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MAESTRÍA EN BIOCIENCIAS

Efecto de la humedad y actividad de agua sobre la producción de ocratoxina A por hongos toxigénicos en café.

T E S I S

QUE PARA OBTENER EL GRADO DE MAESTRÍA EN BIOCIENCIAS

P R E S E N T A:

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APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 10 días del mes de enero de dos mil diecinueve, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Biociencias, para examinar la tesis titulada *"Efecto de la humedad y actividad de agua sobre la producción de ocratoxina A por hongos toxigénicos en café"*, presentada por el alumno **Erick Baruch Estrada Bahena**, para obtener el Grado de Maestría en Biociencias. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Efecto de la humedad y actividad de agua sobre la producción de ocratoxina A por hongos toxigénicos en café.

Esta investigación se llevó a cabo en la Universidad Autónoma de Guerrero (UAGro). La identificación de los hongos y la cuantificación de Ocratoxína A se realizó en el Laboratorio de Toxicología y Salud Ambiental de la Facultad de Ciencias Químico Biológicas en Chilpancingo de los Bravo, Gro. La obtención de la curva de humedad de los granos de café cereza y verde, el modelamiento de las isotermas de adsorción, así como los parámetros físico-mecánicos se realizaron en el Laboratorio de Bromatología y Tecnología de Alimentos de la Facultad de Ciencias Químico Biológicas en Chilpancingo de los Bravo, Gro. Las micrografías electrónicas de barrido se obtuvieron en la Unidad Académica de Ciencias de la Tierra en el laboratorio de Microscopia Electrónica de Barrido y de Microanálisis en el municipio de Taxco de Alarcón Gro.

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ÍNDICE

Effect of water activity and temperature on the Ochratoxin A production by toxigenic fungi in coffee from Guerrero

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Resumen

El café es la bebida más consumida en todo el mundo. Sin embargo, los granos de café son susceptibles a la contaminación por hongos ocratoxigénicos. La actividad del agua (a_w) y la temperatura influyen en el crecimiento de los hongos, así como en la biosíntesis de ocratoxina A (OTA). El objetivo de este estudio fue evaluar la influencia de la a_w y el efecto de la temperatura en el crecimiento de los hongos, así como la producción de OTA y determinar las condiciones críticas de almacenamiento de los granos de café cereza y verde. Las muestras de granos de café fueron colectadas en el municipio de Atoyac Guerrero, México. La identificación de los hongos se realizó mediante las características macro y microscópicas. La cuantificación de la OTA se realizó mediante un análisis inmunoenzimático cuantitativo. Se calcularon las isotermas de adsorción a temperaturas de 15, 25 y 35 °C en un rango de a_w 0.115-0.821, a partir de las cuales se obtuvieron las condiciones optimas de almacenamiento utilizando el criterio de mínima entropía. Las propiedades físicas de las muestras almacenadas (color y fracturabilidad) se determinaron mediante métodos convencionales y los cambios de la microestructura mediante el análisis de microscopía electrónica de barrido. Los resultados obtenidos mostraron que *Aspergillus* sp. y *Penicillium* sp. fueron las principales especies de hongos presentes en los granos de café. Los granos analizados presentaron una alta concentración de OTA (15.49-65.22 μg / kg). Las condiciones bajo las cuales encontramos los cambios físicos y la mayor concentración de OTA son: a_w entre 0.515-0.821 y temperatura de 35 °C. Las isotermas de adsorción y el modelo GAB mostraron que las mejores condiciones de almacenamiento son a_w 0.3 a 35 °C en granos de café cereza y a_w 0.6 a 35 °C para granos de café verde.

Palabras clave: OTA, hongos ocratoxigénicos, café, isotermas de adsorción, a_w y condiciones de almacenamiento.

Abstract

Coffee is the most beverage consumed in worlwide. However, coffee beans are susceptible to contamination with ochratoxigenic fungi. The water activity (aw) and tempearture influences the growth of fungi as well as the biosynthesis of ochratoxin A (OTA). The objective of this study was to evaluate the influence of aw and the effect of temperature on the growth of fungi as well as the production of OTA and determinate the critical storange conditions of the coffee cherries dry beans and green coffee beans. Samples of coffee beans were collected from the municipality of Atoyac Guerrero, México. The identification of the fungi was performed by micro and macroscopic characteristics. The OTA quantification was performed by enzyme immunoassay quantitative analysis. The adsorption isotherms were calculated at temperatures of 15, 25 and 35 ° C in a range of aw 0.115-0.821, from which the optimum storage conditions were obtained using the minimum entropy criterion. Physical property of samples storage (color and fracturability) were determined by conventional methods and the changes on the microstructure by scanning electron microscopy analysis. The results obtained showed that *Aspergillus* sp and *Penicillium* sp were the main species of fungi present in coffee beans. The analyzed grains showed a high concentration of OTA (15.49-65.22 μg/kg). The conditions under which were find the physical changes and the highest concentration of OTA are: aw between 0.515-0.821 and temperature of 35 ° C. The water adsorption isotherms and the GAB model showed that the best conditions of storage are aw 0.3 at 35 ° C in coffee cherries dry beans and aw 0.6 at 35 °C for green coffee beans.

Key Word: OTA, ochratoxigenic fungi, coffee, water adsorption isotherm, a_w and storage conditions.

1. INTRODUCTION

Coffee is one of the most product marketed in the worldwide, approximately 158.901 million bags in 2016/17 and it is the most widely consumed beverage, exceeding billions of cups a year (ICO 2018; Yi-Fang, C 2012). Even today, a significant number of publications describe the potential benefits that this beverage provides for health, such as: antioxidant, antidepressive and antidiabetic (Huxley et al., 2009; Zhang et al., 2011., Yi-Fang, C 2012) as well as for Alzheimer´s disease and Parkinson´s (Cao et al., 2011; Palacios et al., 2012., Umemura et al., 2006; Ochiai et al., 2009; Yi-Fang, C 2012). These activities come from secondary metabolites such as caffeine, chlorogenic, ferulic and caffeic acids (Higdon and Frei, 2006; Cano-Marquina et al., 2013).

However, during different phases of development, harvesting, processing, transport and storage, different fungi and bacteria can contaminate coffee cherries and beans (Batista et al., 2003; Suárez-Quiroz et al., 2004; Masakhwe et al., 2017), and it has been reported that coffee in any form of presentation or marketing (coffee cherries dry, green coffee beans and roasted coffee beans) has a wide prevalence of contamination by Ochratoxin-A (OTA), because OTA is highly resistant to physical and chemical substances. When this toxin is in the food, it persists during the processing and storage conditions (Drunday and Pacini, 2013; Galarce-Bustos et al., 2014; Casal et al., 2014; Masakhwe et al., 2017).

The OTA is a mycotoxin found as a contaminant in different food such as cereals, wine, spices, beans, groundnuts, milk, beer, and coffee (Drunday and Pacini, 2013). OTA contamination has become a public health concern because it has exhibited toxic effects, such as nephrotoxic, hepatotoxic, neurotoxic, immunosuppressive, teratogenic, and carcinogenic (Khoury and Atoui, 2010; Bezerra et al., 2014) and the International Agency for Research on Cancer (IARC) classify OTA as possibly carcinogenic to humans (Group 2B) (IARC, 1993). Given the health risks posed by OTA, the European Food Safety

Authority (EFSA) Scientific Panel on Contaminants in the Food Chain established a Provisional Tolerable Weekly Intake (PTWI) of 120 ng/kg bw (body weight)/week (EFSA, 2006), while the Joint Expert Committee on Food Additives (JECFA) set one of 100 ng/kg bw/week (JECFA, 2007). In addition, the European Commission has established the maximal level allowed of OTA in roasted coffee beans at 5 µg/kg (European Commission, 2006).

The process such as picking of fallen old cherries from the ground during harvesting, contamination of coffee by drying on the ground, storing partially dry coffee for long periods or rewetting during drying influences the growth of fungies on coffee which affects the quality and safety of the final product due to production of mycotoxins (Drunday and Pacini, 2013; Galarce-Bustos et al., 2014; Casal et al., 2014; Masakhwe et al., 2017).

The occurrence of mycotoxins in foods can have profound implications, including health and economic impacts, especially for developing countries. Several studies have shown the influence of moisture content, water activity, temperature and the nature of the substrate, may play an important role in the safety product, quality and the shelf life (Olivera et al., 2019). That is why, the temperature and relative humidity are important parameters to be considered during the storage, because the accurate measurement and prediction of water content assist in decision for correct product storage. Thus, the adsorption isotherms are indispensable to determinate water adsorption changes during storage. Knowledge of thermodynamic properties would help in modeling, designing and optimizing the drying process, evaluating storage stability and microbiological safety of coffee and can give information on microstructure, physical and microbiological changes in the product. The aim of this work was to evaluate the influence of water activity on the physical stability, the thermodynamic properties and production of OTA in the coffee cherries dry and green coffee beans from the state of Guerrero, Mexico. Additionally, we estimate the fungi contaminant to generate data on contamination levels in coffee beans with the objective to preserve the quality of this product during storage conditions.

