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Determination of potentially mycotoxigenic fungi in coffee (*Coffea arabica* L.) from Nayarit

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Abstract A total of fourteen roasted coffee samples were collected from different local markets in Nayarit, Mexico. Twenty-two fungi isolates were related to the genera *Aspergillus* (54.54%) and *Penicillium* (4.5%). The strains R16 (0.33 µg/kg), 6N (1.16 µg/kg) and 11 (0.36 µg/kg) tested positive for OTA (ochratoxin A) production in PDA, the other fungi samples were not toxigenic. According to the sequence analysis of their ITS1-5.8S-ITS2 rDNA region, fungi OTA producers correspond to *A. niger*, *A. versicolor* and *Byssochlamys spectabilis*. These three strains were able to produce OTA when inoculated in roasted coffee in concentrations ranging from 75 to 90 µg/kg, after 21 days. Different production stages of roasted coffee (crop management, postharvest practices and storage) along with environmental conditions do not ensure mycotoxigenic fungi free products. This is the first report of OTA natural occurrence in roasted coffee from Nayarit.

Keywords Roasted coffee · *Byssochlamys spectabilis* · Toxigenic fungi · Ochratoxin A · Immunoaffinity column

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Introduction

Coffee is one of the most widely-consumed food products, with an important economic and cultural role. However, coffee beans, like other crops, can be contaminated by microorganisms during the different stages of growing, harvesting, processing, transport and storage. Many studies have revealed that important toxigenic fungal genera (*Aspergillus* and *Penicillium*) are natural coffee contaminants [1–3]. OTA is a mycotoxin naturally found in various food products including green coffee beans, roasted coffee and instant coffee [2]. This mycotoxin produced by toxigenic fungi has been shown to exhibit hepatotoxic, nephrotoxic, teratogenic, and carcinogenic properties [4]. The International Agency for Research on Cancer (IARC) classified OTA as carcinogenic for humans (group 2B) [5, 6]. According to the European Commission Regulation [7], the maximum levels for OTA are 5 µg/kg for roasted coffee beans and ground roasted coffee, and 10 µg/kg for soluble coffee [8].

One of the main economic activities in Nayarit is coffee production. Environmental conditions such as high temperatures and humidity favor fungal development in coffee beans. De Lourdes et al. [9], reported the presence of OTA in 70% of green coffee samples with an average concentration of 30.1 µg/kg. Franco et al. [10] detected OTA from 4.90 to 37.73 µg/kg and total aflatoxins were found from 1.51 to 1.93 µg/kg. It was found that four out of the 21 samples in Panamanian exportation coffee tested positive for OTA and three tested positive for presence of total aflatoxins. These findings highlight the importance of determining the presence of potential ochratoxin and aflatoxin producing fungi in roasted coffee beans (*Coffea arabica* L.) from Nayarit, an important coffee producer in Mexico.

Materials and methods

Roasted coffee

Experiments were carried out using 14 ground roasted coffee samples (*Coffea arabica* L.) from different local markets in Nayarit, Mexico.

Identification of fungal isolates

Two methods were employed for fungal isolation. In the first method, processed coffee beans were plated directly onto filter paper moistened with sterile distilled water. The beans were collected randomly from each coffee bean sample; they were then disinfected by immersion in a 1% hypochlorite solution for 2 min (w/v). The beans were then put on the surface of potato dextrose agar (PDA) medium and Rose-Bengal (1/15,000) was added as a bacteriostatic agent. Plates were incubated at 28 °C for 7–10 days [11]. In the second method, ten grams of ground roasted coffee were transferred to a wide mouth reagent bottles containing 90 mL sterile distilled water. The bottles were shaken for 15 min. This gave an approximate dilution of 1/10. Sterile dilutions from 1/100 to 1/10,000, were made using test tubes containing 9 mL sterilized distilled water. One mL of each selected dilution was put in a sterile petri dish and 10 mL of the medium (Czapek Yeast Extract Agar, Malt Extract Agar, Czapek Dox Agar) were poured, the plate was then gently moved for homogeneous distribution. The plates were incubated at 28 °C for 7–14 days. Bacterial growth was inhibited by using streptomycin (300 ppm). Sodium bicarbonate ($NaHCO_3$) was added to 50% of the plates in order to substantially inhibit growth of *A. niger* [12].

