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Mycotoxigenic potential of phytopathogenic moulds isolated from citrus fruits from different states of Mexico

T. Sandoval-Contreras[1], A. Villarruel-López[2], R. Torres-Vitela[2], C. Garciglia-Mercado[1], G. Gómez-Anduro[1], A.E. Velázquez-Lizárraga[1], A. Sierra-Beltran[1] and F. Ascencio[1*]

[1]Centro de Investigaciones Biológicas del Noroeste, Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz, BCS, 23096, Mexico; [2]Centro Universitario de Ciencias Exactas e Ingenierías, Universidad de Guadalajara, Marcelino García Barragán #1421 Guadalajara, Jal. 44430, Mexico; ascencio@cibnor.mx

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RESEARCH ARTICLE

Abstract

The main phytopathogenic moulds on citrus fruits, besides from causing great post-harvest losses, are capable of producing mycotoxins that are highly toxic for any human or animal that might eat them. The objective of this study was to determine the mycotoxigenic potential of moulds isolated from the phyllosphere of citrus fruits originating from different orchards and packing-houses in different places around Mexico. In this study, 258 moulds were isolated, finding up to 29 different genera, including host-specific citrus moulds as well as opportunist spoilage moulds. Their pathogenic potential was determined using *Citrus latifolia* T. (Persian lime), finding 17 phytopathogenic strains, including the presence of phytopathogenic species different from the ones most commonly studied, *Penicillium italicum* or *Alternaria alternata*, belonging to the *Aspergillus* genus and with a high degree of pathogenicity. Their mycotoxigenic potential was determined through molecular techniques, finding as a result that *A. alternata, Aspergillus niger* and *Aspergillus carbonarius* have the potential to produce mycotoxins by amplifying specific sequences that partake in the biosynthesis of mycotoxins such as *Alternaria* toxins, fumonisins, aflatoxins and ochratoxin, respectively. When these moulds receive a stimulus under certain environmental conditions, it might trigger production of such secondary and toxic metabolites. Knowledge of the behaviour of these microorganisms will help in developing the necessary strategies for their post-harvest control, thus ensuring the protection of the end consumer against exposure to such mycotoxins. This research also yielded the first register ever made of microbiota in citrus fruits grown in different states of Mexico.

Keywords: citrus fruits, moulds, post-harvest decay, mycotoxigenic potential, health risk

1. Introduction

Fruits provide an entire ecosystem that moulds have been colonising for millennia. Different varieties and features of moulds silently invade the fruits, acidifying and disintegrating them, turning their taste unpleasant, or even making them dangerous (Jay, 2000; Pitt and Hocking, 2009). Due to their low pH, higher humidity, and adequate water activity, fruits are more susceptible to be attacked by moulds than other kinds of microorganisms (Marino *et al.*, 2009). Blue and green rot, caused by *Penicillium italicum* and *Penicillium digitatum*, respectively, are the most common moulds found on citrus fruits, although other species such as *A. alternata* and *Aspergillus* spp. are also capable of causing decay. These diseases can begin while still in the field or can remain latent post-harvest and develop through wounds during later manipulation, thus causing great losses (Pitt and Hocking, 2009; Romanazzi *et al.*, 2016).

Production of mycotoxins is an important part of the development of phytopathogenic moulds. Mycotoxins are fungal secondary metabolic products that may affect exposed vertebrates such as animals in a variety of ways. The role of mycotoxins have not been clearly established. They are believed to play a role in eliminating other

microorganisms and helping parasitic fungi invade host tissues (Bräse *et al.*, 2009). From a phytopathogenicity point of view, toxins are pathogen-produced molecules that are required for disease to occur at all, and on others that are not involved in disease initiation but that are required for a portion of the disease development process (Yoder, 1980).

Although there are hundreds of them, only a few are considered to have food industry and public health implications (Fernández-Pinto, 2008; Marino *et al.*, 2009). The most significant *Aspergillus* mycotoxins in human and animal food are aflatoxins, ochratoxin (OTA), sterigmatocystin, cyclopiazonic acid, patuline, citrinin, and penicillic acid; the latter three are also produced by *Penicillium* species (Barkai-Golan and Paster, 2008). Aflatoxins and OTA receive the most attention due to their carcinogenic, nephrotoxic, teratogenic, and immunosuppressive effects (IARC, 2002).

Depending on a fruit's features, mycotoxins may migrate inside the fruit and stay within the flesh, even after removing the mycelium along with the decay (Restani, 2008). These lower-quality fruits may be used for the production of essential oils, juices, marmalades, and so forth (González and Silva, 2005; Restani, 2008), which has resulted in greater concern about contamination patterns, microbiological quality, and food safety.

The biosynthesis of some mycotoxins involves several genes in related mould species that follow an almost identical order of sequences within the gene cluster. The presence of such genes or their expression has been used as a tool for detecting mycotoxigenic moulds on food products (Gallo *et al.*, 2012). The characterisation of moulds, as well as their possible mycotoxigenic potential, is important for establishing the required measures to protect the final consumer from exposure to high doses of toxic substances such as mycotoxins. The objective of this study was to determine the mycotoxigenic potential of isolated moulds from the phyllosphere of *Citrus sinensis* (L.) Osbeck (Valencia and Washington navel oranges), *Citrus aurantifolia* S. (Mexican lime), and *Citrus latifolia* T. (Persian lime) originating from different sites within Mexico.

2. Materials and methods

Orchard sampling

Fruit samples were collected from orchards that had not been chemically treated. Valencia and Washington navel oranges (*C. sinensis*) were collected from the municipalities of Comondú and San José del Cabo in the state of Baja California Sur (orchard A), and from the municipality of Cabo Corrientes in Jalisco (orchard B). The Mexican limes (*C. aurantifolia*) were from Tecomán, Colima (orchard C), and the Persian limes (*C. latifolia*) were from the municipalities of Atotonilco el Alto (orchard D) and Cabo Corrientes (orchard E) in the state of Jalisco, and from San Pedro Lagunillas (orchard F) in the state of Nayarit. The sampling method was conducted according to the protocol of Zúñiga *et al.* (2004).