2. MATERIAL AND METHODS

2.1 Sampling collection

Coffee samples (1000 g) of *Coffea arabica* coffee cherries dry and green coffee beans were collected according to European Commission regulation (EC) no. 401/2006 and stored at -21 ± 1 ºC and protected from light in December 2017 in Atoyac de Álvarez, in the south the state of Guerrero Mexico (17°12' 33.44’’N (North), and 100°26'0.943’’O (West).

2.2 Isolation and identification of fungi

The identification of the fungal contaminants present in the coffee samples were made using the methodology proposed by Luna et al (2010) with some modifications. Five coffee cherries dry and green coffee beans were also plated directly onto Petri dishes containing 20 mL of Potato Dextrose Agar (PDA) (MCD Lab, S.A.de C.V., Tlalnepantla, Mexico). The plates were incubated at 25 ºC for 7 days. The predominant fungal were identified by micro-and macroscopy methods, using the cotton blue technique of lactophenol. The data were compared whith that reported by López-Jácome et al (2014), Samson et al (2014) and Visagie et al (2014).

2.3 Physical analysis

The color of coffee cherries dry and green coffee beans storage was measured using a spectrophotometer (X-Rite model Ci62-, X-Rite, Inc., Grandville. MI USA), with the CIELAB system and the components L*, a*, b* for the description of the color. Afterwards, the Hue angle (H°) and chromaticity (C*ab) were calculated using the following equations $H° = \tan^{-1} (b^*/a^*)$, where a* = [negative (green) to positive (red)], b* = [negative (blue) positive (yellow)] and $C^*ab = \sqrt{a^2 + b^2}$.

The characterize of fracturability of the samples was measure using a Texture Analyzer, TA-XT2 texturometer (Texture Technologies Corp., N. Y. USA.), with a 4 mm compression disc and velocity of 10 mm/s (50 kg).

The morphology structure of stored coffee samples was studied by Scanning Electron Microscope (ESEM; Philips model XL30) with a beam of 15 kV and a gaseous secondary electron-detector was used. The images were taken in a rango of 35-850X, 1 torr, with a spot size of 3.5 to observe surface sample integrity after storage. The samples were mounted on stainless steel stubs and coated with graphite using a JFC-1100 Sputter Coater (JEOL, 183 Akishima, Japan) before analysis. Five replications were carried out for each evaluated.

2.4 Water adsorption isotherms

Water adsorption isotherms were determinated using a static equilibrium method. In this study we use of saturated salt solutions to maintain a fixed relative humitity. The salts used are LiCl, KC_2H_3O, $MgCl_2$, K_2CO_3, Mg $(NO_3)_2$, $NaNO_3$, NaCl y KCl, the range of water activities of 0.108, 0.215, 0.318, 0.436, 0.515, 0.628, 0.743 and 0.821 (Labuza et al., 1985). The sample holder containing 3.0 g of coffee sample is put in desiccators containing the saturated salt solutions, samples are weighted every 5 days until weight became constant. The experiments were conducted in triplicate replicates at 15, 25, and 35 °C until equilibrium was reached. The equilibrium condition was attained within 20 days, when the differences betwen two consecutive weights were within 0.005 g. The Guggenheim-Anderson- De Boer (GAB) equation (1) was used in the modeling water adsorption (Quirijnis et al., 2005).

$$\boldsymbol{M = \frac{M_0\, C\, K\, a_w}{(1 - Ka_w)(1 - Ka_w + CKa_w)}} \qquad (1)$$

Where $\mathbf{a_w}$ is water activity; **M** is the water content of the simple on dry basis; $\boldsymbol{M_0}$ is the monolayer water content; **C** is the Guggenheim constant, given by $C = c' exp\,(h_m - h_n)/RT$; where c' is the equation constant,

h_m is the heat of sorption of the first layer; h_n is the heat of sorption of the multilayer; *R* is the gas constant; *T* is the absolute temperatura; ***K*** is the constant correcting properties of multilayer molecules with respect to bulk liquid, given by $k=k´\exp(h_1-h_n)/RT$; where *k´* is the equation constant, and h_1 is the heat of condensation of pure water. The parameter values of the GAB equation (M_0, *C* and *K*) where estimated by fitting the matemathical model in the experiment data, using non-linear regression with the Kalediagraph ver. 4.0 software package (Sinergy Software, Perkioment, USA). Accuracy of fitting was evaluated utilizing the average in the difference in relativite percentage between the experimental and predicted values of the moisture content the mean relative deviation modulus (*P*) defined by the following equation (2):

$$\boldsymbol{P}(\%) = \frac{\boldsymbol{100}}{\boldsymbol{N}} \sum_{\boldsymbol{i=1}}^{\boldsymbol{N}} \frac{|\boldsymbol{Me_i} - \boldsymbol{Mc_i}|}{\boldsymbol{Me_i}} \qquad (2)$$

Where

$\mathbf{Me_i}$ is the moisture content at observation *i*; $\boldsymbol{Mc_i}$ is the predicted moisture content at that observation, and ***N*** is the number of observations. It is generally assumed that a good fit is obtained when P < 10% (Lomauro et al., 1985)

2.5 Determination of minimum integral entropy adsorption

Determination of the integral (entropy) thermodynamic propertie and of the water activity-temperature conditions at which the coffee cherries dry and green coffee beans minimum integral entropy occurred, considered as the point of maximal storage stability (monolayer value), was established as indicated by Pascual-Pineda et al., (2014). These authors provided a thorough description of the procedure followed and of the equations employed for this purpose. Briefly, integral enthalpy changes $(\Delta H_{int})_T$ (J/mol) at the water coffee interface and, at different stages of the adsorption process, were determined using the equation of Othmer (1942) as follows (3):

$$\frac{\boldsymbol{dlnP_v}}{\boldsymbol{dlnP_v^0}} = \frac{\boldsymbol{H_v(T)}}{\boldsymbol{H_v^o(T)}} \qquad (3)$$

Where: the desorbed substance is water; $\boldsymbol{P_v}$ (Pa) is the vapor pressure of water over the adsorbent; P_v^0 (Pa) is the vapor pressure of pure water at the temperature of sorption; $\boldsymbol{H_v}(T)$ (J/mol) is the integral molar heat of sorption, and $\boldsymbol{H_v^0}(T)$ (J/mol) is the heat of condensation of pure water. In that all of these terms are temperature-dependent, the equation can be integrated as follows (4):

$$\boldsymbol{lnP_v} = \left[\frac{\boldsymbol{H_v(T)}}{\boldsymbol{H_v^o(T)}}\right]_{\boldsymbol{\phi}} \boldsymbol{ln\, P_v^0} + \boldsymbol{A} \qquad (4)$$

Where: $\boldsymbol{A}$ is the adsorption constant, and Φ (J/mol) is the pressure of diffusion or surface potential. A plot of $\boldsymbol{lnP_v}$ versus $ln\, \boldsymbol{P_v^0}$ yields a straight line if the ratio $\boldsymbol{(T)/\, H_v^0\,(T)}$ is constant within the range of temperatures used.

Molar integral enthalpy $(\Delta Hint)_T$ was calculated using equation (5 and 6), at a constant pressure of diffusion (Nunesand Rotstein 1991) as follows:

$$(\Delta H_{int})_T = \left[\frac{H_v(T)}{H_v^o(T)} - 1\right]_{\phi} H_v^o(T) \qquad (5)$$

$$\phi = \mu_{ap-}\, \mu_a = RT\, \frac{W_{ap}}{W_v} \int_0^{a_w} Md\ lna_w \qquad (6)$$

Where: $\boldsymbol{\mu_{ap}}$ (J/mol) is the chemical potential of the pure adsorbent; $\boldsymbol{\mu_a}$ (J/mol) is the chemical potential of the adsorbent participating in the condensed phase; $\boldsymbol{W_{ap}}$ (g/mol) is the molecular weight of the adsorbent, and $\boldsymbol{W_v}$ (g/mol) is the molecular weight of the water. By calculating $(T)/\, H_v^0(T)$ from equation (4) and substituting it in equation (5), it becomes possible to calculate the integral enthalpy at different temperatures, provided that a good means of estimating is available, such as that proposed by Wexler, (1976) as follows (7):

$H_v^0(T)$ J/mol-K = 6.15 x 104 – 94.14 T + 17.74 x 10-2T2 – 2.03 x 10-4T3 (7)

Using the values obtained for $(\Delta H_{int})_T$ changes, the molar integral entropy $(\Delta S_{int})_T$ can be estimated using the following equation (8) :

$$(\Delta \boldsymbol{S_{int}})_{\boldsymbol{T}} = \boldsymbol{S_1} - \boldsymbol{S_L} = -\frac{(\Delta \boldsymbol{H_{int}})_{\boldsymbol{T}}}{\boldsymbol{T}} - \boldsymbol{R ln a_w} \quad (8)$$

Where: $\boldsymbol{S_1} = S/N_1$ (J/mol K) is the integral entropy of water desorbed in the foodstuff; **S** (J/mol K) is the total entropy of water desorbed in the foodstuff; N_1 is the moles of water desorbed in the foodstuff, and $\boldsymbol{S_L}$ (J/mol K) is the molar entropy of pure liquid water in equilibrium with vapor.