Isolates were purified on Czapek Yeast Extract Agar. The fungi were allowed to grow at 25 °C for 7 days, and were then pre-identified following the traditional morphological methods. Macroscopic (mycelium type, color and growth type) as well as microscopic optical characteristics (at 40×, mycelium type, conidiophores morphology and spore morphology) were considered for identification [13, 14].

Ochratoxin and aflatoxin production ability of the isolates

Mycotoxin production by isolated fungal strains was determined using HPLC following the methodology described by Mounjouenpou et al. [15]. After 10 days of incubation on PDA agar (25 °C), direct extraction was carried out on 3 agar discs taken from the center of the colony. Extraction was carried out in 2.5 mL of solvent (methanol/formic acid 25:1 v/v) for 15 min in an ultrasound bath.

The production of OTA was detected and quantified by reversed phase high (zorbax SB-C18 2.1 × 50 mm id: 1.8 µm) HPLC with electrospray ion (ESI) MS (Agilent Technologies), in ionization mode, positive mode capillary 3500 V, nebulizer 25 psi (nitrogen), dry gas nitrogen at 9 L/min, dry gas temperature 350 °C, fragmentor voltage 95; selected ion monitoring (SIM), m/z 404 (OTA). The mobile phase (5 mM ammonium acetate/acetonitrile, 65:35 at 40 °C) was pumped at a rate of 0.2 mL/min. The injection volume was 20 µL and the retention time was around 4 ± 1 min.

In all cases, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_2 (AFG_2), aflatoxin G_1 (AFG_1) were quantified on extracts by HPLC with fluorometric detection (Shimadzu LC-10 ADVP, Japan) [1]. The operating conditions were as follows: 100 µL injection loop, C18 reverse phase HPLC column, ODS 5 µm (Supelco, Interchim, Montluçon, France) with an identical pre-column thermostatically controlled at 35 °C, an isocratic flow of 1 mL/min, an excitation wavelength of 362 nm and an emission wavelength of 425 nm were used. Aflatoxins were carried out with potassium bromide. Contents were calculated from a calibration curve established from a standard (1 µg/mL; ref PD 226 R. Biopharm Rhône Ltd, Glasgow, UK).

PCR identification

Fungi were grown in Potato Dextrose Agar at 28 °C for 5–7 days. DNA extraction was then performed according to the protocol proposed by Sambrook and Russell [16]. PCR was conducted using the primers ITS1 (ITS1-5, 8s-ITS-2) of ribosomal DNA using universal primers ITS1 (5′ CAACTCCCAAACCCCTGTGA-3′) and ITS4 (5′-GCGACGATTACCAGTAACGA-3′) for molds [17]. Two amplifications were carried out in a Technethermocycler (iCycler Biorad, USA). Thermal cycling parameters for first amplification were: initial denaturation at 95 °C for 2 min, followed by 30 cycles of heat denaturation at 95 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. Thermal cycling parameters for second amplification were: initial denaturation at 95 °C for 5 min, followed by 30 cycles of heat denaturation at 95 °C for 1 min, annealing at 56.5 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. PCR products from these amplifications were separated by electrophoresis on 1% (w/v) agarose gel, stained in syber safe (Sigma-Aldrich), the sample was mixed with buffer TAE 1X. A molecular marker from 100 to 1000 bp was used. Electrophoresis was performed under the following conditions: 1 h and 30 min, 60 V and

400 mA. PCR products were visualized using a UV transilluminator (UVP BioDoc-IT Imaging System, USA). The PCR products were sent to GENEWIZ Inc. (USA), for sequencing and species identification. The results were analyzed using the BLAST (The Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnological Information) with the support of Codon Code Aligner 2.0 editing program sequences.

OTA production by fungi isolates

Isolates of *A. niger*, *A. versicolor* and *Byssochlamys spectabilis* were grown on PDA, at 27 °C for 7 days. Secondly, deionized water (3 mL) was added to each coffee sample (1 g) and autoclaved at 121 °C for 15 min. The coffee was inoculated with 2 culture discs of the strain [18]. After 21 days, OTA production was detected and quantified by HPLC system 1260 series Agilent Technologies model consisting of a quaternary pump, autosampler, a column thermostat and a Quadrupole detector (6120).