Packing house sampling

Sampling was performed in five lime packing houses. Two, in Tecomán, Colima (Mexican lime, packinghouse #1) and San Pedro Lagunillas, Nayarit (Persian lime, packinghouse #2), did not apply any protective treatments on the fruit; the other three were technologically equipped packinghouses in Atotonilco el Alto, Jalisco (Persian limes, packinghouses #3 and #4), and in Tecomán, Colima (Mexican limes, packinghouse #5). Fruits were sampled at the reception or fruit arrival from the field, and after fruit washing. For packing houses that did not apply any protective treatments, fruits were sampled at one point of the packing process.

Quantification and isolation of the mould

Each sampling unit was treated, in the sample bag, with 100 ml of a 1% peptone diluent (Bioxon™, Sparks, MD, USA) followed by 2 minutes of manual ribbing in order to separate the spores adhered to the fruit. From the resulting solution, decimal dilutions were prepared. In triplicate, each dilution was inoculated in Petri dishes with potato dextrose agar (Bioxon™) containing 50 µg/ml ampicillin (Hormona™, Mexico city, Mexico) and 0.5% (w/v) of Bengal rose (Fernández-Escartín, 2008) as inhibitors. The Petri dishes were incubated under aerobic conditions at 25 °C for 5 days (Terlab programmable incubator; Terlab, Guadalajara JAL, Mexico). At the end of the incubation time, formed colonies were counted and the results reported in log ufc/cm^2. Moulds were isolated and purified using Sabouraud dextrose agar (SDA; Bioxon™). All recovered moulds were characterised both macro- and microscopically.

Molecular identification of the moulds

Mould DNA was extracted according to Sambrook and Russell's protocol (Sambrook and Russell, 2001) with a modified buffer and enzymes (crushing buffer, chitinase and proteinase K). Identification of all isolates, besides the morphological identification, was performed using the molecular method suggested by White *et al.* (1990). PCR was performed to amplify the ribosomal rDNA internal transcribed spacer (ITS) regions (ITS1-5, 8S-ITS2) using the universal primers ITS1 and ITS4. The PCR products were sent to Genewiz, Inc. (South Plainfield, NY, USA) for sequencing, and the results were analysed and the species identified using the Basic Local Alignment Search Tool from the National Center of Biotechnological Information

(NCBI). The strains were stored in 15% glycerol at -80 °C, and their respective DNAs were stored at -20 °C.

Phytopathogenicity tests

After identifying the moulds, representative samples were tested. Their phytopathogenicity grade was determined on Persian limes by using the infection protocol from Yao *et al.* (2004). Limes were then incubated in an environmental chamber at 25 °C and a relative humidity of 85% for 5-15 days and were checked daily. The degree of pathogenicity was determined by measuring the diameter of the damage as an indication of the severity, and the infected fruit to total inoculated fruit ratio indicated the incidence.

Mycotoxigenic potential investigation

PCR was utilised to investigate the presence of genes involved in some steps of mycotoxin biosynthesis for the identified strains of phytopathogenic moulds. PCR assays were carried out using sets of primers forward/reverse, respectively: AaPKS (5'tgggtgttgatgtttccg3' and 5'atcttcgggtccattggc3'), AaPKS-TE (5'ttccatgcgtgaggctactg3' and 5'ctgggccgaaaccttagca3') (Tseng *et al.*, 2011) for *Alternaria* toxins; Ao-PKS (5'atggatgtgagaagcgcagtagagaa3' and 5'tgttgacctcttatggct3') (O'Callaghan *et al.,* 2003) for OTA-A; Gm-FUM1 (5'cgaggcccgagcgagcactgg3' and 5'gatcaagctcggggccgttcatag3') (Baird *et al.*, 2008) for fumonisin and AfVerB (5'ttcagcggaaatggtgta3' and 5'accttgtcccaaaagtcg3') (this study) for aflatoxins.

Real-time PCR analysis

Genes that tested positive to PCR reaction and synthesis of mycotoxin involved in a culture medium (method explained later) were investigated with real-time PCR. A Rotor Gene Amp 6000 detection system (R080762; Corbett Life Science, Mortlake, Australia) was used to perform the real-time PCR cycle reactions, using 'SYBR® Green PCR Master Mix' (Applied Biosystems, Waltham, MA, USA). The primer/probe set used had the following nucleotide sequence: EF1AqPCR151F, 5'-tcc ccc aag ttc atc aag tc-3'; EF1AqPCR151R, 5'-ccttgataacaccgacagca-3' for elongation factor gene, VerBpPCR204F, 5'-caactttactcacgccgaca-3'; VerBqPCRR, 5'-ccagtcttgcccaca tcc-3' (keto reductase gene), FUM1qPCR185F, 5'-agggtcagcgtcaactcg-3' and FUM1qPCR185R, 5'-cgcacttccatcttcatcg-3' (poliketosynthase gene). These probes were designed based on mRNA sequences reported in NCBI public database. SDA cultures of the *Aspergillus niger* 24 strain were carried out for five days in two different conditions: room temperature (25±3 °C, pH 4.5 as problem treatment and 30 °C, pH 5.5 as control treatment). RNA extraction was performed with the FastRNA Red Kit® (MP Biomedicals, Sanata Ana, CA, USA), while the cDNA was obtained with the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The $2^{-\Delta\Delta CT}$ method proposed by Livak *et al.* (2001) was used to analyse the relative change in gene expression

Production and quantification of mycotoxins

Mycotoxin production was determined by triplicate on dextrose Sabouraud broth (DSB) (Bioxon™). Each replicate was inoculated with 20 µl of a spore suspension (concentration 10^6 spores/ml) of mould that resulted positive to a sequence for mycotoxin biosynthesis, in an Erlenmeyer flask with 250 ml of broth, and cultivated in static mode at 25 °C for 10 days (Brzonkalik *et al.*, 2011). For mycotoxin extraction, a 20 ml aliquot of cell-free culture for replicate was placed into a separating funnel. It was extracted twice with 35 ml of chloroform. Solvent was chosen in order to extract a large number of mycotoxins (Tournas *et al.*, 2001). The mixture was vigorously shaken for three minutes. The chloroform phase was separated and evaporated to dryness at room temperature, in a flow cabinet (Brzonkalik *et al.*, 2011). The residue was redissolved in 500 µl methanol (high-performance liquid chromatography (HPLC) grade) for a second extraction, and filtered with a syringe filter (Acrodisc™ PTFE, Darmstadt, Germany) according to the method of Vishwanath *et al.* (2009).