2.6 Analysis of OTA in coffee beans storage

The methodology employed was based on the procedure described by the enzyme immunoassay for quantitative analysis of OTA (RIDASCREEN®FAST Ocratoxin A; R-Biopharm AG, Darmstadt, Germany). Linearity was determined by utilizing five standard calibrators in the range of 5-40 µg/kg of OTA. The extraction procedure included the weighing of 5 g of a ground sample a suitable container, to which we added 12.5 mL of 70% methanol, with shaking vigorously manually for 3 min. We then filtered the extract through Whatman No.1 filter paper (Sigma-Aldrich), and later diluted 1 mL of the filtrate obtained with 1 mL of distilled water. Fifty mL of standard or prepared sample was placed into separate wells, to which we added 50 µL of enzyme conjugate and 50 µL of anti- OTA antibody solution, after this, we mixed the plate manually and incubated it for 10 min at room temperature (20-25 ^{o}C). Subsequently, we emptied the liquid out of the wells and filled the latter with distilled water (repeating the washing step two additional times), added 100 µL of substrate/chromogen mix, and incubated this for 5 min at room temperature in the dark. Then, we added 100 µL of stop solution and mixed this. We read absorbance at 450 nm in a microplate reader (Stat Fax-2100; Awareness Technology, GMI-Inc.).

The OTA concentration (µg/kg) corresponding to the extraction of each sample can be read from the calibration curve.

2.7 Statistical analysis

A descriptive analysis was realized and the data expressed as the mean of three repeats ± the standard deviation. The ANOVA was carried out with Tukey's posterior test. It was considered a statistically significant difference with values of $p< 0.05$.

3. RESULTS AND DISCUSSION

Isolation and identification of fungi in samples of coffee

All coffee samples analyzed showed contamination by fungi. Table 1 depicts the percentage of occurrence of *Aspergillus* sp., *Penicillium* sp. and other fungi from coffee cherries dry and green coffee bean samples. Identification of the fungies isolated revealed that *Aspergillus* was the most common species found in coffee cherries dry and green coffee bean. It was also noted that *Penicillium* sp. and other fungi, as *Rhizopus* and *Fusarium* were isolated. These species of fungi were identified in the coffee cherries dry and green coffee beans samples from their macro and microscopic characteristics according to the information provided by López-Jácome et al (2014), Samson et al (2014) and Visagie et al (2014). In the Figure 1, the morphology of the isolated species is shown, where the specie *Aspergillus* sp. is indicated by the development of black and cottony mycelium without formation of pigments in the adverse of the plate (AI), *Penicillium* by the presence of greenish color mycelium, without production of pigments in the adverse of the plate (AII) and the genus *Rhizopus* by the growth of white, cottony mycelium, without production of pigments (AIII). With respect to the microscopic characteristics, the presence of structures such as conidiophores, microconidia, vesicle and sterigmas in *Aspergillus* sp. (BI and CI), as well as conidiophores and microconidia for the genus *Penicillium* (BII and CII), and finally

sporangia, rhizoide and sporangiophores in *Rhizopus* (BIII and CIII). Some studies have confirmed that filamentous fungi are the most common contaminats in coffee cherries dry beans and coffee green beans after harvesting and during drying, which can increase their biomass during storage (Silva et al., 2008: Lamanaka et al., 2014; Broissin- Vargas 2017). The occurrence of these fungi species on green coffee beans has been reported before, and the genus *Aspergillus* sp. was the most common contaminat reported in coffee from Brazil 96% (Batista et al., 2009); Saudi Arabia 12.9- 77.2 % occurrence (Fardos 2007); Ethiopian at 79 % occurrence (Geremew et al., 2016); Phillippines 21.6 % (Barcelo y Barcelo 2018). In other hand, Viegas et al (2017) analysed twenty-eight simples coffee from different countries of origin (Brazil, Timor, Honduras, Angola, Vietnam, Costa Rica, Colombia, Guatemala, Nicaragua, India and Uganda) reported that *Aspergillus* sp. was the most common fungal contaminant were detected with 96.4 % of ocurrence also Taniwaki et al (2018) report the ocuurence of *Aspergillus* genus (*ochraceus, steynii, carbonarius, niger* and *westerdijkiae*) in food commodities (peanuts, dry fruits, cocoa, almonds, pistachios, walnuts, hazelnuts), among themselves coffee. In the coffee cherries dry beans, there are few studies, since this type of coffee does not have an economic importance, because coffee it is not marketed in this way. However, a high degree of contamination by *Aspergillus* genus is reported in coffee cherries dry beans from Ivory Coast 50-100% of contamination (Kouadio et al., 2012), the Philippines 98.4% (Culliao and Barcelo 2015) and Brazil 48% (Sousa et al., 2018). On the other hand, *Penicillium* and *Fusarium* are other genus of fungi that contaminate coffee, in coffee beans from Brazil have been found 42% of *Penicillium* (Batista et al., 2003); in Ethiopia, these genus are reported in 5% and 8% respectively (Geremew et al., 2016); in Saudi Arabia 42.5-46.5% and 20% respectively (Fardos, 2007) and Viegas et al (2018) report the presence of *Penicillum* in the variety of Arabica and Robusta coffee.

In Mexico, there are few studies that shown contamination by toxigenic fungi in coffee, only the studies of Luna et al (2010), who reported 88-100% of contamination by *Aspergillus niger* and poor growth of *Penicillium* sp. in green coffee beans from Coatepec, Veracruz; Casas-Junco et al (2018) who found a

contamination of 54.54% by *Aspergillus* and 4.5% *Penicillium* in roasted coffee samples from Nayarit; as well as Broissin-Vargas et al (2017) who reported a contamination of 1.8% by *Aspergillus flavus* and poor growth of *Fusarium* in green coffee beans from Chiapas. This being the fourth work to report the contamination of coffee cherries dry and green coffee beans by these fungal species in Mexico and the first time reporting the contamination of coffee beans from Atoyac de Álvarez, Guerrero.

The coffee beans are susceptible to contamination with ocratoxigenic fungi; the temperature, a_w and the type of substrate influences the growth, and germination of these fungi. The effect to temperature appeared to be strongly influenced by a_w, suggesting a clear interaction between a_w and temperature, resulted in better growth of the fungi species (Gil- Serna et al., 2014; Sousa et al., 2018; Oliveira et al., 2019). The genus *Aspergillus*, *Penicillium* and *Fusarium* are the three fungi genus best adapted to growth in the tropics (growth strongly at 37°C or above and relative humidity of between 70 - 90%), with a_w between 0.80- 0.990 (Pitt and Hocking 2009; Temiz Perez et al., 2011; Taniwaki et al., 2018; Oliveira et al., 2019). Also, the type of substrate influence the growth of these fungi. In this sense, the pericarp of the coffee cherries dry beans is constituted mainly by water (moisture), carbohydrates (7%), pectin (galaturonic acid, 2.6%), other sugars (50%) and cellulose (17%), while the composition of green coffee beans consists of caffeine (1.2%), chlorogenic acids (8%), proteins (13%) and polysaccharides (55%) and it has been found that some of the compounds present in green coffee beans have antifungi activity (Temiz Perez et al., 2011; Lou et al., 2018: Kwasniewska-Sip et al., 2019; Mirón- Merida et al ., 2019).

On the other hand, the conditions of harvest, post-harvest and storage also have an important role in the development of contaminating fungi of coffee. Kouadio et al (2012) reported a range of contamination by toxigenic fungi (*A. niger, A ochraceus, A. flavus* and *A. fumigatus*) of 1.7-63% in coffee cherries dry stored from Ivory Coast, observing that when coffee cherries were stored at longer time after harvesting, they became more infected inside, increasing the percentage of infected samples over the course of the days; Broissin-Vargas et al (2018) correlated the increase in green coffee contamination from Chiapas,

Mexico, by *Aspergillus flavus* with storage time and other variables such as chromaticity, a_w, moisture content, concentration of reducing sugars, fungal infection and production of ochratoxin A.