OTA production was analyzed according to Mounjouenpou et al. [19] with some modifications. Samples were extracted for 50 min (60 °C) with a solution of methanol/3% sodium bicarbonate (20:80), the extracts were filtered and diluted with phosphate-buffered saline and applied to an immunoaffinity column (Ochrastar R). OTA was eluted with 6 mL HPLC grade methanol. The eluate was evaporated to dryness (3 min) under an oven at 70 °C, re-dissolved in 1 mL of HPLC mobile phase water/acetonitrile (50/50, v/v) and then quantified by LC–MS.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a zorbax SB-C18 (2.1 × 50 mm id: 1.8 µm) column (Agilent Technologies) using a mobile phase of 5 mM ammonium acetate/acetonitrile (65/35, v/v) at 40 °C with a flow rate of 0.2 mL/min for 3 min run. The detection of OTA was performed in LC–MS system model 6120 series LC quadrupole equipped with an electrospray ion (ESI) MS (Agilent Technologies), in ionization mode, positive mode (capillary 3500 V, nebulizer 25 psi (nitrogen), dry gas nitrogen at 9 L/min, dry gas temperature 350 °C, fragmentor voltage 95); selected ion monitoring (SIM), m/z 404 (OTA) [20].

Table 1 Relationship between levels of contamination of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_2 (AFG_2), aflatoxin G_1 (AFG_1) and ochratoxin A (OTA) and toxigenic filamentous fungi in roasted coffee beans

Strain	AFG_1 (µg/kg)	AFG_2 (µg/kg)	AFB_1 (µg/kg)	AFB_2 (µg/kg)	OTA (µg/kg)
A	ND	ND	ND	ND	ND
A-B	ND	ND	ND	ND	ND
2B	ND	ND	ND	ND	ND
R5	ND	ND	ND	ND	ND
R10	ND	ND	ND	ND	ND
R11	ND	ND	ND	ND	ND
R14	ND	ND	ND	ND	ND
R15	ND	ND	ND	ND	ND
R16[a]	ND	ND	ND	ND	0.33
R17	ND	ND	ND	ND	ND
R18	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND
6N[b]	ND	ND	ND	ND	1.162
7	ND	ND	ND	ND	ND
8-2	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND
11[c]	ND	ND	ND	ND	0.36
V	ND	ND	ND	ND	ND

ND Not detectable

[a] *A. niger*

[b] *Byssochlamys spectabilis*

[c] *A. versicolor*

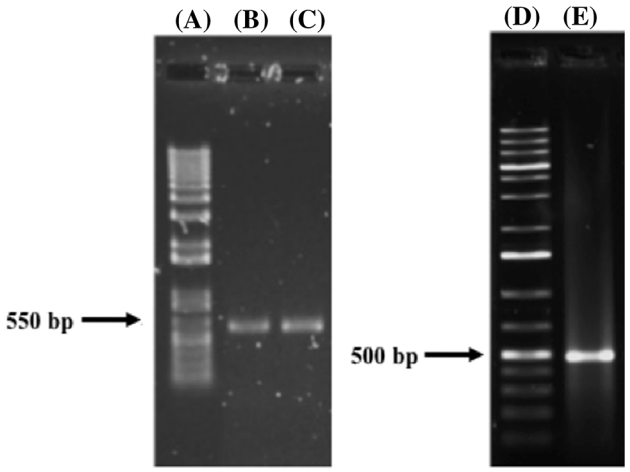

Fig. 1 Amplification of ITS1-5.8S-ITS2 rDNA region from mycotoxigenic fungi isolated from roasted coffee from Nayarit, Mexico. (**A**) Molecular weight marker, 1KB plus DNA ladder (Invitrogen)., (**B**) *A. niger*., (**C**) *A. versicolor*., (**D**) molecular weight marker, 1KB plus DNA ladder (Invitrogen)., (**E**) *Byssochlamys spectabilis*

Results and discussion

Isolation and identification of fungi

Macroscopic and microscopic presumptive identification revealed two fungal genera: *Aspergillus* and *Penicillium*. *Aspergillus* was predominant in roasted coffee samples with a recovery of 95.43% and *Penicillium* with 4.54%. From the 22 isolates, the predominant species determined by dichotomous characters were *Aspergillus ochraceus* (4.54%), *Aspergillus carbonarius* (4.54%), *A. niger* (27.27%), *Aspergillus fumigatus* (4.54%) and other Aspergillus spp. (54.54%). *Aspergillus* spp. propagules get on grain in different ways, most often with dust from soil, from the surface of plant remnants during harvesting, transportation, storage, and processing [21]. It has great metabolic versatility and ability to disperse conidia in the environment to such an extent that it can sustain growth

