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REGULAR ARTICLE

Effect of pH and temperature in production of mycotoxins and antibiotics by phytopathogenic moulds for Persian lime (*Citrus latifolia* T.) in a complex lime pericarp-base medium

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ABSTRACT

Post-harvest decay of citrus fruits is caused by moulds capable of producing mycotoxins with great impact on health. The objective of this study was to determine the effect of pH and temperature in synthesis of mycotoxins and antibiotics in Persian lime pathogenic and mycotoxigenic moulds, and to investigate the diffusion of mycotoxins inside the citrus fruit. As a result, several mycotoxins were identified, such as aflatoxins and fumonisins. In most of the analysed moulds, synthesis of the secondary metabolites was observed on acidic pH at 20 °C; some antibiotics were found as well. Diffusion of *A. niger* mycotoxins and antibiotics was observed in the interior of the lime fruit, achieving highest concentration in the septa. Knowing the behaviour of these moulds is useful for evaluation of health risk due to mycotoxins and control of pathogenic and mycotoxigenic post-harvest moulds.

Keywords: *Alternaria*; Antibiotics; *Aspergillus*; Citric fruits moulds; Mycotoxins; Persian lime

INTRODUCTION

Besides from decay, the main fruit contaminant moulds have the ability to produce mycotoxins that represent a threat to human and animal health (Barkai-Golan and Paster, 2008). Mycotoxins are secondary metabolites of contaminant moulds throughout the food chain (Sanzani et al., 2016). *Penicillium, Aspergillus and Alternaria* species are major mycotoxigenic fungi that attack harvested fruits, and the mycotoxins produced by these moulds in the host tissues include patulin, aflatoxins (AF), ochratoxin A (OTA), and fumonisins (FB) (Barkai-Golan and Paster, 2008; Sanzani et al., 2016). Some of these mycotoxins receive greatest attention due to their carcinogenic, nephrotoxic, teratogenic and immunosuppressive effects (IARC, 2002).

Contamination of citrus fruits by decay moulds is a problem that concerns the worldwide citrus production industry. *Penicillium italicum* and *Penicillium digitatum* are the major cause of post-harvest diseases of limes that cause economic losses (Romanazzi et al., 2016). *P. italicum* and *P. digitatum* have not been considered mycotoxigenic moulds, however, these moulds have been found to produce secondary metabolites that are toxic to bacteria, plants and animals (Frisvad et al., 2004).

The "brown stain" caused by *Alternaria alternata* is also another citrus-specific disease. Damage caused can range from a small speck to a large lesion (Magnani et al., 2007). This mould also synthesises mycotoxins: Altenuene (ALT) and altertoxins (AT), which have been implicated in human and animal disorders (Zao et al., 2014, Sanzani et al., 2016); as well as alternariol (AOH) and alternariol monomethyl ether (AME), which are individually not as toxic, but are mutagenic (Jackson and Al-Taher, 2008).

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On the other hand, several investigations have shown that under experimental conditions, citrus fruits can withstand growth of *A. flavus* and formation of aflatoxins (Varma and Verma, 1987; Ariza et al., 2002; Bamba and Sumbali, 2005). Aflatoxins have been found naturally in citrus fruits, dates and figs, in regions that show the high temperature conditions required by aflatoxigenic moulds to develop (Jackson and Al-Taher, 2008). Perhaps the *Aspergillus* genus represents a greater problem, being one of the most common food contaminant moulds (Sanzani et al., 2016). OTA-A, produced by species of *Aspergillus*, is a mycotoxin found naturally in a wide variety of fresh products throughout the world (Esteban et al., 2004), and moulds such as *Aspergillus* have been demonstrated to survive and develop in oranges and their juice, producing mycotoxins (Marino et al., 2009).

Second-quality fruits may be used to produce derivatives (González-Sánchez and Silva-Echeverría, 2003; Restani, 2008). Should decay exist, the fruit is usually discarded partially or completely. However, mycotoxins can diffuse in adjacent tissues, and will remain in the final product and contaminate it even after having removed the decayed part (Restani, 2008; Zhao et al., 2014). Natural occurrence of mycotoxins has been reported in grains and grain-based products, tomato and tomato products, sunflower seeds and sunflower oil, fruits and fruit products, beer, wine and so on, especially from several processed foodstuffs manufactured with damaged raw materials (Fan et al., 2016). There is little research about incidence of mycotoxins produced by Persian lime phytopathogenic moulds, and information about health risks caused by the presence of these moulds in the fruit or its derivates is also scarce (Chen et al., 1992; Faid et Tantaoui-Elaraki, 1989; Logriego et al., 1990; Bamba and Sumbali, 2005; Marino et al., 2009). On the surface of citrus fruits, we can find a wide variety of moulds such as *Aspergillus* sp, *Penicillium* sp, *Alternaria* sp or *Fusarium* sp. (Ochoa et al., 2007; Palou et al., 2001; Sandoval-Contreras, 2011). Epiphytic microbiota makes lime susceptible to development of moulds, both pathogenic and mycotoxigenic. On the other hand, the fruit may act as a vector for contamination of derivatives where moulds can develop (Esteban et al., 2004).