The results found in this study showed high contamination of the coffee cherries dry beans and green coffee beans by fungi of the genus *Aspergillus*, *Penicillium* and *Fusarium*. This degree of contamination could be associated to the dry process that is made to the coffee beans in Atoyac de Álvarez, this process, (also known as natural method), is a simple process and requires little machinery, and consists in drying the cherries in large playground of dirt or cement, exposing them to a greater risk of contamination. Being this stage the most important of the process, since the results affect the final quality of green coffee beans, this because a coffee that has not dried enough will have too much moisture and will be susceptible to rapid deterioration caused by fungi and bacteria (Alvarado and Rojas 2007; Kouadio et al., 2012).

Physical analysis of coffee beans after storage

The characteristics that determine the quality of food include physical, chemical or nutritional parameters, which allow verifying that it complies with a specific standard, which is directly related to food safety and acceptance by consumers, so it is necessary their determination (Badui, 2006). The color is one of the main physical properties of food and farmn products, since it has been shown to correlate with the quality of the product (Mendoza et al., 2006). The results of the CIELAB color parameters, are shown in Table 2. The luminosity or variation of the energy content in the food that gives rise to the light and dark colors (L * 100 = white and L * 0 = black), it was shown that all the samples present an opaque luminosity (in interval of 7.62-30.53). However, an increase of this parameter was observed in the coffee cherries dry beans subjected to an a_w of 0.821 showing a light tendency to a lighter value, which could be due to the visible presence of white mycelium on the surface of the beans (Fig. 3, a_w 0.821).

On the other hand, the chromaticity is a parameter that determine the variation of the color intensity (C0 = zero intensity and C100 = high intensity). In the table 2 is shown the chromaticity from the coffee

samples. The coffee cherries dry beans showed little intensity of color (chroma 2.45-6.86) in comparison with green coffee beans that showed medium intensity (chroma 13.09-19.45). This behavior may be due to oxidation and enzymatic darkening processes that occur within the coffee beans, which causes the change in their pigmentation. In the relation to the Hue° that is characterized by the wavelength of the radiation and differentiates one color from another, there was only a difference in the coffee cherries dry beans in the a_w of 0.743 and 0.821 showing a trend of variation from a dark red color to yellow, this could be due to the visible presence of mycelium in the structure of the coffee beans (Table 2 and Fig. 3), in this context, Broissin-Vargas et al., (2017) corelated the loss of color and chromaticity of coffee beans whit the presence of a fungal mycobiota during storage.

The textural parameters are also important for the acceptability of foods; these include physical sensations such as gumminess, hardness and fracturability, which depend on the structural elements of the material and are directly related to deformation, disintegration and flow through the application of a force (Foegeding et al., 2011 Akwetey and Knipe, 2012; Zhuab et al., 2013; Paula and Conti-Silva, 2014). In these tests, the maximum force required to produce a break or total fracture of the product structure is evaluated, understanding that the higher the force values, the greater the resistance of the food (Rosenthal, 2010; Zhuab et al., 2013 Milde et al., 2014).

The fracturability of coffee cherries dry beans and green coffee beans are shown in figure 2. In the graphs obtained, it can be observed that the coffee cherries dry beans have greater resistance to the fracture force in the range of a_w 0.515-0.743 (Fig. 2, AII) while in the range of a_w 0.108-0.435 they showed lower resistance to fracturability showing a brittle behavior (Fig.2, AI). Likewise, the coffee cherries dry beans subjected to the condition of a_w 0.821 showed a 50% reduction in resistance to fracture strength with respect to the conditions of 0.515-0.743. This may be because an increase in moisture content causes the structure to become softer, since foods with a high a_w (> 0.5) have a juicier, tender and more chewy

texture whereas when the a_w of the products decreases, texture attributes appear as hardness, dryness and hardening resulting in more crisp foods (Badui, 2010).

On the other hand, in the green coffee beans an opposite behavior was observed, the resistance to the fracture force was gradually decreasing as the a_w increased, the greater resistance was obtained in a_w 0.108 (Fig.2 BI) and the lowest resistance was observed in a_w 0.743-0.821 (Fig.2 BII), this effect and that observed in coffee cherries in the a_w 0.821 is attributed to the fact that the higher the a_w is and the closer it gets to 1.0, which is the value of the pure water, the greater its structural instability (Badui, 2006).

These results may be due to the structural differences between the two, the green coffee beans only have the endosperm (coffee bean) formed mainly of polysaccharides, proteins, lipids and caffeine, making this type of food do not flow against pressure efforts, but they are fragile and brittle (Romero et al., 2014), while the coffee cherries dry beans in addition to this structure have tegument (silver skin), endocarp (parchment), a layer of pectin (cell wall), mesocarp or pulp (mucilage) and pericarp (outer skin) giving them a greater number of protective anatomical fractions (Rojo-Jiménez, 2014). This allows a greater adsorption of moisture to be present in its structure, giving it gumminess properties. This effect has been described by Rodríguez et al (2005), who atribute these parameters in high carbohydrate food products, as is the case of coffee cherries dry beans, is particularly difficult due to its heterogeneous composition and its little uniform structure.

Morphology structure of coffee samples after storage

The effect of a_w on the microbilogical characteristics and morphology in coffee cherries dry and green coffee beans are shown in figure 3 and 4.

In the microbiological changes, we observed that a water activity of 0.108-0.515 the coffee cherries dry beans and green coffee beans do not present the growth of fungi (Fig 3). However, the increasing in water

activity to 0.628-0.821 showed the presence of growth the fungi, characterizad by the presence of white mycelium (Fig 3). In order of evaluate changes in the microestructure of the samples, in this study we perform an analysis of the microstructure of cherries coffee dry beans and green coffee beans, SEM images are showed in figure 4. The water activity had a pronounced effect on the microstructure in coffee cherries dry and green coffee beans. The figure 4a corresponding to a_w 0.108 shows the characteristic morphology of the coffee cherry dry beans that consists of oval and compact cells, this same behavior was observed in a range of a_w of 0.108-0.515 where there were no apparent changes in its structure. In contrast, at a higher a_w (0.628-0.821) the cell structure was modified, showed developing folds with wide crests giving a less rough surface appearance, in addition, the growth of fungi mycelium was observed (Fig. 4 b-d). The characteristic microstructure of the samples of green coffee beans at a_w 0.108 consist of cells of a polyhedral shapes and it can seen cell distribution in coffee endosperm is heterogeneous (Fig. 4 e), but an increase in the a_w (0.628-0.821 and 35 ° C) they suffer a deformation in their architectura with the growth of fungi mycelium was observed (Fig. 4 f-h).

It has been reported that aw (0.7-0.1), and temperature have a very important role in the growth of filamentous fungi and yeasts in food (Badui, 2006, Taniwaki et al., 2018). Oliveira et al (2019), who observed an increase in the growth of the fungi *A. carbonarius* and *A.ochraceus* in a_w conditions of 0.935-0.965 and at temperatures of 21-30° C, also Sousa et al (2019) stimated the best conditions for germination, growth, and synthesis of OTA whith increases the temperature.

Water adsorption isotherms of coffee samples after storage

Water activity is an important tool for the prediction of food stability, since being its major component, there will always be a constant transfer of this compound to the environment, affecting its quality and physical and organoleptic properties. It also indicates the availability of water of a given medium for chemical, biochemical and transfer reactions through semipermeable membranes there of, which directly

interferes with the speed with which certain deterioration changes such as microbial growth and appearance of contaminating fungi (Aksil et al., 2019; Pascual-Pineda et al., 2014).

The water adsorption are useful thermodynamic tools to determine the interactions of water and food substances and thus provide valuable information to ensure food processing operations such as drying, mixing and storage. These values can be used to select the most appropriate storage conditions and packaging systems that optimize or maximize the retention of aroma, flavor, color, texture, nutrients and biological stability (Beristain et al., 2002; Diosady et al., 1996; Gabas et al., 2000).

The adsorption process was modeled with the Guggenheim-AndersonDe Boer (GAB) equation, which is a model with three parameters which have physical significance. It has been suggested as the most versatile model to explain the process of adsorption in food (Lomauro et al., 1985). The GAB model was adjusted satisfactorily to the experimental data, the average relative deviation (P) module for coffee cherries dry beans was less than 10% while for green coffee beans it was less than 4%. The values of the parameters of the GAB equation (M0, C, K) were determined by a non-linear regression analysis with the Kalediagraph software see 4.0 (Table 3). The coefficcients of determinarion (R^2) were over 0.99 for all temperatures. The value of the monolayer (M_o) shows the amounth of water is strongly adsorbed to specifics sites and is considered as the optimum value at which a food is more stable. The values of M_o of coffee cherries dry beans and green coffe beans were in the range of 3.78-7.96 and 4.32-6.38 g H_2O/100 g d.s respectively and decreased as temperature increased from 15 at 35 °C. This phenomenon can be attributed to reductions in the number of aviable sites for water binding due to excitation state of molecules, an increase in kinetic energy leading to an increase in the distance between them. Therefore, water molecules whit a low motion at low temperatures bound more easily to situable binding sites on surfaces (McLaughlin and Magee, 1998; Alpizar-Reyes et al., 2018).