Table 2 Sequence homology of toxigenic fungi isolated from coffee (*Coffea arabica* L.), according to N.C.B.I. (National Center for Biotechnology Information)

Strain	Homology	Sequence	Access number
R16	95% *A. niger*	1 tacgagcgcg aggtctttgg gccacctccc atccgtgtct attgtaccct gttgcttcgg	JN226991.1
		61 cgggcccgcc gcttgtcggc cgccgggggg gcgcctctgc cccccgggcc cgtgcccgcc	
		121 ggagacccca acacgaacac tgtctgaaag cgtgcagtct gagttgattg aatgcaatca	
		181 gttaaaactt tcaacaatgg atctcttggt tccggcatcg atgaagaacg cagcgaaatg	
		241 cgataactaa tgtgaattgc agaattcagt gaatcatcga gtctttgaac gcacattgcg	
		301 ccccctggta ttccgggggg catgcctgtc cgagcgtcat tgctgccctc aagcccggct	
		361 tgtgtgttgg gtcgccgtcc ccctctccgg ggggacgggc ccgaaaggca gcggcggcac	
		421 cgcgtccgat cctcgagcgt atggggcttt gtcacatgct ctgtaggatt ggccggcgcc	
		481 tgccgacgtt ttccaaccat tctttccagg ttgacctcgg	
11	99% *A. versicolor*	1 tccgtaggtg aacctgcgga aggatcatta ctgagtgcgg gctgcctccg ggcgcccaac	NR_131277.1
		61 ctcccacccg tgactaccta acactgttgc ttcggcgggg agccctctcg ggggcgagcc	
		121 gccggggact actgaacttc atgcctgaga gtgatgcagt ctgagtctga atataaaatc	
		181 agtcaaaact ttcaacaatg gatctcttgg ttccggcatc gatgaagaac gcagcgaact	
		241 gcgataagta atgtgaattg cagaattcag tgaatcatcg agtctttgaa cgcacattgc	
		301 gccccctggc attccggggg gcatgcctgt ccgagcgtca ttgctgccca tcaagcccgg	
		361 cttgtgtgtt gggtcgtcgt ccccccgggg ggacgggccc gaaaggcagc ggcggcaccg	
		421 tgtccggtcc tcgagcgtat ggggctttgt cacccgctcg atttagggcc ggccgggcgc	
		481 cagccgacgt ccaaccattt ttcttcaggt tgacctcgga tcaggtaggg ataccgctg	
		541 aacttaagca tatcaataag cggaggaaaa gaaaccaacc gggattgccc c	
6N	100% *Byssochlamys spectabilis*	1 ctgcggaagg atcattaccg agtgagggtc cctcggggcc caacctccca tccgtgttgt	KC157703.1
		61 cctgacacct gttgcttcgg cgggcccgcc gtggttcacg ccccggccgc cgggggttc	
		121 acgcccccgg gcccgcgccc gccgaagacc cctggaacgc tgcctggaag gttgccgtct	
		181 gagtatacaa tcaatcaatt aaaactttca acaacggatc tcttggttcc ggcatcgatg	
		241 aagaacgcag cgaaatgcga taagtaatgt gaattgcaga attccgtgaa tcatcgaatc	
		301 tttgaacgca cattgcgccc cctggcattc cgggggcat gcctgtccga gcgtcattgc	
		361 taaccctcca gcccggctgg tgtgttgggc cgccgtcccc ccccccgggg gacgggcccg	
		421 aaaggcagcg gcggcgtcgc gtccggtcct cgagcgtatg gggctttgtc acacgcttca	
		481 gtagaaccgg ccggcttgct ggccacacga ccttcacggt cacctatatt tctcttaggt	
		541 tgacctcgga tcaggtaggg atacccgctg aacttaagca tatcaataag cg	

even under adverse conditions such as low humidity and low water activity [22].

Table 1 shows the production of mycotoxins by isolates from roasted coffee beans inoculated in PDA. Three fungi isolates produced OTA. Strain 11 with a concentration of 0.36 µg/kg, R16 with 0.33 µg/kg and 6N with 1.16 µg/kg mycelium. No OTA or aflatoxin were detected in the rest of the isolates.

