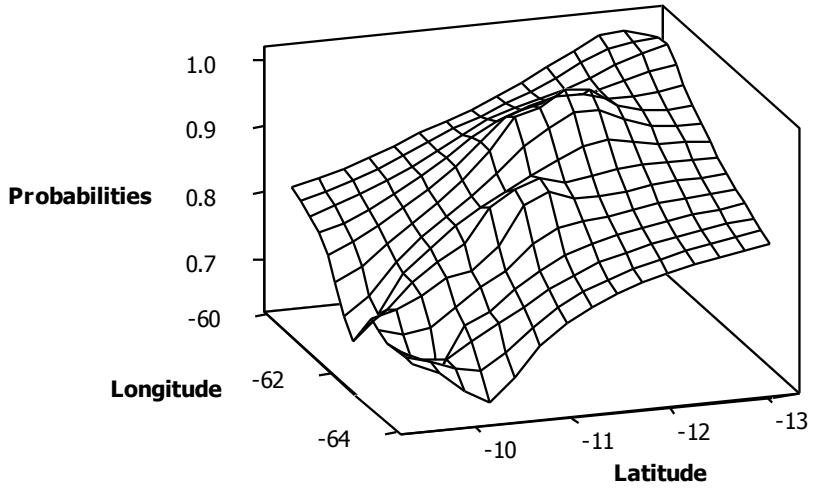

Figure 1. Response surface for the probability of occurrence of total fungi on localities characterized by different ranges of latitude and longitude in Rondônia, Brazil.

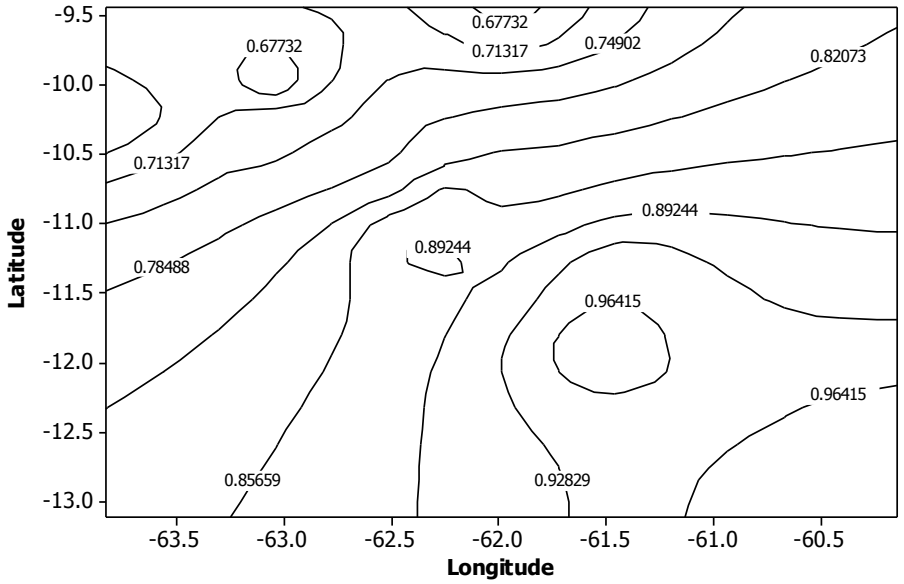

Figure 2. Contour lines obtained from the response surface with percentages of probability of occurrence of total fungi in Rondônia, Brazil.

production of OTA, *A. carbonarius* produced the most elevated levels at 15 or 20°C (Esteban et al., 2004; Passamani et al., 2014), this temperature range below that found in Rondônia which suggests a reduced risk of contamination of the cocoa beans OTA produced by this species.

The uniformity among probabilities predicted by the model adjustment in relation to the geographical coordinates and temperatures may be related to the limited number of collected samples of cocoa beans, thus decreasing the possibility of isolating toxigenic fungi. We highlight that results were obtained in function of geographical coordinates and temperature, and then the inclusion of new variables may identify other municipalities with high index of toxigenic fungi.