Mycotoxin analysis by HPLC/MS-MS

All mycotoxins standards were supplied by Sigma Aldrich (St. Louis, MO, USA). All concentrate solutions of mycotoxins were dissolved in methanol 50% and stored at -80 °C. Composite working standard solutions were prepared in appropriate dilutions to prepare calibration curves: fumonisin B_1 (FB_1), (130.0 to 4,166.5 mg/ml); aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) (26.0 to 833 ng/ml); aflatoxin B_2 and aflatoxin G_2 (7.8 to 250.0 ng/ml). Pure water (Optima LC-MS; Fisher Scientific, Bridgewater, NJ, USA), methanol, acetonitrile and formic acid (Sigma Aldrich), HPLC-MS grade, and glacial acetic acid, reactive grade (Sigma Aldrich) were used.

High performance liquid chromatography – tandem mass spectrometry system

Chromatographic separation was performed with an ultra-fast liquid chromatography Shimadzu Prominence System (Shimadzu Scientifics Instruments, Inc., Columbia, MD, USA) coupled to a triple quadrupole mass spectrometer (MS/MS) TSQ Vantage equipped with heated-electrospray ionisation AB/Sciex API 3200 QTRAP (Sciex, Framingham, MA, USA) with an electrospray ionisation (ESI) interphase. In duplicate, an aliquot of 20 µl was injected at 45 °C on Kinetex 2.6 m biphenyl 100A 50×2.1 mm column (Phenomenex, Inc., Torrance, CA, USA) with a security

guard cartridge of the same characteristics. A gradient consisting in eluent A (5 mM ammonium acetate and 1% acetic acid in water) and eluent B (5 mM ammonium acetate and 1% acetic acid in methanol) was used in a flow of 0.5 ml/min. The settings of the ESI-source were as follows: source temperature 550 °C, curtain gas 10 psi, ion source gas 1 and 2: 50 psi, ion-spray voltage -4,000 V and +4,000 V, respectively, collision gas (nitrogen) high. Analysis parameters for the presence and concentrations of each fungal metabolites were performed according to Vishwanath *et al.* (2009). The software Analyst® (Sciex) was used for controlling chromatography and mass spectrometer.

Statistical analysis

Unifactorial, comparative and inferential variance analysis was performed using Statgraphics Centurion IV software (Warrenton VA, USA), with a confidence level of 95%. Fisher's least significant difference (LSD) multi-range test was used for the LSD analysis. Descriptive statistical analysis for mycotoxin quantification was performed using Excel (Microsoft, Redmond, WA, USA).

3. Results

A total of 162 samples from the different sampling sites and citrus fruit were analysed: 43 Valencia orange samples, 18 Washington navel orange samples, and 20 Persian lime samples; five from artisanal packinghouses, 10 samples before protection process and 10 samples after protection process; 25 Mexican lime samples: six from artisanal packinghouses, 12 samples before protection process and 13 samples after protection process.

Mould quantification

Surface mould levels on the limes from the orchards fluctuated between 2.0 and 3.5 log cfu/cm^2 (Figure 1). Similar levels were found in artisanal packinghouses #1 and #2, as well as in technified packinghouses #3, #4 and #5, before the protective treatment. Regarding the technified packinghouses, mould levels were different after protective treatment. In packinghouse #4, the pre-treatment values of nearly 2.5 log cfu/cm^2 were reduced to less than 1 log cfu/cm^2, and packinghouse #5 decrease the moulds level to 0.5 log cfu/cm^2 with statistical difference (P<0.05). In contrast, packinghouse #3 shows a higher moulds level after protective treatment (P>0.05). As for the oranges, no mould counts from the samples originating from orchards A and B were performed.

Molecular identification of the isolated moulds

A total of 258 strains of mould were identified, belonging to a wide variety of genera and species. The most widespread genera identified from all sampling sites were, in decreasing order: *Aspergillus* sp., *Fusarium* sp., *Alternaria* sp. and *Penicillium* spp. (Table 1). *A. niger* aggregate and *Penicillium* genus were recovered from all of the sampled sites, while *Fusarium equiseti* was recovered from more than half of the orchards and Persian lime packinghouses, both before and after the protective treatment. *A. alternata* was recovered from all sampling sites. *Aspergillus aculeatus* was the second most relatively abundant species. In smaller proportions, we found species such as *Alternaria citri*, *Geotrichum citri-auranti*, *P. italicum* and *Penicillium citrinum.*