The variation of the value of the monolayer (M0) with the temperature indicates that there are more active sites of water adsorption with the increase of this, which can be the result of structural changes, such as

the swelling of the beans. The values of *K* present interactions between water molecules and adsorbent (coffee cherries dry beans and green coffee beans). Values of K <1 indicates a structured state of the adsorbate in the adjacent to the monolayer (Lewicki 1997). In the table 3 showed the values of *K* for coffee cherries dry beans and green coffee beans, we observed range between 0.82- 0.94 in both samples, which implies there were fewer interactions between water molecules and type of coffee in the multilayer, and a tendency of increased as the temperatura increase.

In the figure 5 and 6 we observed that the integral entropy decreased until reaching a minimum point and then increased as a_w increased. The decrease in integral entropy represents a disminution on mobility of water molecules promoting the saturation of available sites. Higher energy is required to continue the adsorption phenomena. Thus, the increase implies that water molecules are to form multilayers. The integral entropy is a thermodynamic function used to describe the degree of arrangement of water molecules during the adsorption process and is considered as the maximum stability point, indicated this, water is less available to carry out spoilage reactions (Viganó et al., 2012).

The integral thermodynamic properties of the coffee cherries dry beans and green coffee beans (entropy) at 15, 25 and 35 ° C were determined; and the a_w and temperature conditions at the point of minimum integral entropy was considered as the best condition for the storage of coffee beans, keeping it free from contamination by fungi or bacteria and delaying deterioration reactions (Fig. 5 and 6). For coffee cherries dry beans, a_w 0.3 (2 g H_20/100 g of ds) at 35 °C (Fig. 5) and by green coffee beans a_w 0.6 (8.4 g H_20/100 g of ds) at 35 °C (Fig. 6) are the best conditions of storage.

Analysis of OTA in coffee beans after storage

The results obtained from the analysis of ochratoxin A from samples of coffee cherries dry beans and green coffee beans from Atoyac Álvarez are shown in Table 4. All the analyzed samples presented OTA levels above the permitted values established by the European Commission (5 μg/kg) and it was observed

the concentration of OTA was higher in samples of coffee cherries than in green coffee. However, it can be seen that in the a_w range of 0.108-0.515 the concentration of OTA remained (40.73-46.17 μg/kg in coffee cherries dry beans and 15.49-17.77 μg/kg in green coffee beans) without showing a significant difference between the samples. On the other hand, at a_w of 0.628 and 0.743, a significant increase was observed with respect to the other conditions de a_w, for both samples (65.22 μg/kg in in coffee cherries dry and 22.54 μg/kg in green coffee beans) exceeding the permissible values of OTA to 12.9 and 4.5 times respectively. The most important observation in this study was that in a_w of 0.628 and 0.734 at a temperature of 35 ° C the optimal conditions for the production of OTA by *Aspergillus, Penicillium* and *Fusarium* for both samples of coffee were presented. There are few bibliographic reports that demonstrate that a_w and temperature conditions are important limiting factors for the growth and production of OTA and may vary according to the substrate and toxigenic fungi strain (Sousa et al., 2018, Oliveira et al., 2019); Moss (1991) reported the optimal conditions for *Aspergillus ochraceus* to produce OTA, including temperatures between 12 and 37 ° C and a_w of 0.85-0.97; Oliveira et al (2019) observed that *Aspergillus carboanarius* (CCDCA 10288 and 10293) showed higher growth and OTA production at a_w 0.940-0.990 and temperature between 21-30 ° C.

On the other hand, the presence of OTA in coffee has been documented during pre- and post-harvest processing, such as Palacios-Cabrera et al (2004), in which they reported a range of 1.90-8.200 μg/kg in green coffee from Campinas, Brazil, also Kouadio in 2012 reported concentrations between 0.92 and 118.47 μg/kg in coffee cherries dry beans from Bingerville Ivory Coasty; Franco et al (2014) who reported concentrations of 4.90-37.73 μg/kg in green coffee from Panama; Hesseltine et al (2015) that found concentrations of 953-1262 μg/kg in green coffee from Peoira, Illinois; Casas Junco et al (2018) showed OTA concentrations between 76.74-91.03 μg/kg in green coffee beans from Nayarit; Bessaaire et al (2019) analyzed green coffee from several countries (Vietnam, Brazil, Colombia, Ethiopia, Cote d'Ivoire, China, Indonesia, Mexico and Guatemala) that reported OTA concentrations in a range of 0.2

to 12.2 μg/kg. This may be due to the fact that said activities refer to humidity conditions of 63.30 and 84.32%, respectively, reported as optimal for the growth of fungi and the production of mycotoxins, which in turn are influenced by numerous parameters abiotic and biotic and their complex interactions. The development and biosynthesis of OTA by toxigenic fungi depends on environmental factors. Water availability is probably the most important factor affecting the germination, growth and establishment of fungi on nutrient-rich substrates. The next most important factors for mycotoxin production and fungi growth are high moisture content (20-25%) and high relative humidity (70-90%), and because Atoyac de Álvarez is a region that has these conditions and also it is known that the impact of climate changes causes a significant increase in damage to crops, it is suggested that this favors a greater colonization of coffee beans by mycotoxigenic fungi and, as a consequence, a greater production of mycotoxins (Bebber et al. , 2013; 2014; Medina et al., 2015; Oliveira et al., 2019).

4. CONCLUSION

It is concluded that the Atoyac Guerrero coffee has higth levels of OTA contamination, were the most common fungal species was *Aspergillus* sp. and *Penicillium* sp. In addition, it was found that the highest risk of fungal contamination and OTA production is found when we have values of a_w 0.628, 0.743 and temperature of 35°C. Trough the models obtained, it was possible to predict the best storage conditions. Thus, producers should maintein good agricultural practices during the processing of coffee in order to avoid contamination and OTA production by fungi.

5. CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

6. ACKNOWLEDGMENTS

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7. TABLES AND FIGURES

Table 1. Occurrence of fungi species in coffee cherries dry beans and green coffee beans from Atoyac Guerrero.

	Occurrence (%)		
Beans sample	***Aspergillus***	***Penicillium***	***Others fungi***
cherries dry	88.8	16.6	38.8
green coffee	100	27.7	16.6

Table 2. CIELAB colour parameters of coffee cherries dry beans and green coffee beans from Atoyac Guerrero after storage (35 ^{0}C and different range of a_w).

	Cherries coffee				**Green coffee**			
a_w	L^*	b^*/a^*	chroma	Hue	L^*	b^*/a^*	chroma	Hue
0.108	10.75±1.6^a	2.18/1.89	2.53±0.5^a	41.75^a	22.78±0.6^a	13.38/2.17	13.56±0.9^a	80.85^a
0.215	7.62±2.0^a	2.64/2.01	3.34±0.3^a	53.23^a	23.03±3.1^a	12.90/2.25	13.09±0.5^a	80.06^a
0.318	11.25±2.4^a	4.56/4.33	6.14±2.1^a	43.96^a	24.975±0.6^a	13.82/2.00	13.97±0.4^a	81.70^a
0.436	10.39±2.4^a	2.98/6.05	6.86±5.2^a	33.28^a	22.045±4.1^a	13.09/2.72	13.37±0.8^a	78.20^a
0.515	8.31±1.9^a	1.49/1.95	2.45±1.2^a	37.3^a	26.96±3.1^a	15.28/2.62	15.50±2.1^a	80.25^a
0.628	11.86±3.1^a	2.95/2.80	3.85±1.5^a	49.11^a	17.79±1.0^a	12.75/4.96	13.68±0.3^a	74.02^a
0.743	19.56±7.1^a	5.83/1.63	6.05±2.3^a	74.10^b	30.535±9.1^a	16.93/3.78	19.45±5.0^b	77.32^a
0.821	23.23±2.9^b	3.58/0.74	3.65±1.5^a	78.44 ^{b}	25.655±5.2^a	15.06/4.19	15.64±1.3^a	74.30^a

Lightness (L^*), green/red parameter (a^*), blue/yellow parameter (b^*), Hue angle (Hue). The values represent the mean of trhee repeats ± the standard deviation. An ANOVA test was performed with Tukey's multiple comparison test. Different letters represent statistically significant difference $p < 0.05$.

Table 3. Estimated GAB parameters for coffee cherries dry beans and green coffee beans from Atoyac Guerrero.