The objective of this study was to determine the effect of pH and temperature in production of mycotoxins and antibiotics by pathogenic moulds for Persian lime (*Citrus latifolia*, T.) on a complex citrus pericarp-based medium, and to establish whether they diffuse on the Persian lime.

MATERIALS AND METHODS

Aspergillus niger (strain 24), *Alternaria alternata* (strain 5a), and *Aspergillus carbonarius* (strain 40) were used in this research. All of them are native moulds previously isolated from the surface of Persian limes (*Citrus latifolia,* T.) obtained from a citrus orchard in Baja California Sur, Mexico. Characterization and identification of all isolates was carried out at the Laboratory of Microbial Pathogenesis (Northwest Biological Research Center, La Paz, Baja California Sur, Mexico). Moulds were cultured on potato dextrose agar (Bioxon® BD, New Jersey, USA) at 25 °C for 5 to 7 days, using suspensions of about 6 log spores/ml prepared in an aqueous solution (0.05% w/v) of Tween-80 (Pose et al., 2010). The spores were counted in a Neubauer chamber.

Culture medium

Mycotoxin and antibiotic synthesis was determined on a lime agar medium (LA) specifically designed for this purpose. LA was elaborated with lime pericarp, in which dry-base composition contains about 33% cellulose and 20% pectic substances (Stechina, 2005). LA contain 57.5 g of finely ground Persian lime pericarp (*Citrus latifolia*, T) and 15 g of agar per litre. The LA was adjusted to different pH with 1 molar hydrochloric acid or 1 molar sodium hydroxide in order to determine optimal acidity conditions. For kinetics studies, lime broth (LB, pH 5.5 ± 0.2) was prepared in the same proportions as the LA medium.

Kinetics of mycotoxin and antibiotic synthesis in lime broth

The previously described LB medium was prepared and inoculated with 15 µl of mould inoculum suspension. Batches were incubated statically for 21 days at different temperatures: 20, 25 and 30 °C (three replicates per batch). Every 7 days, 20 ml of cell-free samples were taken for extraction. Mycotoxin and antibiotic were extracted twice with 25 ml chloroform, shaken for 3 minutes, and let sit for separation and filtration of the chloroform phase (Whatman No. 4 paper, Sigma-Aldrich, St. Louis, MO, USA). The phases were then evaporated to dryness. Extracts were diluted in 1 ml of extraction solvent (acetonitrile/water/acetic acid (79:20:1, v/v/v)), filtered (Acrodisc® PTFE, Sigma-Aldrich, St. Louis, MO, USA), and directly injected into the HPLC-MS/MS instrument (Brzonkalik et al., 2011; FDA 2001).

HPLC/MS/MS analysis

Chromatographic separation was performed with an UFLC SHIMADZU Prominence System (Shimadzu Scientifics Instruments, Inc., Columbia, MD) coupled to a triple quadrupole mass spectrometer (MS/MS) TSQ Vantage equipped with heated-electrospray ionization API3200 Q TRAP (SCIEX, Framingham, MA) with an ESI interphase. Aliquots of 20 µL were injected at 45° C on Kinetex 2.6m Biphenyl 100A 50x2.1 mm column (Phenomenex, Inc., Torrance, CS, 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-1. 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 metabolite were performed according to Vishwanath et al. (2009). The software Analyst (SCIEX, Framingham, MA) was used for controlling chromatography and mass spectrometer.

Evaluation of pH and temperature conditions for mycotoxin and antibiotics synthesis

The previously described LA plates were prepared at different pH (4.0, 5.5 and 7.0). They were inoculated, three plates per replicate, with 15 µL of spore suspension of each mould. Batches of plates were incubated at 20, 25 and 30° C for 10 d in all cases. At the final of incubation time, each sample was transferred to a blender cup for mycotoxin and antibiotic extraction, with chloroform (FDA 2001). The chloroform phase was filtered and evaporated to dryness for HPLC-MS/MS analysis, previously described.

Mycotoxins and antibiotics production on Persian lime.