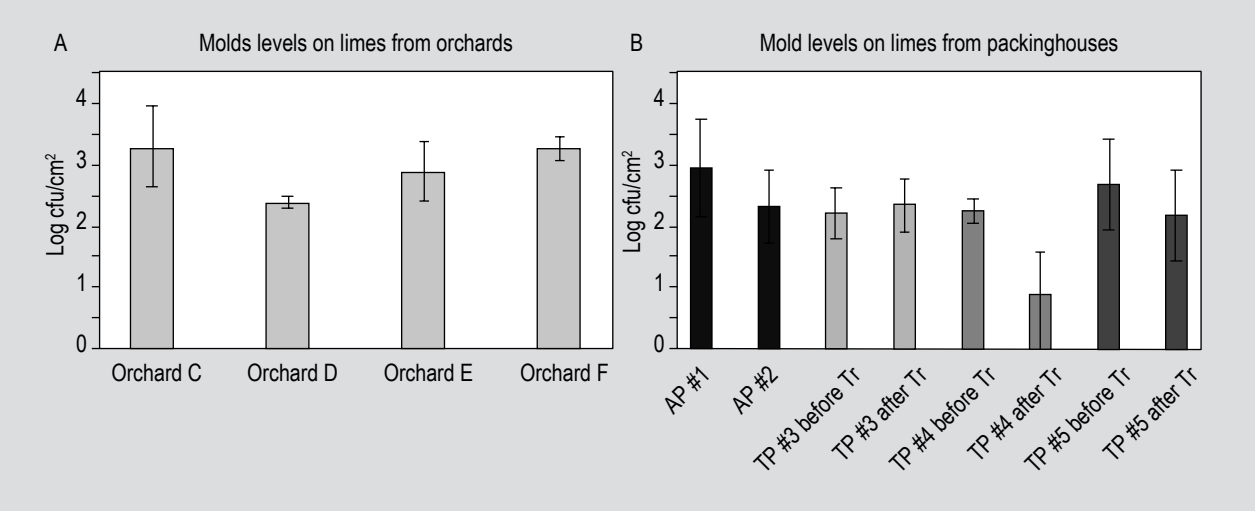

Figure 1. Moulds levels found on limes fruits in (A) different orchards and (B) different packinghouses (AP = artisanal packinghouse; TP = technologically equipped packinghouse; Tr = treatment).

Table 1. Relative frequency of moulds recovered from the phyllosphere of different varieties of citrus: Valencia and Washington navel orange, Mexican and Persian lime.

Species isolated	Relative frequency (%)	Recovery fruit[1]		
		Orange	Mexican lime	Persian lime
Aspergillus niger	12.50	O	O, AR	O, BP
Fusarium equiseti	8.20	O	AR	O, BP, AP
Penicillium sp.	7.75	O	AR	O, BP, AP
Alternaria alternata	5.47	O	O	O, BP, AP
Lasiodiplodia theobromae	4.30	O	O, AR	
Aspergillus aculeatus	3.91	O		O, BP
Colletotrichum gloeosporioides	3.13	O		
Aspergillus nidulans	1.95	O	O	
Mucor fragilis	1.56	O		O
Alternaria citri	0.78	O		
Penicillium citrinum	0.78			O
Geotrichum citri-aurantii	0.39	O		
Penicillium italicum	0.39	O		
Others[2]	56.63	O	O, AR, BP, AP	O, AR, BP, AP

[1] O = orchard; AR = fruits from artisan citrus packinghouse; BP = fruits from lime packinghouses before protection process; AP = fruits from lime packinghouses after protection process.
[2] Ratios equal to or less to 0.39.

Phytopathogenicity tests

Of the 258 strains identified, 17 exhibited pathogenicity in the Persian lime model (Table 2). Damage became apparent from the fourth to sixth days onwards, and on subsequent days, some strains showed significant differences in the severity of the damage. Among such species, *P. italicum* was identified as the most virulent species due to having the earliest display of symptoms, a larger diameter of damage, and a greater incidence (Figure 2). *A. niger* aggregate showed different grades of pathogenicity depending on the strain, with the damage severity fluctuating between diameters of 5 and 35 mm and an incidence of 70-100% of the infected fruit. At a lesser degree of virulence, different strains of *Aspergillus* and *A. alternata* were observed on eighth day, which had important increases in the severity and incidence in the subsequent days. *A. aculeatus* displayed a low damage severity, but had a high infection incidence.

Mycotoxigenic potential investigation

Of the 17 phytopathogenic strains found, four had genes involved in mycotoxin biosynthesis. One was *A. alternata* strain 35a isolated from the technified Persian lime packinghouse before the protective treatment. This strain contained a positive match for the polyketide synthase gene (*PKS*), which codes for polyketide condensation in melanin production (Figures 3A and 3B). Another strain,

Table 2. Phytopathogenic moulds for the Persian lime.

Isolates[1]	GeneBank accession
Alternaria alternata S35a	HQ014678.1
Aspergillus aculeatus S3	EU833205.1
A. aculeatus S2	JF439460.1
Aspergillus brasiliensis S5	FJ629324.1
Aspergillus carbonarius S40	FJ629325.1
Aspergillus japonicus S34	GQ359412.1
Aspergillus melleus S60	FM986321.1
Aspergillus nidulans S8	FJ878642.1
Aspergillus niger S24	GU082483.1
A. niger S29	HM461860.1
A. niger S31	HQ170509.1
A. niger S32	HQ401273.1
Aspergillus tamarii S38	GU362011.1
Aspergillus tubingensis S12	GQ461899.1
Mucor fragilis S14	JN198474.1
Penicillium italicum S36	EU128647.1
Penicillium steckii S23	EU833226.1

[1] S = strain; the number next to the 'S' is the assigned number of the tested isolate that result positive for pathogenicity.