	Coffee cherries dry beans			Green coffee beans		
	15°C	25°C	35°C	15°C	25°C	35°C
Mo (g H2O/100 g d.s.)	7.96	6.90	3.78	6.38	4.76	4.32
C	0.94	0.94	0.91	2.94	4.21	2.95
K	0.82	0.82	1.03	0.83	0.92	0.94
R^2	0.99	0.99	0.99	0.99	0.99	0.99
P (%)	8.47	8.69	9.51	2.86	2.27	3.37

Table 4. Concentration of OTA in coffee cherries dry beans and green coffee beans from Atoyac Guerrero after storage (35 ^{0}C and different range of a_w).

	Concentration of OTA (μg/kg)	
water activity (a_w)	cherries dry	green coffee
0 .108	45.29[a]	16.17[a]
0 .215	40.73 [a]	16.83[a]
0 .318	43.51 [a]	16.62[a]
0.436	41.58[a]	15.49[a]
0 .515	46.17 [a]	17.77[a]
0.628	64.36[b]	22.54[b]
0.743	65.22 [b]	20.40[b]
0.821	43.94[a]	16.48[a]

The values represent the mean of trhee repeats ± the standard deviation. An ANOVA test was performed with Tukey's multiple comparison test. Different letters represent statistically significant difference $p < 0.05$.

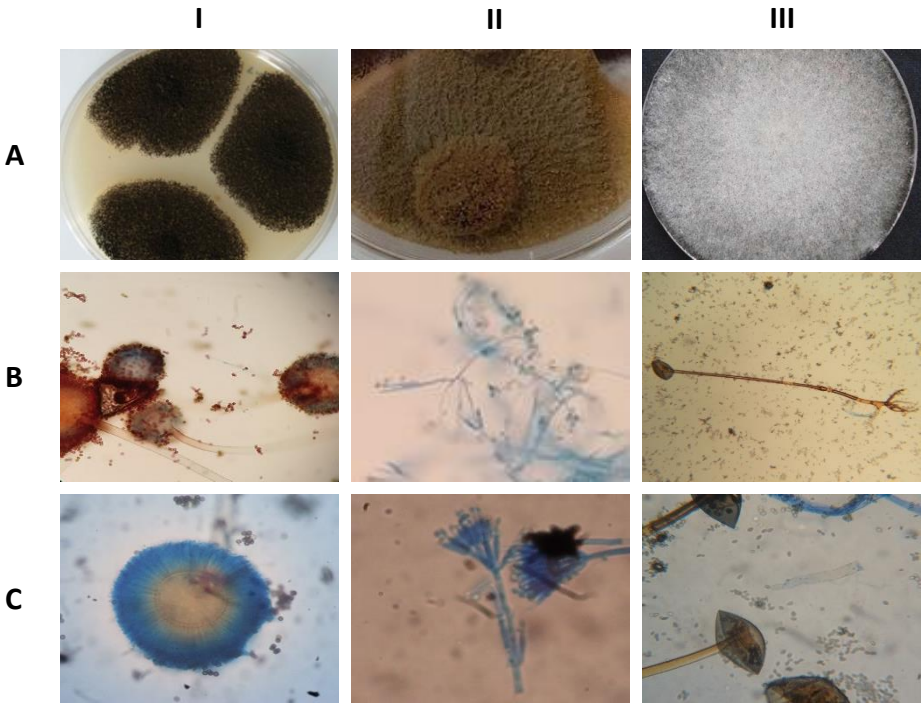

Figure.1 Fungi species isolated from coffee cherries dry beans and green coffee beans from Atoyac Guerrero. I) *Aspergillus*, II) *Penicillium* y III) *Rhizopus.* Macroscopy morphology (**A**); Microscopy morphology (**B** and **C**) optical microscopic 10x and 40x.

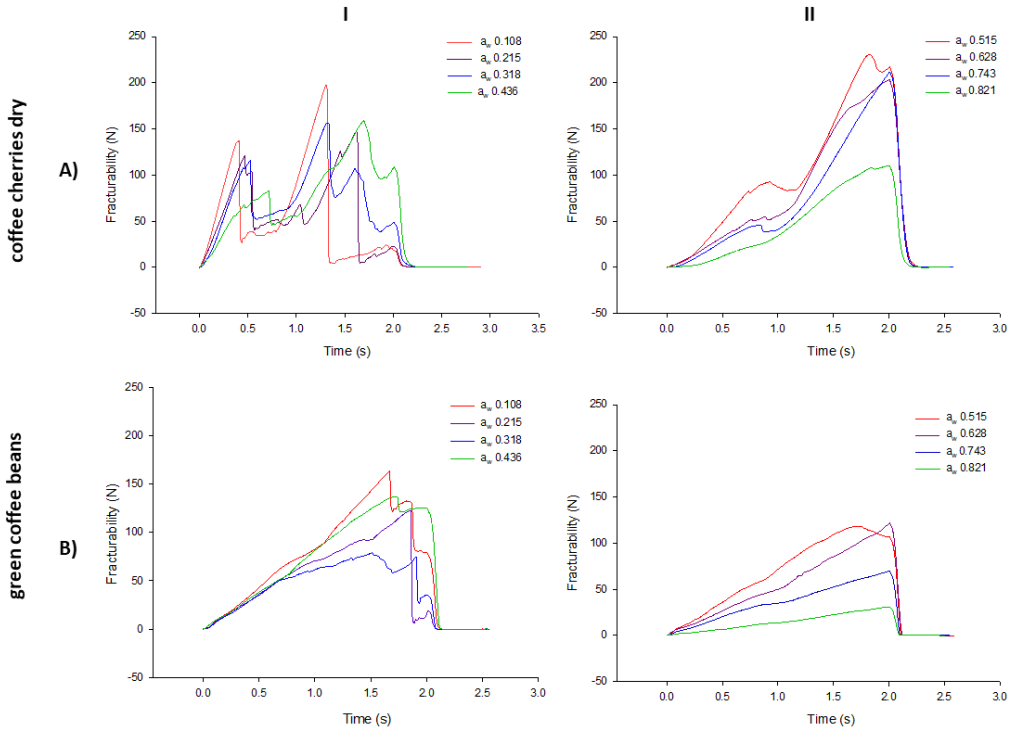

Figure 2. Changes in fracturability propierties of coffee cherries dry beans and green coffee beans. A) Fracturability values of coffee cherries dry beans, B) Fracturability values of green coffe beans (I water activity range 0.108-0.426, II water activity range 0.515- 0.821).

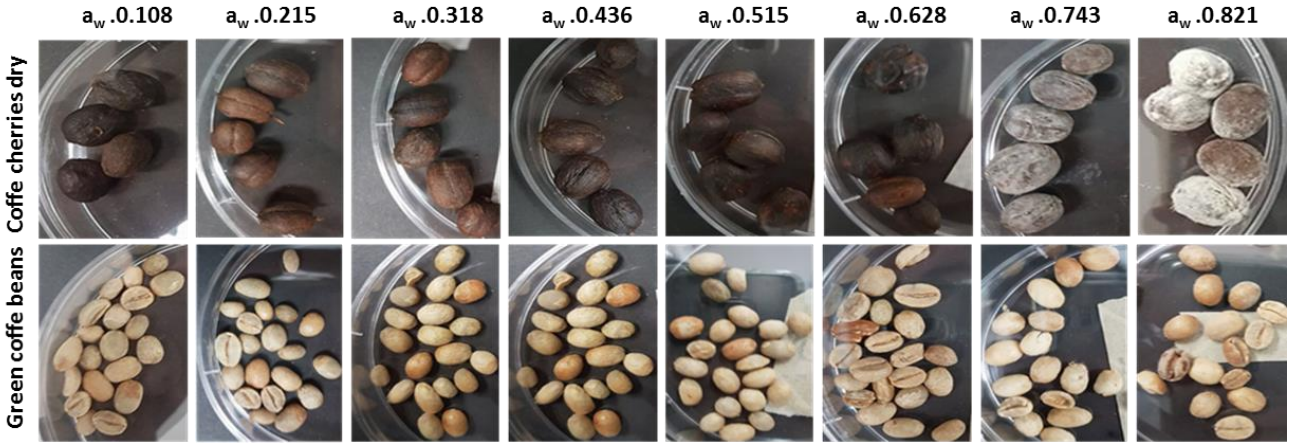

Figure 3. Physical changes in coffee cherries dry beans and green coffee beans from Atoyac Guerrero after of storage (35 ^{0}C and different range of a_w).