Aspergillus niger 24 and *A. carbonarius* 40 were used for this step. Persian limes (*Citrus latifolia*, T.) were purchased at a local market without any postharvest protection treatments. They were washed and disinfected by immersion and gentle stirring in a solution of NaClO (1% v/v) for two minutes. Limes were rinsed with sterile water and allowed to dry at room temperature for 1 h. A wound (3 mm long and 1 mm deep) was performed on each fruit with a sterile scalpel, followed by inoculation of 15 mL of the mould spore suspension (6 log spores/mL) and incubated for 10 d at room temperature (25 ± 3 °C). After incubation time, the infection for each mould was characterized and the different lime parts were separated and weighed: Flavedo, albedo, septa, and trichrome. They were placed in a flask for the extraction of mycotoxins and antibiotics as described above. The incidence of disease was measured based on the number of infected limes from the total inoculated limes, using the formula:

$$I = (Li/Lt)*100$$

where I (%) is the incidence of infection, calculated after the proportion of decayed fruit; Li is the number of limes with decay, and Lt is the total number of limes used (ten limes per trial). The severity of disease was evaluated in relative terms from lesion diameter: 2 to 10 mm: Low severity; 11 to 30 mm: Medium severity; a diameter greater than 30 mm: High severity.

Statistic analysis

Due to the asymmetric distribution of our quantities, we used comparison of averages with Levene's test to determine statistic significance. Multiple linear regression was used for correlation analysis between concentrations, using the StatGraphics Centurion statistics package (Maryland, USA) with a significance factor of 95%.

RESULTS

Kinetics of mycotoxin and antibiotic synthesis in lime broth (LB)

HPLC-MS/MS analysis allowed for identification and quantification of more than one mycotoxin of alimentary interest produced by the analysed moulds, which were aflatoxin B_1 (AFB_1), AFB_2, AFG_1, AFG_2, fumonisin B_1 (FB_1), as well as antibiotics penicillin G (PG), cycloheximide (Cy), and chloramphenicol (Cl). Fig. 1 shows the chromatograms obtained from some quantified mycotoxins and antibiotics, along with their respective fragment ion.

Alternaria alternata (Fig. 2-A1) shows the highest concentrations of AFG_1 and G_2 at 20º C at earlier stage of incubation ($AFG_{1:}$ 340 ng/mL and $AFG_{2:}$ 530 ng/mL), whereas AFB_1 and AFB_2 (148 and 950 ng/mL, respectively) were observed at most at the 14th day. All AF decreased at the end of incubation ($P > 0.05$). FB_1 (7170 ng/mL; $P < 0.05$) was observed at 25 °C at the end of incubation time. *Alternaria alternata* also synthesized the antibiotics cycloheximide, penicillin G, and chloramphenicol at 20º C at an earlier stage of incubation ($P < 0.05$) (Fig. 2-A2). A positive correlation ($R^2 = 73.22$; $P < 0.05$) between AF_t and the sum of the antibiotics synthetized was observed.

In *A. carbonarius* (Fig. 2-B1), the highest concentration of mycotoxins was observed at 20º C ($AFB_{1:}$ 113 ng/mL, $AFB_{2:}$ 71 ng/mL; $AFG_{1:}$ 1155 nm/mL and $AFG_{2:}$ 400 ng/mL). The levels of AF_t decrease over time, but reappear and reach maximum value at the end of incubation time. Antibiotics (penicillin G and chloramphenicol: 4300, 6250 ng/mL, respectively) (Fig. 2-B2) were also observed at 20º C at the end of incubation time. Cycloheximide (1070 ng/mL) was observed on the middle of incubation time at 20ºC. At this temperature we also observed a positive correlation between the sum of AF and the sum of the antibiotics produced ($R^2 = 90.54$; $P < 0.05$).

In cultures of *A. niger* (Fig. 2-C1) the maximum aflatoxin synthesis occurred at 20º C at earlier stages ($AFB_{1:}$ 1340 ng/mL; $AFB_{2:}$ 3760 ng/mL and $AFG_{1:}$ 4780 ng/mL) ($P < 0.05$). Smaller amounts were observed as the incubation time advanced. FB_1 synthesis was also produced by *A. niger* (18500 ng/mL) at 20º C.