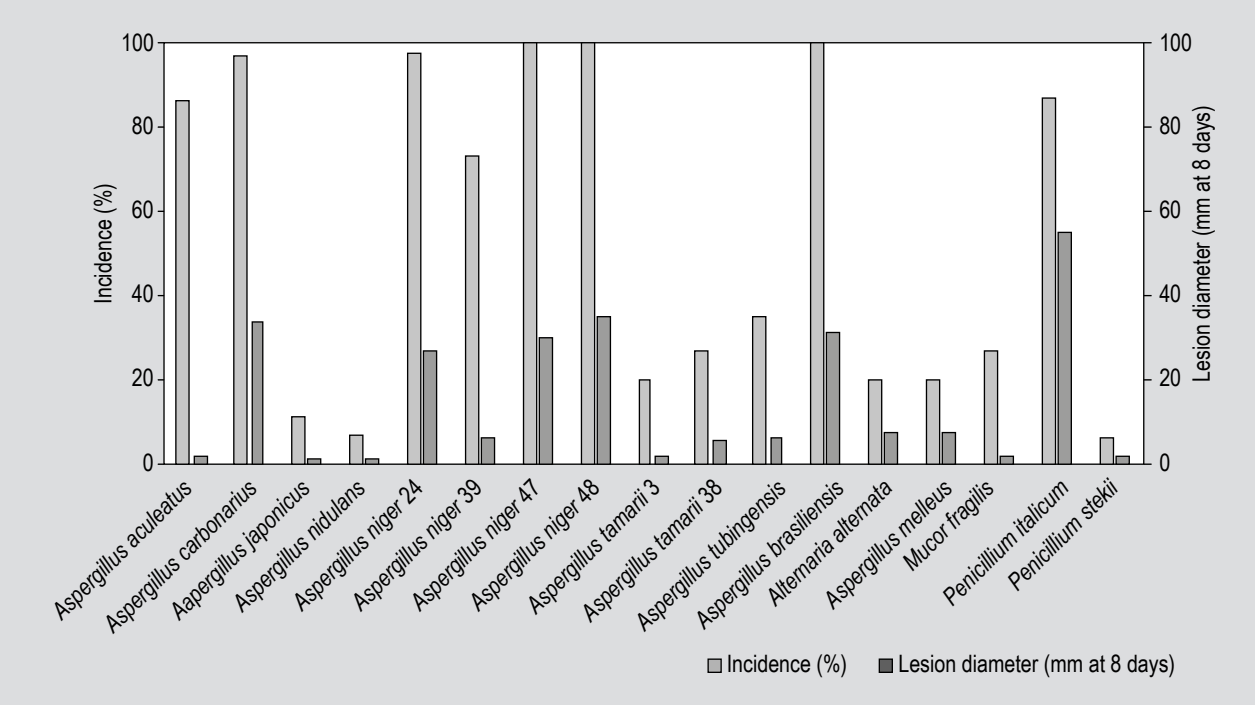

Figure 2. Phytopathogenic degree of moulds isolated from citrus fruit from different states of Mexico. In grey: incidence; in black: lesion diameter at 8 days of inoculum.

A. niger S24, from the orange orchard, was positive for the *FUM1*, another polyketide synthase gene involved in fumonisin biosynthesis (Figure 3C), as well as for the *VerB* gene (desaturase) involved in aflatoxin biosynthesis (Figure 3D). *A. carbonarius* strain 40, from an orange orchard, was positive for the sequence encoding the PKS enzyme involved in OTA biosynthesis (Figure 3E).

Expression of VerB and FUM1 genes

Figure 4 shows the relative expression of *VerB* and *FUM1* in *A. niger* S24 strain. Values obtained for both genes were normalised with the values obtained for the *EF1A* gene. The plot value represents the times the *VerB* and *FUM1* gene were expressed compared to their calibrator or control (*A. niger* culture at 30 °C and pH 5.5). In both cases the expression were positive and close to zero, indicating that the gene is expressed in a basal form with respect to the constitutive gene *EF1A*.

Mycotoxin production

After 10 days of incubation, *A. niger* S24 showed some level of mycotoxins (Figure 5). Concentration detected was 0.023±0.03 ng/l of media culture for aflatoxin B_1, and 81.33±70.55 ng/l of media culture for fumonisin. *Alternaria* toxins from *A. alternata* and OTA from *A. carbonarius* were not detected.

4. Discussion

Being part of the ecosystem, mould levels of around 3 log cfu/cm^2 in orchards can be considered normal, given that these moulds are dispersed throughout the air and ground and are deposited on the surfaces of citrus fruit by wind or rain (Palou *et al.*, 2001). Maximum mould levels were observed in orchards B and C, probably due to the higher relative humidity of these zones. In 2001, Palou *et al.* found similar values when investigating the mycobiota present in tangerine fields. Moulds were found at higher levels during the season with the greatest rainfall. In Mexico the best citrus-producing regions have warm and humid climates throughout most of the year (SAGARPA, 2005). Citrus fruit have great commercial importance. Handling the fruit inadequately can stimulate greater mould development and facilitate the production of mycotoxins in these products, as well as in their derivatives. This has scarcely been considered a problem in this kind of fruit. The results suggest that the fields' mycobiota are those most likely to be prevalent in the packing plants (Palou *et al.*, 2001).

The degree of contamination by moulds becomes more important for the packinghouses when the existing equipment is compared. In 2005, González and Silva (2005) reported that the degree of equipment in a packinghouse determines the quality control of citrus fruit and their shelf life. Mould counts can verify if the procedures of a standard manufacturing operation and its maintenance are adequate, as large variations could imply an incorrect