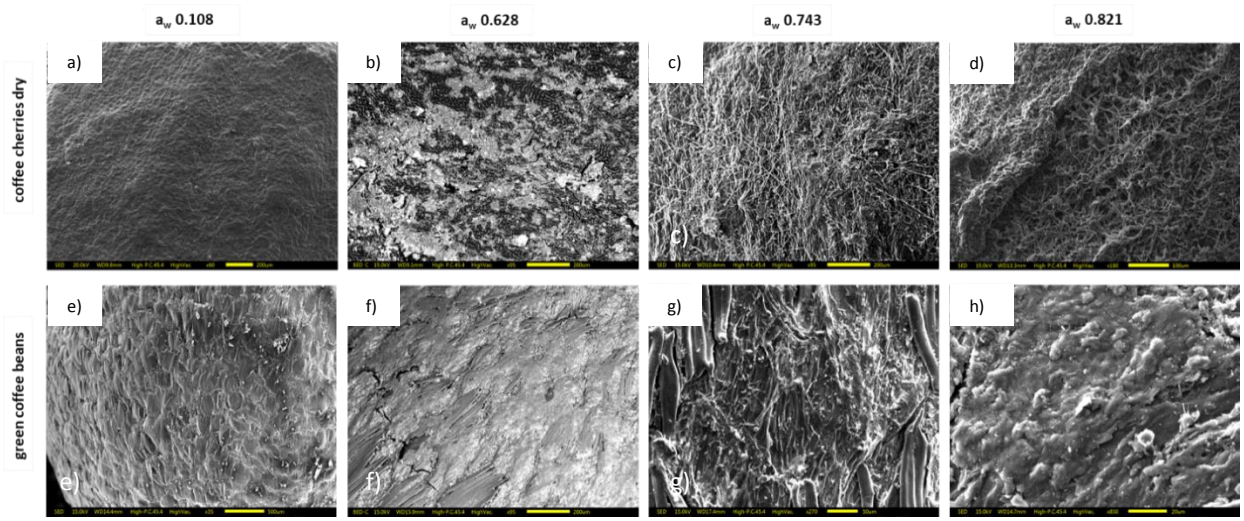

Figure 4. Microstructure changes in coffee cherries dry beans (a-d) and green coffee beans (e-h) from Atoyac Guerrero, after of storage (35 ^{0}C and different range of a_w).

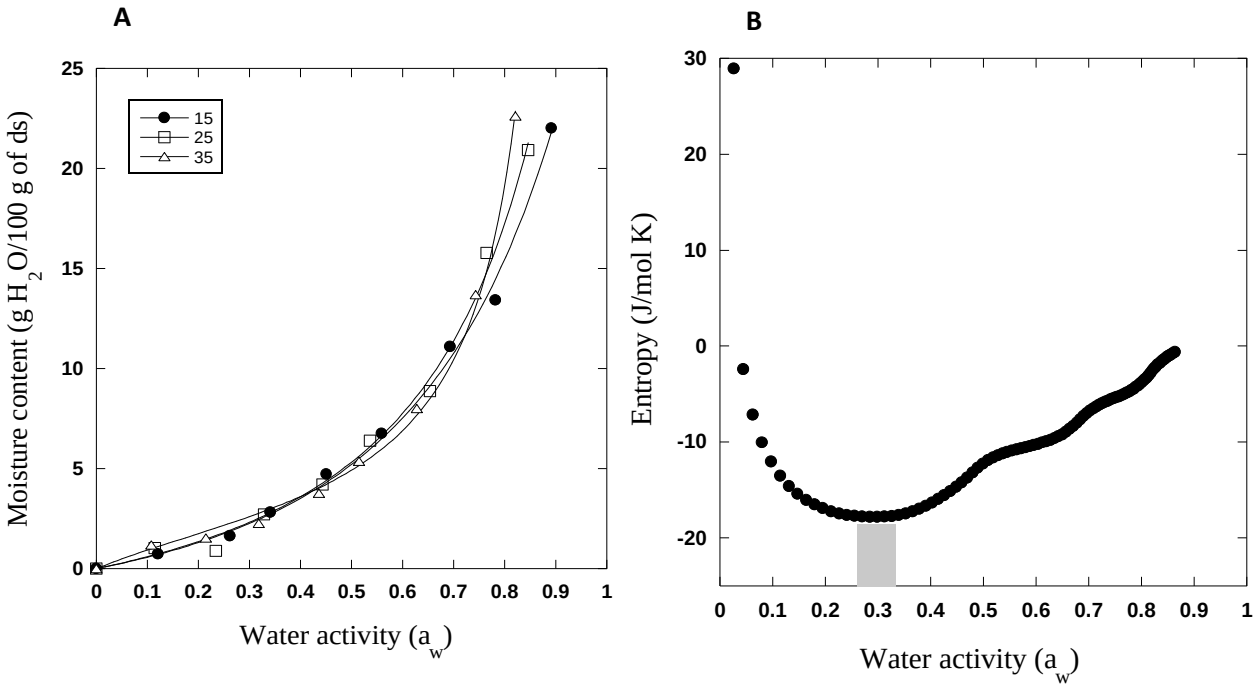

Figure 5. Adsorption isotherms of coffe cherries dry beans from Atoyac Guerrero at different temperatures (**A).** Differential (□) and integral (●) entropy changes as a function of water activity for coffe cherries dry beans at 35 °C (**B**). The arrows indicate minimum integral entropy.

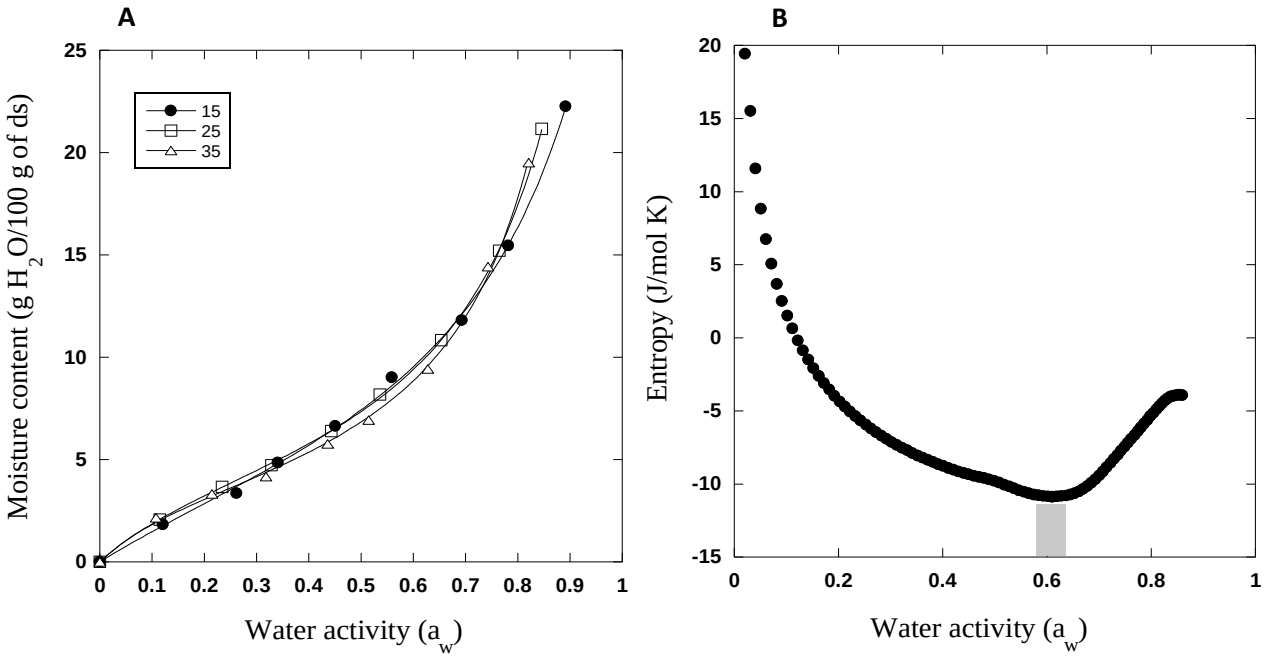

Figure 6. I) Adsorption isotherms of green coffe at different temperatures. A) Differential (□) and integral (●) entropy changes as a function of water activity for green coffe at 35 °C B). The arrows indicate minimum integral entropy.