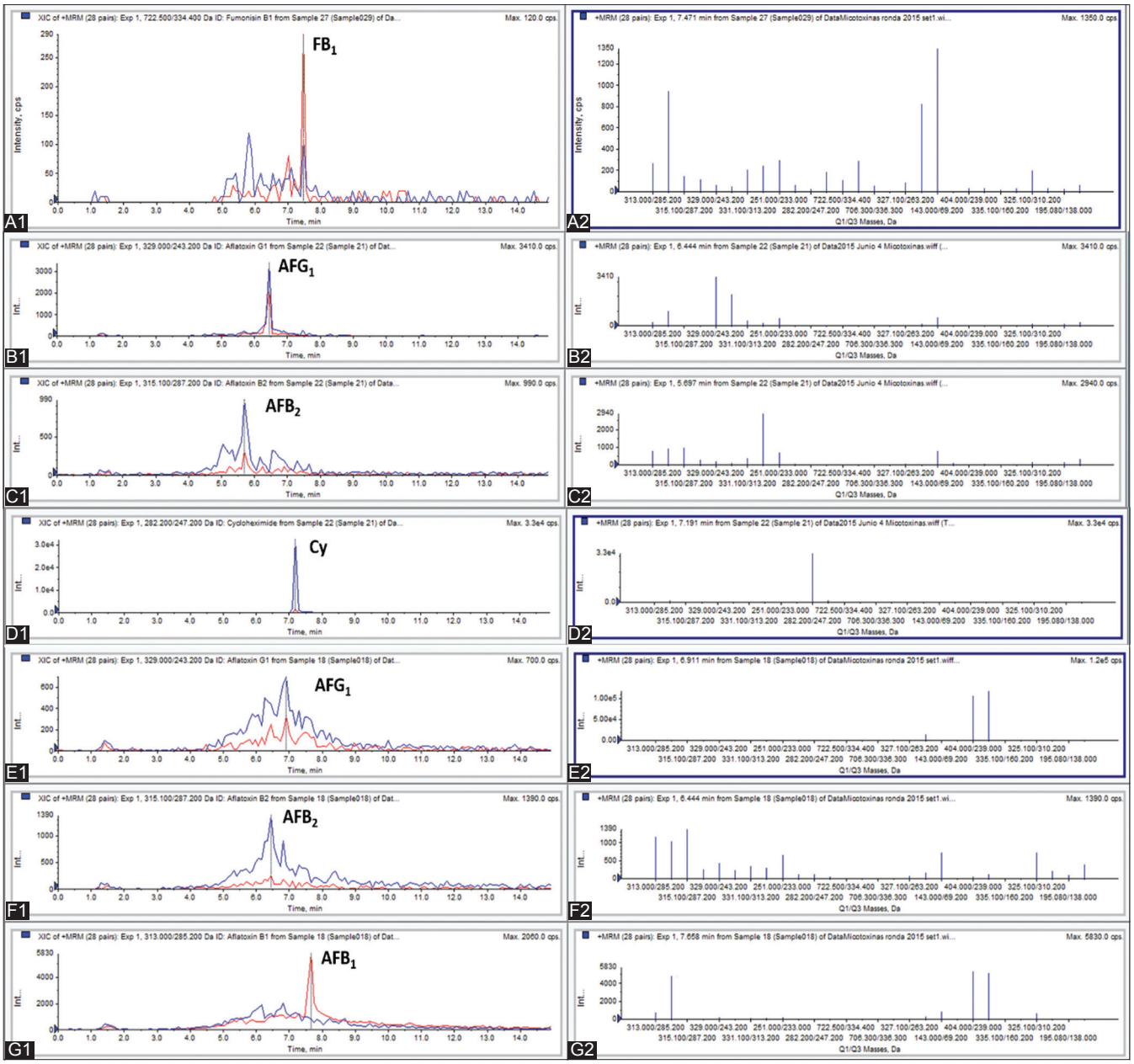

Fig 1. Chromatograms obtained by High- performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) of mycotoxins from extracted agar lime samples. HPLC chromatograms (*left*), and their respective extraction chromatograms of the mayor fragment ions (*right*). A): *Alternaria alternata*. The peak is marked for fumonisin B1. B), C) and D): *Aspergillus niger*. The peak is marked for mycotoxins aflatoxin G_1, B_2 and, for antibiotic cycloheximide (Cy) respectively. E), F) and G): *Aspergillus carbonarius*. The peak is marked for aflatoxins G_1, B_2 and B_1 respectively.

Antibiotics (cycloheximide: 88900 ng/mL, and penicillin G: 1350000 ng/mL) (Fig. 2-C2) also showed their maximum synthesis at 20° C ($P < 0.05$).

Evaluation of pH and temperature conditions for mycotoxin and antibiotics synthesis

In all the analyzed moulds, more than one mycotoxin (reported as AF_t) and antibiotics (reported as sum of antibiotics PG, Cy and Cl) were identified. In a multivariate analysis, no correlations were found between mycotoxins and antibiotics for any of moulds studied at any condition.

In *A. alternata* extracts, AF_t (110 ng/g of AL) was observed at 20° C and pH 5.5 (Fig. 3-A1) ($P < 0.05$). Antibiotics (52200 ng/g of AL) were also detected in a wide range of pH and temperature ($P > 0.05$), where the maximum concentration was at 30° C and pH 4.0 (Fig. 3-A2).