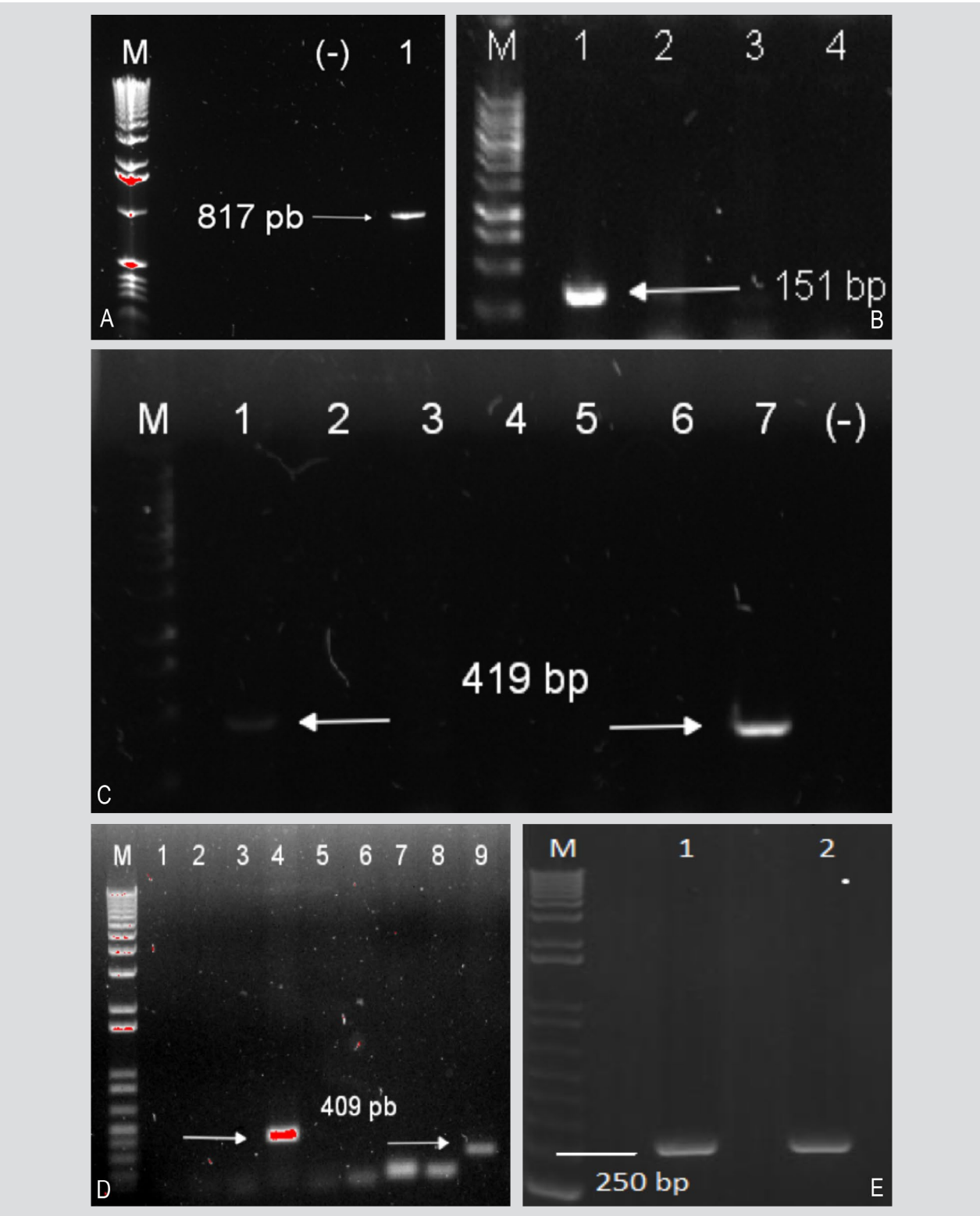

Figure 3. Identification of *PKSA*, *FUM1*, *VerB* and *PKS* genes by electrophoresis in agarose gel at 1% of (A and B) *Alternaria alternata*; (C and D) *Aspergillus niger*; and (E) *Aspergillus carbonarius.* Lane M: molecular weight marker 1 Kb (Promega®) in all cases. Arrows indicate the size of the expected product. (A) lane 1: *A. alternata* and negative control, primer AaPKS-TE. (B) lane 1: *A. alternata*; lane 2: *Alternaria* sp.; lane 3: *Alternaria tenuissima*; lane 4: negative control, primer AaPKS. (C) lane 1: *A. niger* 24; lane 2: *A. niger* 29; lane 3: *A. niger* 31; lane 4: *A. niger* 32; lane 5*: Fusarium equiseti* 18; lane 6: *F. equiseti* 19: lane 7: *Mucor fragilis,* primer GmFUM1. (D) lane 1 and 6: *A. niger* 31; lane 2 and 7: *A. niger* 32; line 3 and 8: *Aspergillus flavipes*; lane 4 and 9: *A. niger* 24, primer AfVerB. (E) lane 1 and 2: *A. carbonarius*, primer AoPKS.

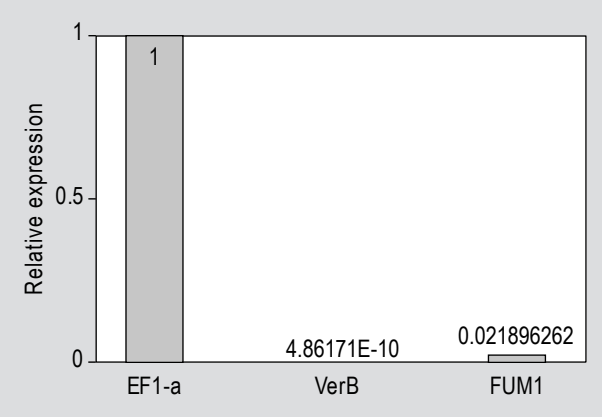

Figure 4. Graphic representation of the relative expression of *VerB* and *FUM1* in *Aspergillus niger* S24. The expression obtained in both genes is relative to the data obtained for their calibrator: the culture of *A. niger* S24 at 30 °C, pH 5.5. Standard deviation for: *EF1-A* = 0.08; for *VerB* = 9.33E-08; for *FUM1* = 0.14.

operation (Droby *et al.*, 1996). Those procedures were not uniformly observed in two of the technified packinghouses that were analysed. However, several factors other than the equipment determine the safety and sanitary quality of the fruit, as we also have good manufacturing practices. It is important to point out that, based on the bibliographical review performed by the authors of this research, this is the first time the mycotic levels in citrus fruit phyllosphere have been measured.

In this study, a large variety of fungal species was recovered including species that have been reported as phytopathogenic or mycotoxigenic. These species could have contaminated the fruit as they passed the successive stages of processing. Other studies of the mycobiota present on equipment surfaces and the environments of citrus fruit centres in Tarragona, Spain and São Paulo, Brazil emphasise the use of an integrated production system that includes effectively physically separating the fruit on arrival from the field, sanitising the processing lines, and disinfecting the equipment, chambers, floors, and so forth (Fischer *et al.*, 2009; Palou *et al.*, 2001).