Therefore, as was observed, the strains vary in their capacity to produce OTA, as well as in the quantity produced. This is because fungal growth occurs under favorable environmental conditions and is associated with the production of a wide range of secondary metabolites. There are more important factors that rule the growth of fungi and the production of mycotoxins such as the amount of nutrients available, the ambient temperature, water activity and available oxygen [23]. In spite of OTA being a stable metabolite, in some cases OTA content decreased considerably as incubation time increased or just at the final incubation time. Some authors have suggested that this may be due to the fact that the strains remove and assimilate the phenylalanine moiety from the OTA molecule as well as nitrogen sources in the culture media when they become exhausted [24].

Fungal growth and mycotoxin production are influenced by numerous abiotic and biotic parameters and their complex interactions. Water availability is probably the single most important factor affecting germination, growth and establishment of fungi on nutrient rich substrates. The second most important is temperature (22–30 °C) [8, 25]. It has been shown that both factors influence interactions between different mycotoxigenic and non-mycotoxigenic fungi [26]. The next most important factors for mycotoxin production and mold growth are high moisture content (20–25%) and high relative humidity (70–90%) [8].

The strains that tested positive for OTA production were molecularly analyzed, the amplification of the ITS1-5.8S-ITS2 region of rDNA from the two relevant *Aspergillus* strains generated PCR products of 550 bp and were detected as *A. niger* and *A. versicolor* (Fig. 1). Another PCR product of 500 bp was observed and identified as *B. spectabilis* (Fig. 1). Sequences reported at BLAST v2.3.0 (Basic Local Alignment Search Tool http://www.ncbi.nlm.nih.gov/) at the National Center for Biotechnology Information, NCBI, USA, were used to compare the obtained sequences (Table 2).

The strain *A. niger* presented a colony on CYA 65.6 ± 1.75 mm (7 days/25 °C); conidia coffee brown to black and white mycelium. Its texture is irregular, plane and velvety; reverse pale yellow; it formed radial furrows very close to each other. The conidial heads were smooth and pigmented brown, vesicle is globose and produces phallus around it, phialides are biseriate and conidia are globose and smooth. Conidiophore size is 538.03 ± 113.34 µm × 6.5 ± 0.30 µm, phialides 7.9 ± 1.00 µm × 3.36 ± 0.25 µm, vesicles 44.7 ± 10.25 µm, conidia 4.5 ± 0.05 µm (Fig. 2).

The morphological characteristics of *A. versicolor* show colony diameter on CYA, 14 ± 1.80 mm (7 days/25 °C). The colonies were initially colored white, though they gradually turned grayish-green with white edges, granular shape, convex elevation, rough texture. Reverse is brownish orange or reddish brown. No exudates. Conidiophore were smooth to slightly rough walls. The conidia head is biseriate, phialides radially cover the vesicle. Conidia are

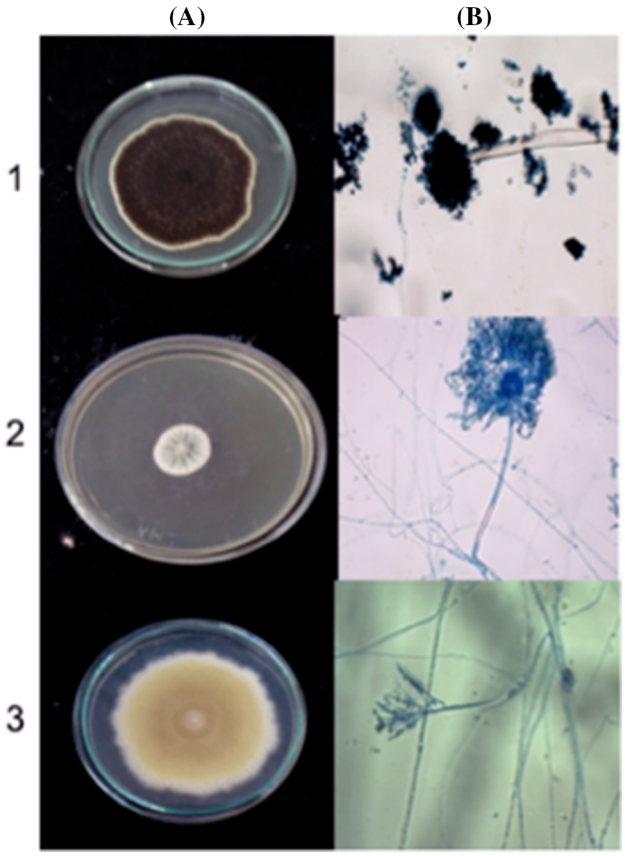

Fig. 2 Mycotoxigenic fungi isolated from roasted coffee. (*1*) *A. niger* colonies on CYA, (*2*) *A. versicolor* on CYA, (*3*) *Byssochlamys spectabilis* on CYA. (**A**) macroscopic morphology, (**B**) optical microscopic ×40