In *A. carbonarius* (Fig. 3-B1), the maximum synthesis of AF_t (2837 ng/g of AL) was at 25° C and pH 7 ($P < 0.05$). Antibiotics were also observed in all conditions ($P > 0.05$), being the maximum concentrations at pH 4 and 20° C (Fig. 3-B2).

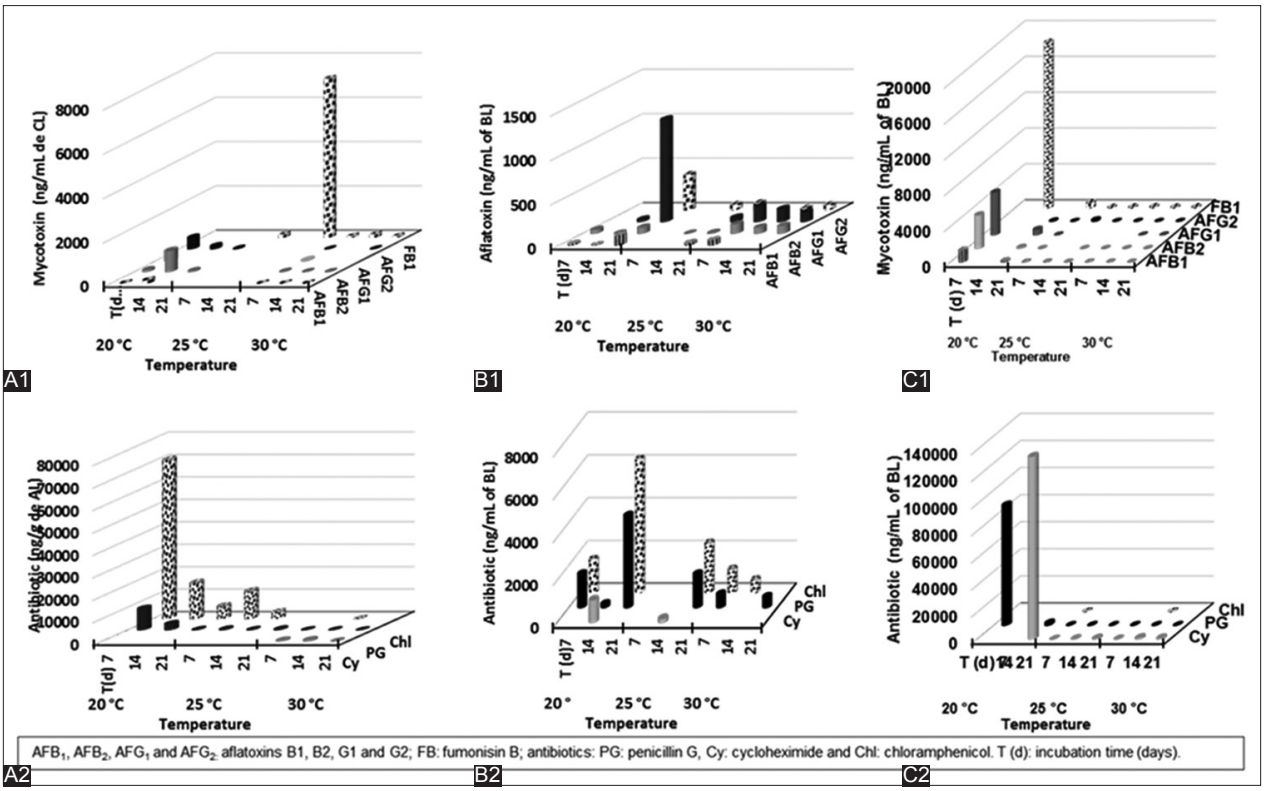

Fig 2. Synthesis of mycotoxins in lime broth (LB) at different temperatures and incubation times. A1) *Alternaria alternata,* mycotoxins: Significant differences exist ($P < 0.05$) for BF_1 at 25 °C (21 day). A2) Antibiotics: Significant differences exist ($p < 0.05$) for Chl at 20 °C at 7th day of incubation. B1) *Aspergillus carbonarius,* aflatoxins: Significant differences exist ($P < 0.05$) for AFG_1 at 20 °C (day 21). B2) Antibiotics: Significant differences exist for PG and Chl ($P < 0.05$) at 20 °C, 21 days of incubation. Coefficient of correlation (CC) between total aflatoxin and antibiotics production: 0.9 ($P < 0.05$). C1) *Aspergillus niger*: Significant differences exist ($P < 0.05$) at 20 °C for AFB_1, AFB_2 and AFG_1 at 7th day, and FB_1 at 14th day. C2) Antibiotics: Significant differences exist at 20 °C ($P < 0.05$) for PG (day 7) and Cy (day 21).

For *A. niger* extracts, the maximum of AF_t were quantified at 20 °C and pH 4.0 (Fig. 3-C1) ($P < 0.05$). Antibiotics (34840 ng/g AL) were detected in high levels at pH 7 and 25° C (Fig. 3-C2).