Results obtained in the present study suggest a risk of contamination by toxigenic fungi for cocoa beans, once *A. carbonarius, A. niger* and *A. flavus* were isolated and identified. The higher probability of fungi occurrence was estimated for municipalities located between the parallels 11 and 23° S and 60 and 62° W, thus confirming the

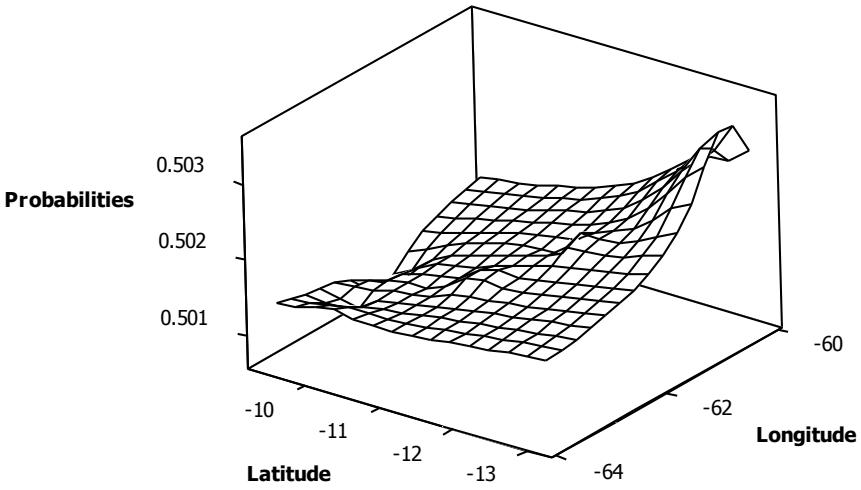

Figure 3. Response surface for the probabilities of occurrence of toxigenic fungi on localities characterized by different ranges of longitude and latitude in Rondônia, Brazil.

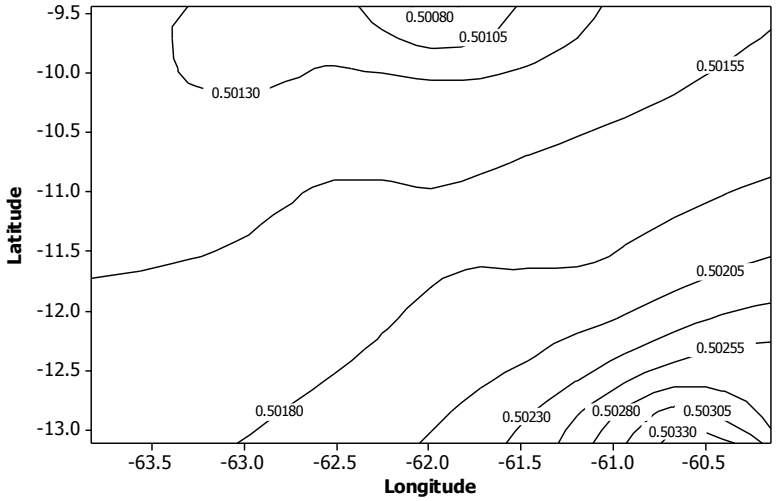

Figure 4. Contour lines for the probabilities of occurrence of toxigenic fungi on localities characterized by different ranges of longitude and latitude, in Rondônia, Brazil.

effect of geographic localization and temperature on the incidence of fungi of the genus *Aspergillus.*

However, some key questions still must be answered to minimize the contamination by toxigenic fungi in cocoa beans processed in these municipalities. We suggest: (a) identify which step of processing represents a greater risk of contamination by these toxigenic fungi; (b) characterize which step of processing provide optimum conditions for the production of toxins by fungi; (c) optimize methods of field control, for example, use of fungicides; (d) create systems of geographic information and maps of risk as support material to producers and researchers.

Conflict of interests

The authors did not declare any conflict of interest.

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