Table 3 Level of OTA production (µg/kg) for *A. niger*, *A. versicolor* and *Byssochlamys spectabilis*

Isolate	Coffee medium
OTA production (µg/kg)	
A. niger	91.03
A. versicolor	76.74
Byssochlamys spectabilis	81.97

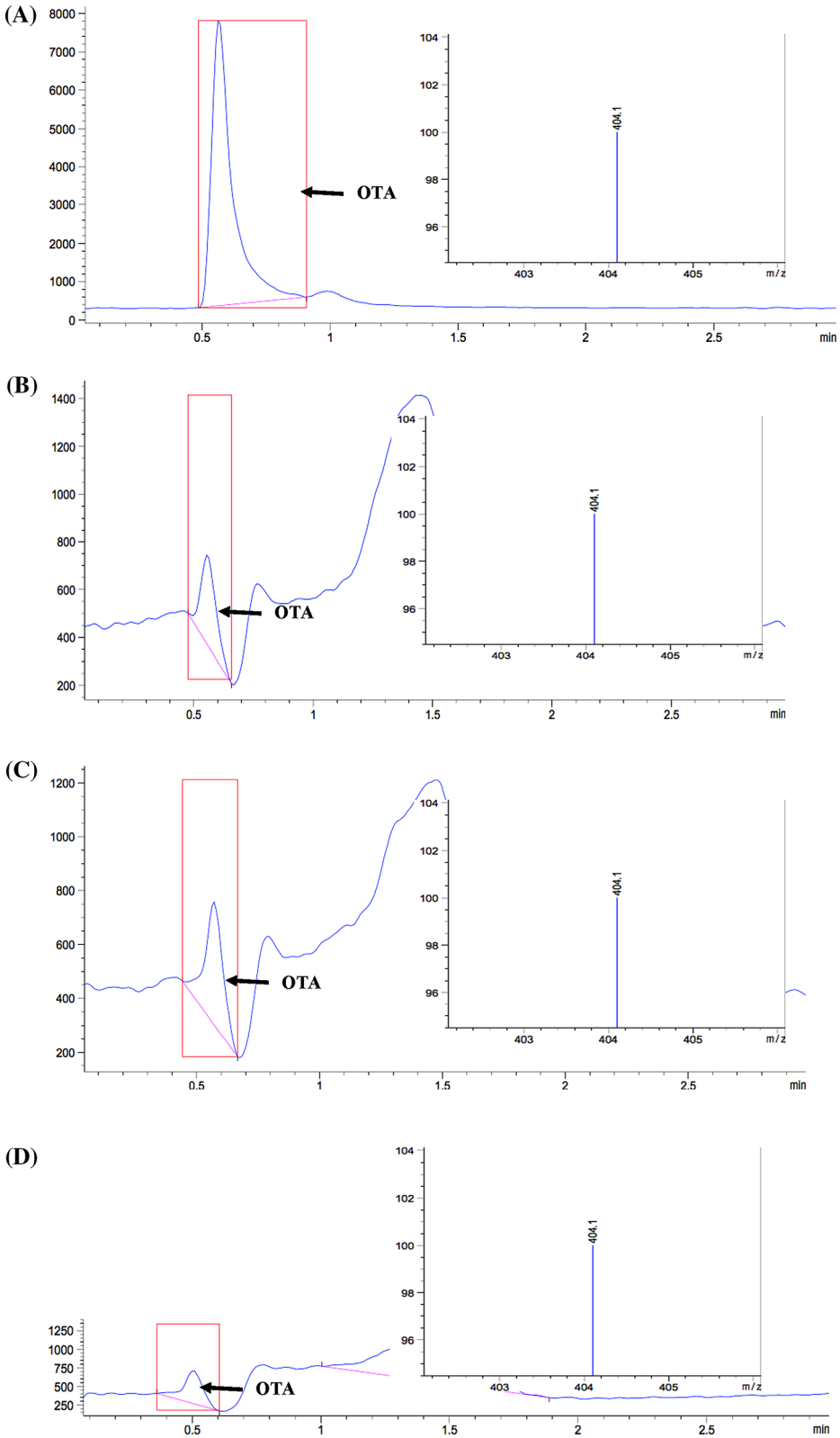

Fig. 3 LC–MS chromatograms: (**A**) standard solution (OTA 0.021 µg/mL), roasted coffee sample contaminated with: (**B**) *A. niger*, (**C**) *A. versicolor,* (**D**) *Byssochlamys spectabilis*