Evaluation of the production of mycotoxin on Persian lime

During the infection of Persian lime with *A. niger*, the incidence and severity of the disease was low (30%). At the end of experiment, all fruits were rotten. In most cases, mould remained attached to the flavedo; the albedo was softened and the septa were degraded in the most severe infections. The trichomes changed their appearance from turgid to very smooth.

The mycotoxin analysis of the flavedo extracts shows low or undetectable levels. Concentrations increased in the albedo, and the maximum levels were observed in the septa (AF_t: 2892 ng/g, antibiotics, Cy and Cl: 90451 ng/g);these levels then decrease in trichomes ($R^2 = 99$; $P < 0.05$). (Fig. 4-A)

In the infection of Persian lime with *A. carbonarius* (Fig.4-B), disease incidence was 100% at the beginning of the experiment. The appearance of the infection was similar to that observed in *A. niger*, however, the highest concentration of mycotoxins was observed in the flavedo (AF_t: 9814 ng/g; antibiotics Cy and Cl: 40000 ng/g). At the interior of the fruit, the concentrations gradually decreased ($R^2 = 99$; $P < 0.05$).

DISCUSSION

Citrus are a highly consumed product in Mexico with great international commercial importance. Inadequate handling makes them an optimal substrate for the development of pathogenic moulds (Ochoa et al., 2007) that can also produce mycotoxins that are toxic for human health (IARC 2002).

The pH of fruits and temperature are the most important parameters to determine the ability of the mould to grow and synthesize mycotoxins (Pitt and Hocking, 2009). Citrus are produced in different regions of Mexico. The range of temperatures selected in our research represent the average temperatures on which citrus can be handled on different regions: Cool zones (20° C), temperate zones (25° C) and

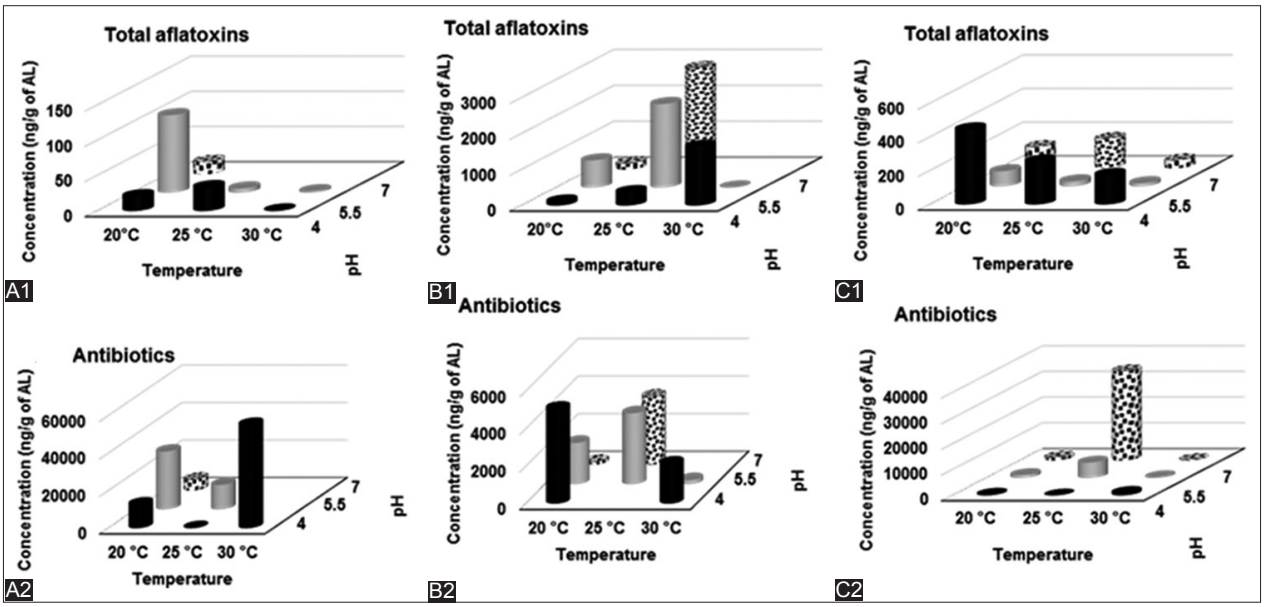

Fig 3. Synthesis of mycotoxins on lime agar (LA) at different pH and temperatures. A1) *Alternaria alternata*: Significant differences exist ($P < 0.05$) at 20 °C, pH 5.5 for total aflatoxins; A2) antibiotics: ($P > 0.05$). B1) *Aspergillus carbonarius:* Significant differences exist ($P < 0.05$) at 25 °C, pH 7.0 for total aflatoxins; B2) Antibiotics: ($P > 0.05$). C1) *Aspergillus niger: S*ignificant differences exist ($P < 0.05$) at 20 °C, pH 4.0 for total aflatoxins; C2) Antibiotics, significant differences exist ($P < 0.05$) at 25 °C, pH 7.0.