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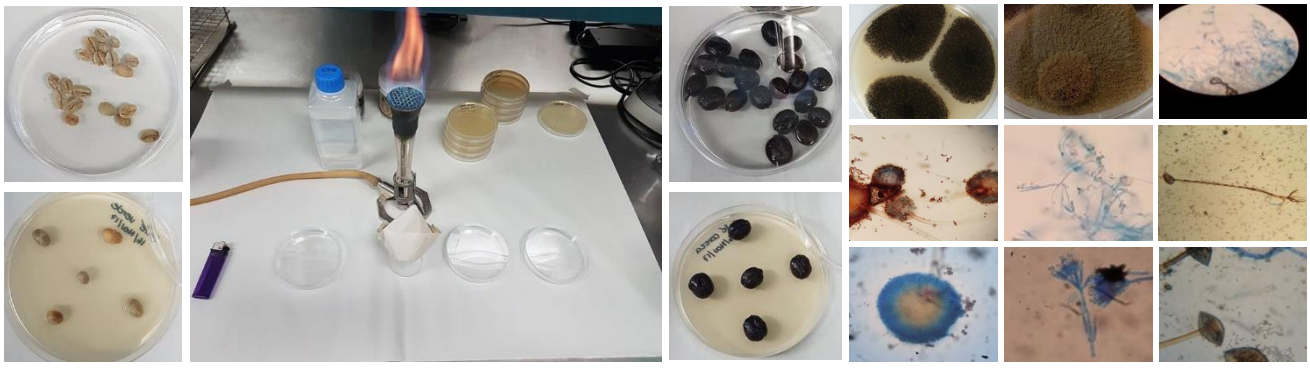
9. ANNEXES

Anexo 1. Identificación de los hongos

La identificación de los hongos contaminantes de las muestras de café cereza y verde se realizó de acuerdo a lo reportado por Luna et al (2010). Los granos fueron sometidos a un protocolo de desinfección (cloro 10%/5 min y enjuagues con agua estéril), posteriormente se colocaron granos de café en cajas Petri con agar papa dextrosa (PDA; DIFCO, Becton Dickinson and company), en relación de 5 granos por caja, y fueron incubadas durante 7 días a 27 °C. Para la identificación de las especies fúngicas se observaron características macroscópicas y microscópicas de los cultivos. El estudio macroscópico se realizó a simple vista, observando color y forma de crecimiento de los micelios. Inmediatamente se procedió a realizar el estudio microscópico, donde se observó la morfología característica a partir de tejido micelial teñido con azul de lactofenol. La comparación de las estructuras se realizó tomando como referencia lo reportado por López-Jácome et al (2014) y Bonifaz Trujillo (2010). Las características que se consideraron fueron la presencia de estructuras especializadas como: conidio, conidióforo, esterigmas, métula, microconidio, rizoide, vesícula, esporangióforo y esporangio.



Anexo 2. Determinación de color

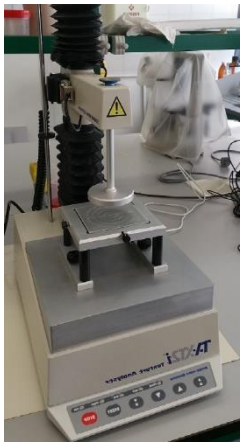
Para la caracterización del color de los granos de café cereza y verde, se determinaron las coordenadas correspondientes a luminosidad (L), matiz o tono (a) y saturación (b), utilizando el sistema X-RITE Ci62. Los granos de café cereza y verde se colocaron en la cámara de medición del equipo y se prosiguió con la lectura. Esta medición se basa en el sistema estandarizado por el Comité Internacional de Iluminación, que determina el color de acuerdo a la posición en el espacio en tres dimensiones; L* representa la luminosidad y varia de un valor de 100 para blanco perfecto a 0 para negro, a* es la variación de rojo (+) a verde (-) y gris (0), b* es la variación de amarillo (+) a azul (-) y gris (0), y la relación a*/b* permite calcular los ° Hue. Posteriormente se calcularon el ángulo de tono y la cromaticidad (C * ab) usando las siguientes ecuaciones H ° = tan-1 (b * / a *), donde a * = [negativo (verde) a positivo (rojo)], b * = [negativo (azul) positivo (amarillo)] y C*ab =$\sqrt{a^2 + b^2}$.



Anexo 3. Fracturabilidad

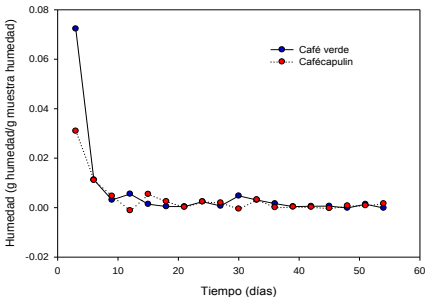
Las propiedades mecánicas de oos granos de café cereza y verde, fueron obtenidas mediante el empleó del texturómetro Stable Micro Systems®, modelo TA-XT2i, en modo de compresión con una celda de carga de 50 kg, la velocidad del bastidor fue de 10 mm/s, hasta una deformación de 4 mm de la muestra. Los ensayos se realizaron para cada una de las 8 condiciones de a_w. La información registrada de fuerza (N) y deformación (mm) fue procesada usando el software "Texture Expert Exceed" del texturómetro. A

partir de la curva de compresión registrada se determinó la fuerza de fractura y la de formación unitaria (deformación/dimensión del fruto).



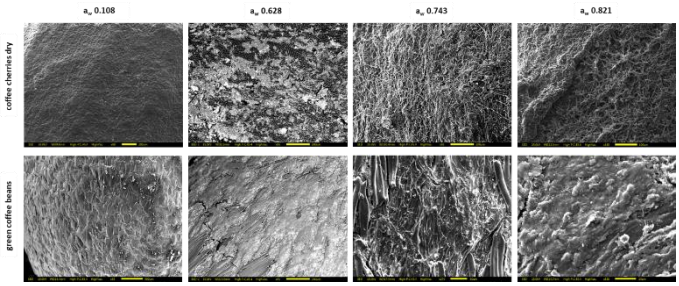
Anexo 4. Curva de secado

Para la obtención de la curva de secado, se realizó la preparación de los granos de café cereza y verde de acuerdo con lo reportado por Tinoco y Ospina (2010). Se pesaron en charolas de aluminio 3 g de las muestras y se colocaron dentro de desecadores con vacío que contenían en el fondo 10 g de agente deshidratante pentóxido de fósforo (P_2O_5). Las muestras fueron pesadas cada 5 días durante 4 semanas (hasta alcanzar un grado de estabilidad en el que no perdiera peso) y finalmente se obtuvo la cuerva de secado.



Anexo 5. Microscopía Electrónica de Barrido

Las imágenes obtenidas por microscoía electrinica de barrido, fueron obtenidsas con el microscopio electrónico de barrido Jeol T300. Los granos de café cereza y verde se colocaron sobre el soporte y fueron recubiertos con grafito. Una vez recubiertas las muestras se colocaron en el compartimiento del microscopio y se observaron a 500 y 1,000 x.



Anexo 5. Isotermas de adsorción

Se elaboraron las isotermas de adsorción según lo reportado por Brunauer *et al.,* (1940). Se pesaron 3 g de muestra y se colocaron en soportes de aluminio dentro de celdas de adsorción de vidrio con soluciones saturadas de sal, que proporcionan diferentes actividades de agua (a_w) en un rango de 0.1 - 0.8 y porcentajes de humedad relativa (%HR) de 11 – 95% (Lang *et al.,* 1981). Las isotermas se realizarán por triplicado a 15, 25 y 35 °C y se obtendrán al graficar el contenido de humedad en el equilibrio contra la actividad de agua correspondiente.

Reactivo	Humedad relativa (%)	Sal (g)	Agua (mL)
Cloruro de litio (LiCl)	11.15	10	10
Acetato de potasio (CH_3COOK)	22.60	24	8
Cloruro de magnesio ($MgCl_2$)	32.73	20	4
Carbonato de potasio (K_2CO_3)	43.80	13	13
Nitrato de magnesio ($MgNO_3)_2$	52.86	20	6
Nitrato de sodio ($NaNO_{2)}$	63.30	13	10
Cloruro de sodio (NaCl)	84.32	20	20
Cloruro de potasio (KCl)	90.26	20	20

Anexo 6. Determinación de OTA mediante el método inmunoenzimático

Preparación de la muestra

5 g de la muestra molida de café cereza y verde se colocaron en un tubo Falcon (50 mL) y se adicionó 12.5 mL de metanol (70 %), se agitó vigorosamente durante 3 min, transcurrido el tiempo se filtró el extracto a través de un papel Whatman no.1. Posteriormente se preparó la solución de trabajo diluyendo 1 mL del filtrado con 1 mL de agua destilada.

Cuantificación de Ocratoxina A

La concentración de OTA se determinó con el KIT RIDASCREEN FAST OCHRATOXIN A®. Inicialmente se realizó una curva de calibración de OTA de acuerdo a lo reportado por el fabricante y se determinó la ecuación de calibración y el coeficiente de correlación. Para las muestras se agregaron 50

μL en los pocillos previamente identificados, utilizando una punta nueva para cada muestra, después se agregaron 50 μL del conjugado de ocratoxina A-enzima, posteriormente se adicionaron 50 μL de anticuerpo anti-ocratoxina A y se mezcló suavemente durante 10 min, transcurrido el tiempo se vaciaron los pocillos sobre un papel absorbente limpio, se lavaron con agua destilada y este paso se repitió dos veces más, posteriormente se agregaron 100 μL de substrato/cromógeno mezclando el contenido y se dejó reposar por 5 min en condición de oscuridad a temperatura ambiente y finalmente se le agregaron 100 μL de la solución stop mezclando el contenido de la microplaca y se determinó la absorbancia en un espectrofotómetro (Stat Fax 2100) a 450 nm.