spherical. Conidiophore size is 370.91 ± 220.01 × 9.82 ± 3.73 μm, vesicle 24.33 ± 0.90 μm, conidia 2.6 ± 0.2 μm. Another fungal species detected was *B. spectabilis* whose colony diameter on CYA was 78.43 ± 3.30 mm, plane and filamentous (7 days/25 °C). The colonies are pale yellow–brown, dusty texture with white border. Reverse is white color. No exudates. Conidiophores biverticilate and have smooth and thick wall. Conidia are ellipsoidal. Conidiophore size is 173.09 ± 110.01 × 3.1 ± 1.1 μm, phialides 21.30 ± 3.06 μm × 2.5 ± 3.0 μm, conidia 4.23 ± 0.25 μm (Fig. 2).

Gautam and Bhadauria [27] characterized at the molecular level the identification of mycoflora associated with *Aspergillus* species in samples of *Triphala churn* and ingredients (mixture of dry fruits of medicinal plants: *Embilica officinalis*, *Terminalia bellirica*, *Terminalia chebula*) and obtained the amplified product of 500, 700 and 1110 bp for *A. versicolor*.

According to the identification, only the strain of *A. niger* produced OTA in the analyzed sample of roasted coffee, the production of OTA in coffee by *A. versicolor* has not been reported before, but it has been reported in wheat, where the strain produced 0.01–0.07 μg/g [28].

In this study, *B. spectabilis* was first identified in roasted coffee using primers ITS1 and ITS4 amplified with 500 bp approximately. *Byssochlamys* species are abundant in soil and recognized as important spoilage molds in fruit and fruit products [29]. *Byssochlamys spectabilis* (anamorph *P. variotii* s.s.) commonly occurs in air, compost, various foodstuffs (including pasteurized fruit juices, rye bread) [30]. In general, this fungus can survive considerable periods of heat above 85 °C and can grow under very low oxygen conditions, produce mycotoxins such as vomitoxin and deoxynivalenol; the production of OTA by this strain had not been reported before [30, 31].

The occurrence of OTA in all inoculated roasted coffee samples demonstrates fungi isolated may produce this mycotoxin in this kind of food product. A mycotoxin may be produced by several different fungi (Table 3, Fig. 3). The mycotoxigenic potential depends on species and strain of fungus, matrix composition and environmental factors (temperature and moisture).

According to these results, it can be concluded that the fungi with the potential to produce OTA in roasted coffee samples (*Coffea arabica* L.) are *A. niger*, *A. versicolor* and *B. spectabilis*. It was also observed that once a toxigenic strain was isolated from a coffee sample, they were able to produce OTA in roasted coffee samples. The presence of toxigenic strains implicates a great risk of OTA presence.

OTA is a stable compound not destroyed by common food preparation procedures. Temperatures above 250 °C for several minutes are necessary to reduce the concentration of this toxin [32]. Roasting treatment for green coffee at 200 °C for 20 min reduced levels of OTA by only 0–12% in the dried whole beans [33]. Therefore, there is a high chance that the population get contaminated by this toxin, since the roasting of coffee does not assure its total destruction. Consequently, a cup of coffee could contain high amounts of ochratoxin. Because of its high affinity with plasma proteins, their persistence in the organism (average 840 h) is ensured [34]. OTA is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine and distributed via the blood, mainly to the kidneys, with lower concentrations found in liver, muscle and fat. Specific transporters may be involved in the cellular uptake of OTA into the kidney, where it accumulates. OTA could be implicated in the pathogenesis of some renal diseases including kidney tumors and chronic interstitial nephropathy [35].

Good manufacturing practices and hygiene throughout the coffee production and processing chain is highly recommended in order to reduce the risk of contamination of processed coffee. Furthermore, the OTA presence risk implies the necessity to develop effective technologies to detoxify coffee products.

In Mexico, there are no legal limits established for ochratoxin contamination and there are few studies on the occurrence of OTA in foods and beverages. More research is necessary to evaluate the real exposure of the population to this mycotoxin through the ingestion of contaminated food.

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