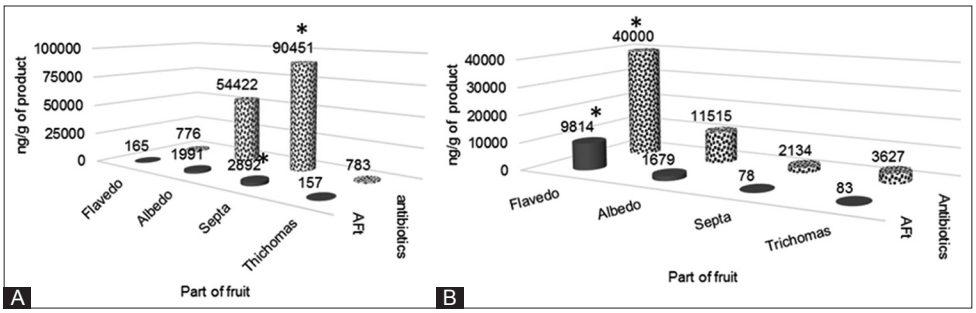

Fig 4. Persian lime artificially infected with mycotoxigenic moulds. AF_t is the sum of AFB_1, AFB_2, AFG_1 and AFG_2. Other toxins: Antibiotics are the sum of chloramphenicol and cycloheximide. A) Infection with *Aspergillus niger: S*ignificant differences exist ($P < 0.05$) in septa. B) Infection with *Aspergillus carbonarius: S*ignificant differences exist ($P < 0.05$) in flavedo.

warm zones (30° C) (Sandoval-Contreras, 2011; Sandoval-Contreras et al., 2017). Our results showed that moulds analyzed synthesize more than one mycotoxin mainly at 20° C, whereas *A. niger* synthesizes FB_1 at 25° C. This indicates that the risk of contamination increases on cool and temperate citrus production zones (Passamani et al., 2014).

On kinetics of synthesis, positive correlations were observed between mycotoxins and antibiotics production for *A. carbonarius* and *A. alternata*, indicating that synthesis of mycotoxins and antibiotics occurred at same conditions. In this case, the risk become higher because the presence of multiple toxins at the same time. This fact have been demonstrated in feedstuff (Borutova et al., 2012; Zachariasova et al., 2014).

Synthesis and degradation patterns were observed for *A. niger*, similar to those obtained by Huynh and Lloyd (1984). They observed that the young mycelium synthesized maximum values of AF on day 14 of incubation, and it began degradation, caused by the old mycelium, as it gets older. It was also observed that the addition of cycloheximide to growths prevented aflatoxin degradation (Huynh and Lloyd, 1984). In our study, this pattern was observed in *A. carbonarius* at 20° C. The difference in our research was that Cy has not been added to the culture; it was produced by the mould itself. This phenomenon could have been one of the causes for the observed variability.

Our results suggest additional studies on PG, Cy, and Cl, antibiotics detected in *A. alternata* and *A. carbonarius* extracts, because their presence may pose a food risk (Graham et al., 2014). Anti-microbial resistance of bacteria generated by indiscriminate use of antibiotics has become a global health issue. Excessive and often empirical use of antimicrobials to treat different clinical situations has conducted to

modifications of bacterial ecology (Cires–Pujol 2002). Exposure to low concentrations of the antibiotic selects bacteria that have acquired the resistance allowing them to develop more favorably than those who had not acquired it (Phillips et al., 2004). The presence of antibiotics synthesized by moulds on agro-alimentary products are a source of contact for bacteria to acquire resistance. Even though there are not considered an alimentary risk, their consumption, thus, requires a strict supervision on the antibiotic content, due to their natural occurrence in foods (Barkai-Golan and Paster 2008).

The effect of pH and temperature in mycotoxin and antibiotics production for all moulds in this study was similar to those obtained by Passamani et al. (2014). They evaluated the influence of temperature, pH and a_w on the growth and mycotoxin production on *A. carbonarius* and *A. niger* on a grape-based medium, finding that optimal values of *A. carbonarius* ranged from 20 to 33° C and pH levels from 5 to 6.5 (0.96 – 0.98 a_w). For *A. niger* they determined temperatures from 24 to 37° C with pH levels from 4 to 6.5 (0.95 a_w).