When ensuring food safety, the contaminant itself needs to be identified. In the case of moulds, it is particularly important to know the genera and species present, because they are related to the production of mycotoxins in the food itself. Identifying the mycobiota present in the orchards and citrus packinghouses also offers an opportunity to learn the role of identified species and develop strategies to fight them (Ochoa *et al.*, 2007). Among the recovered strains, host-specific species for citrus fruits as well as opportunistic species were found (Barkai-Golan and Paster, 2008; Fischer *et al.*, 2009).

Agronomic management of the orchard and pre-harvest treatments that can reduce pathogens may influence the abundance of particular species (Ochoa *et al.*, 2007; Romanazzi *et al.*, 2016). Mycobiota of storage citrus in Pakistan were identified based on morphological characteristics, like *A. niger, Aspergillus flavus, Penicillium verrucosum, Fusarium oxysporun* and *A. alternata* (Akhtar *et al.*, 2013). Moulds in our research have been identified by molecular methods (White *et al.*, 1990), now accepted as an official barcode for fungi identification (Samson *et al.*, 2014). In this study, *Aspergillus* was the most widely identified genus. This genus dominates contamination in tropical areas, has been reported as a major source of post-harvest decay in various products including fruits like apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes, and melons, and some vegetables,

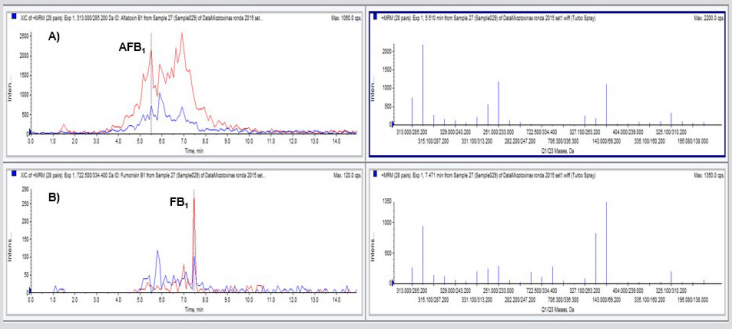

Figure 5. Chromatograms obtained by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) of mycotoxins from extracted cultures of *Aspergillus niger* S24 strain that result positive to *VerB* and *FUM1* genes. HPLC chromatograms (left), and their respective extraction chromatograms of the major fragment ions (right). Spectrum for (A) AFB_1 and (B) FB_1. Between the positive strains of mycotoxigenic genes, *A. niger* S24 was the only one that result positive to mycotoxins analysis. Limit of detection = 6/15 and 10/108 µg/l for AFB_1 and FB_1, respectively. Values are given in the order in which external standards were diluted in solvent/spiked samples. Number of evaluated concentration levels: 3.

especially onions, garlic, and yams (Barkai-Golan and Paster, 2008; Filtenborg *et al.*, 1996; Perrone *et al.*, 2007; Pitt and Hocking, 2009). *Aspergillus* section Nigri species are opportunistic pathogens whose penetration mechanism is through wounds inflicted on the fruits, naturally or due to inadequate post-harvest handling (Agrios, 2005). *Aspergillus* have a high enzymatic activity that can cause severe damage to the pericarp reducing shelf life (Bamba and Sumballi, 2005; Filtenborg *et al.*, 1996). In warm grapevine production areas, *Aspergillus* section Nigri rot is particularly severe, surviving mainly on soil (Battilani *et al.*, 2008). *Alternaria* and *Penicillium* species, the second most abundant host-specific recovered mould in our investigation, are well known for their post-harvest pathogenesis because of inadequate storage handling (Romanazzi *et al.*, 2016).

Although the isolates were in the phyllosphere (as a result of many factors, such as air transmission), these could not be infecting the fruit, and could not represent a danger. Exposure of unripe hosts to pathogens initiates defensive signal-transduction cascades that limit fungal growth and development. Opportunistic postharvest pathogens may also be located within the fruit in an inactive mode, awaiting fruit wounding, ripening or senescence. The length of each period may vary among pathogens, hosts, and host developmental stages. Pathogens such as *Colletotrichum*, *Monilinia*, *Botrytis* and *Alternaria* may remain quiescent for long periods in developing fruit tissues, but initiate immediate necrotrophic development on ripening and senescing fruits (Prusky *et al.,* 2009).

However, there is a host-specific interaction of germinating spores of some plant pathogenic moulds with early toxin synthesis in the pathogenesis process. That has been documented in *Helminthosporium victoriae* and *A. alternata*. In the case of *A. alternata,* the host-specific toxins AK (alternaric acid) and AF-toxin, wich assigned names AK-toxin and AF-toxin due to their closely related and complex structure derived from epoxy-decatrienoic acid (9,10epoxi-8-hydroxi-9-methil-decatrienoic acid moiety), act in that way (Hayashi *et al.*, 1990). External factors also regulate mycotoxigenesis and sporulation. During these phases, in moulds such as *Aspergillus* and *Fusarium*, the profile of secondary metabolism changes. Secondary metabolism and sporulation are temporally and functionally associated, including the synthesis of mycotoxins once the mould sporulates. For example, some secondary metabolites protect the spores or have antimicrobial activity as a chemical defence. In *Aspergillus nidulans*, for example, the sterigmatocystine is present as a protective agent (Brodhagen and Keller, 2006). This means that the spores already contain mycotoxins once they are deposited in the fruit to infect – which may also be toxic to humans – and then begin the chemical signalling necessary for germination and subsequent development of rot. However, this process is not yet visible to the human eye. This increases the risk of exposure to mycotoxins, or another secondary metabolite such as antibiotics; a situation that has been poorly evaluated in fruits and vegetables (IARC, 2002).