Even though the *Aspergillus* species is not considered a major cause of citrus disease, these fungi are responsible for several disorders in various fruits, plants, and plant products including citrus or their derivatives (Stinson et al., 1981, Ariza et al., 2002; Bamba and Sumbali 2005; Perrone et al., 2007). In a similar research, *Aspergillus* strains isolated from spoilage in oranges (*Citrus reticulata*) have produced AFB_1 on a semi-synthetic medium, showing also a decrease of levels with incubation time (Varma and Verma 1987). In India, strains of *A. flavus* have been found to cause deterioration of Mexican limes (*Citrus aurantifolia S.),* and have the ability to produce AFB_1 and cyclopiazonic acid (CPA) on fruit tissue (Bamba and Sumbali 2004). Alemandri et al. (2004) determined the mycotoxigenic capacity on strains of *A. clavatus* and *A. flavus* isolated from citrus juices, confirming the production of aflatoxins on a citrus juice-based medium. Xing et al. (2015) developed a method of extraction and analysis for HPLC to determine the presence of aflatoxins B_1, B_2, G_1, and G_2 simultaneously from orange extracts (*Citrus reticulata*) with a quantification limit of 0.044 to 1.2 μg/kg. In another research related to the safety of foods containing citrus pulp, aflatoxin levels of up to 10 μ/kg were detected, proving also that the ingest of mycotoxins can be on an indirect form (Paulsch et al., 1988).

In this research, FB_1 was detected in *A. niger* extracts. Since the gene cluster for fumonisins in *A. niger* was discovered and it was proven that some isolates have the ability to produce it (Frisvad et al., 2007), studies on black *Aspergilli,* as potential producers of fumonisin on several products has increased. Varga et al. (2010) reported the synthesis of FB_1 by *A. niger* isolated on raisin (5.16 mg/kg average) for the first time. On similar studies, Morgensen et al. (2010) reported lower concentrations (FB_2: 171 - 7841 μg/kg and FB_4: 14 - 1157 μg/kg on grapes).

A recurrent association was observed between aflatoxin and fumonisins. This association has been qualified with synergetic or additive effect (Grenier and Oswald 2011). This is important for risk evaluation of mycotoxins in food.

The studies performed on citrus are primarily with *Alternaria* and its mycotoxins (Zhao et al.; 2014). In our study, we identified FB_1, PG and CY, metabolites that not have been reported for *A. alternata*. This is why systematic research and vigilance is suggested on fresh products and its derivatives with the purpose of providing scientific bases for the risk evaluation on food exposure of these substances. (Sanzani et al., 2016).

Diffusion of mycotoxins from an infected area of fruit towards a healthy one has consequences for the final product. It has been proved on processed apples and pears (Laidou et al., 2001; Rychlik and Schieberle 2001). Due to the great thermal stability, the mycotoxins that diffuse on fruits remain intact and pass to the finished product (Rychlik and Schieberle 2001). Our results showed that in the infection of Persian lime with *A. niger,* mycotoxins apparently migrate towards the depth of the fruit. These results differ from those reported by Marino et al. (2009) where orange was infected with ochratoxigenic *A. westerdijkiae*, and they found the highest concentration of OTA on the surface. This behavior was similar to that observed on the infection with *A. carbonarius* where the highest values of mycotoxins was observed on the flavedo. Magnani et al. (2007) detected AOH and AME, 0.90 and 17.40 g/kg respectably, on tangerine flavedo infected by *Alternaria.* These mycotoxins were not found on the albedo, suggesting that the flavedo acted as a barrier. Rychlik and Schieberle (2001) separately studied the diffusion of mycotoxins on several alimentary matrixes, concluding that diffusion is facilitated on products with a greater amount of water (melon, grape, and tomato) than in viscous matrixes (apple) and it seems impeded by the presence of structural polysaccharides. In our study, the difference on the diffusion of mycotoxins of *A. niger* and *A. carbonarius* could have been the result of the differences between them as they were different species with a different behaviour (Restani, 2008).

CONCLUSION

This study indicates a potential hazard for development of pathogenic and mycotoxigenic moulds on % citrus

and the possibility of contamination of its derivatives. Knowing their behavior under different environmental factors is useful to determine the adequate control to reduce the risk of contamination through the whole process. Considering the frequency of these moulds as part of the normal microbiota of citrus fruits and their mycotoxigenic potential, their analysis would be beneficial for health risk evaluations. However, further investigations are necessary to continue studying the presence of moulds and mycotoxins reported in this study and their diffusion on the fruit.

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Authors' contributions

T.S.C. conducted the experiment, work, analyzed the results, and drafted the manuscript. A.V.L. and F.A. designed, supervised, and discussed the results with the research group. A.P.S.B. was involved in the HPLC-MS/MS analysis and interpretation. R.T.V. supervised certain scientific approaches and advised the research.

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