In this study, we found different degrees of pathogenicity and disease incidence in the *A. niger* aggregate strains. Leyva *et al.* (2003) also found common wheat to have different degrees of susceptibility to 10 different strains of *Fusarium graminearum.* In addition to the intrinsic factors that determine the pathogenicity of a strain, such the strain itself, many other factors in the field can contribute and trigger fruit infection during the early stages of development and continue through harvest, including the influence of climate (Agrios, 2005). The development of a pathogen also depends on the incidence of latent infections that usually start in the field or during harvest, on the effectiveness of treatments applied after harvest, and on the storage and sale conditions (Themis *et al.*, 2010). Many preformed substances such as phytoalexins or essential oils are synthesised by citrus fruits and may act *per se* as fungistatic or fungicides. However, sometimes they may not be able to prevent infection by *Penicillium* sp. in citrus (Musalem *et al.,* 1985).

With some exceptions, the phytopathogenic isolates on the Persian limes recovered in our investigation have been reported to produce one or more mycotoxins under storage conditions in a great variety of fresh produce (AFSSA, 2009; Barkai-Golan, 2008; Frisvad *et al.*, 2007b; Pitt and Hocking, 2009; Zachariasova *et al.,* 2014). Mycotoxins can be produced after the mould receives an external stimulus that activates the expression of the mycotoxigenic genes (Reverberi *et al.*, 2010). In this study, we amplified two different sequences for *A. niger* S24 strain: *FUM1* for fumonisin, and *VerB*, for aflatoxin (Yu *et al.*, 2002). This means that *A. niger* S24 has the potential to produce two of the highest-risk mycotoxins. Basal expression of these genes indicates that the possibility of synthesis of mycotoxins exist. Schmidt-Heydt *et al.* (2009) have found similar results. They performed a microarray analysis to study the effect of varying combinations of water activity and temperature on the activation of aflatoxin biosynthesis genes in *A. flavus*. In conditions favourable for growth of *A. flavus*, the cluster genes are expressed at a basal level with the strain producing high amounts of aflatoxin.

In this research it has been demonstrated that *A. niger* S24 is capable of producing AF and FB_1 in DSB medium. In other study in our laboratory the same strain *A. niger* S24 was able to produce AF in a wide variety of conditions of pH, temperature and incubation time, being the highest concentrations at their optimal synthesis conditions. In citrus fruit it was observed that *A. niger* S24 grows and produces AF that diffuses inside the fruit: a mayor health hazard if present in citrus fruit and transferred to their citrus derivatives (Sandoval-Contreras *et al.*, 2017). The

ability to produce fumonisin B_1 by *A. niger* have been demonstrated by Frisvad *et al.* (2007a) and some of these strains may produce another kind of mycotoxins like OTA. Similar results were found by Gallo *et al.* (2012) who also developed a rapid method to detect aflatoxigenic *Aspergillus* strains based on the detection of genes participating in the biosynthesis of mycotoxins, because the detection of mycotoxigenic genes has a positive correlation with synthesis of the mycotoxin.

A. niger is an important species used as production organisms in industrial fermentations and it has been affirmed as generally recognised as safe by the FDA (2017) in 1994. However, production of OTA by a few isolates of *A. niger* was confirmed (Blumenthal, 2004; Pitt and Hocking, 2009) as well as fumonisin (Frisvad *et al.*, 2007a). According to the review by Schuster *et al.* (2002), 'this species does not have the ability to produce aflatoxins' and they state, 'Previous reports may result from errors in detection methodology' (Blumenthal, 2004). Reports on some levels of production of aflatoxins by *A. niger* date from at least 30 or more years (Glinsukon *et al.*, 1979; Kulik and Holaday, 1966). Methodologies at the time were less precise. Further researches were focused on looking only for ochratoxigenic *A. niger* (Barberis *et al.*, 2012; Frisvad *et al.*, 2011; Ouf *et al.*, 2015) failing to give importance to aflatoxins. With the improvement of technology, methodologies are more specific and accurate, even in low quantities. Turner *et al.* (2015) in their review reported that thin-layer chromatography analyses have insufficient sensitivity compared to liquid chromatography methods. At present, HPLC-MS is accepted by AOAC (2000) as a standard method against which all other methods are compared. Rubert *et al.* (2012) reported detection limits of various mycotoxins in baby food by HPLC/MS-MS of 0.05-50 µg/K. It is important to emphasise that due to the controversy of whether *A. niger* produce aflatoxins or not, further studies are required, including more isolates from *A. niger* aggregates from citrus and other fruits, grains or vegetables.

A. carbonarius is another mycotoxin-producing mould, which in our research had sequences participating in OTA biosynthesis. In this study OTA was not detected for *A. carbonarius S40*; the culture media or the growth conditions were probably not suitable for mycotoxin production (Frisvad *et al.*, 2011), however, the risk is still present. Studies performed on oranges inoculated with *Aspergillus westerdijkiae* and their juice showed the mould was able to produce OTA on the peel. In addition, production of the mycotoxin increased at temperatures higher than 26 °C, conditions that are easily met during the storage of citrus fruit in Mexico (Marino, 2009). The presence of this mould and its capacity to produce mycotoxins are important and suggest a search for measures aimed at diminishing their presence on citrus fruit is necessary.

Regarding *A. alternaria* S35a strain, this mould has been found to have mycotoxigenic potential, like *A. carbonarius* S40. *Alternaria* species has the ability to produce the greatest variety of secondary metabolites; however, little information exists regarding their effects when present on food (Lou *et al.*, 2012). Nevertheless, some of these mycotoxins participate in the fruit colonisation process, which suggests mycotoxins are produced that can compromise human and animal health before any rot is evident (Barkai-Golan, 2008).

This study indicates there is a potential hazard for the post-harvest development of damaging and mycotoxigenic moulds on citrus fruit. Knowing the adverse effects of mycotoxins and those they can remain in citrus products, continued research is necessary to develop methods that ensure food safety and contribute to safeguarding the health of consumers. Knowing the life form of the mycotoxin-producing mould, its survival on different citrus crops in the field throughout the different seasons of the year, its population density in different regions of Mexico, will help to establish field control strategies against such moulds. Importantly, this research has contributed the first registry of the mycobiota present on citrus fruits grown in the different states of Mexico.

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