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Mycotoxigenic potential of *Alternaria alternata* isolated from dragon fruit (*Hylocereus undatus* Haw.) using UHPLC-Qtof-MS

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ABSTRACT

The occurrence of mycotoxins synthesized by spoilage fungi has been reported in various crops. Despite being a crop susceptible to attack by microorganisms, such as *Alternaria alternata*, there are no reports on the production of mycotoxins in *Hylocereus undatus* (Haw.). The objective of the present study was to identify mycotoxins produced by *A. alternata* previously isolated from *Hylocereus undatus* (Haw.) fruit in the postharvest phase, and *A. alternata* ATCC 46,582, *in vitro* and *in vivo*. The compounds were identified using liquid chromatography-mass spectrometry (LC–MS/MS). Alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), altertoxins (ATX) I, II and III were detected *in vitro* for *A. alternata*, and ATX I and II were identified for the reference strain, *A. alternata* ATCC 46,582, which was used as a positive control. Mycotoxins characteristic of *A. alternata* were not detected *in vivo* in the fruit stored for 8 d, which is its estimated shelf life. The use of LC–MS/MS facilitated rapid and efficient mycotoxin identification, contributing significantly to the evaluation of food safety.

1. Introduction

Hylocereus undatus (Haw.), or dragon fruit, is a fruit of the *Cactaceae* family which is native to South and Central America (Arul et al., 2017). *H. undatus* is susceptible to attack by various pathogenic and spoilage microorganisms, including *A. alternata* (Ortiz-Hernández and Carrillo-Salazar, 2012; Castro et al., 2017).

Alternaria alternata, a filamentous and spoilage fungus, is an important pathogenic and toxigenic microorganism in fruit (Vilaplana et al., 2017). *Alternaria* species are widely distributed in the soil and are pervasive in humid and semi-arid regions, surviving in extreme climatic conditions. Some strains produce survival structures due to adverse conditions, such as crop, climate and chemistry composition, while others need stimulation for sporulation and infecting growing plants (Troncoso-Rojas and Tiznado-Hernández, 2014; López et al., 2016).

A. alternata has been isolated from different plant crops and has been reported as a contaminant in fruits such as tomatoes (Yang et al.,2017), pears (Pan et al., 2017), apple (Estiarte et al., 2017), citrus (Gabriel et al., 2017), *Hylocereus undatus* and *Selenicereus megalanthus* (dragon fruit) (Castro et al., 2017 and Vilaplana et al., 2017). It causes pre-and post-harvest decay (Armitage et al., 2015; Cabral et al., 2016) and important economic and industrial losses (Estiarte et al., 2016).

Moreover, such contamination progressively worsens as food maturity and storage time increase (Troncoso-Rojas and Tiznado-Hernández, 2014). Although *H. undatus* (Haw) fruits which are infested are not consumed, deterioration ensues in the epidermis and pulp with no visible symptoms, which leads many consumers to discard the epidermis and consumes the pulp. This is of great concern to human health in consuming foods with a possible presence of mycotoxins (FAO, 2017).

A. alternata is involved in the production of mycotoxins in foods, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN), tenuazonic acid (TeA), and altertoxins (ATX) (Troncoso-Rojas and Tiznado-Hernández, 2014; López et al., 2016). Although studies have shown that *Alternaria* prevalence is low in various by-products, as tomatoes, dried figs, sunflower seeds and wine (López et al., 2016), adverse factors such as climate, preharvest conditions differing from regions, states and countries may affect the production and quantities of these metabolites. The genus it is notable for producing a wide range of mycotoxins with different degrees of

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toxicity (Vaquera et al., 2016).

The presence of mycotoxins in foods is of concern to human and animal health. Furthermore, the deterioration and the occurrence of mycotoxins can lead to economic losses and food safety issues (Sivagnanam et al., 2017). Therefore, the determination of these mycotoxins in dragon fruit, which are widely consumed in countries like China, Malaysia, and Brazil, is extremely important for food safety and food quality. The objective of this study was to evaluate the mycotoxigenic potential of *A. alternata* isolated from *Hylocereus undatus* (Haw.) fruit, as well as *A. alternata* ATCC 46582, *in vitro* and *in vivo*, an 8 d shelf life study.

2. Material and methods

2.1. Chemicals and reagents

A. alternata isolate and *A. alternata* ATCC 46582 cultivated in potato dextrose agar (PDA) (Merck, Darmstadt, Germany). Acetonitrile (≥99.9 - Fluka, Castellar del Vallès, Barcelona, Spain) and water with 0.1% formic acid (Sigma, St. Louis, MO, USA) were used for extraction and LC–MS analysis. Ultrapure-grade water was obtained from a MilliQ water purification system (Millipore, Billerica, MA, USA).

2.2. Fungal strains and growth conditions

Two strains of *A. alternata* were used for this study: *A. alternata* isolated from *H. undatus* (Haw.) fruit collected in Marialva, Paraná, Brazil (coordinates: 23°46′35.51″S, 51°79′71.10″W), in March 2015 (Castro et al., 2017) and *A. alternata* ATCC 46582 donated by the Oswaldo Cruz Foundation (FIOCRUZ) in Rio de Janeiro, Brazil, which was used as a positive control. The strains were grown in potato dextrose agar (PDA) and incubated at 28 °C for 10 d, then extracted and used for *in vitro* experimental assays. The identification of the isolated strain was performed and confirmed from a previous study, in accordance with Castro et al., (2017).

2.3. In vitro mycotoxin extraction

The *A. alternata* isolates and ATCC 46582 were cultured in PDA at 28 °C for ten d. After incubation, three slides of each strain were cut into 1 × 1 cm cubes using a sterile scalpel, transferred to sterile falcon centrifuge tubes, and kept at −80 °C for three h. The subcultured hyphae were transferred to Erlenmeyer flasks and 60 mL of acetonitrile/water/formic acid (84:16:1, v/v/v) was added (López et al., 2016). The extract was sonicated for 60 min, then agitated in a Shaker agitator for 1 hour at 28 °C and 150 rpm. The extract was then filtered through a qualitative filter with a rotary evaporator vacuum pump (TE-210, Tecnal, Brazil) and centrifuged for 20 min at 13,000 rpm. The supernatant was diluted in ultrapure water (1:1) in vials, and stored at −20 °C until injection (Ntasiou et al., 2015, with modifications). The extraction was carried out under light protection.

2.4. In vivo inoculation in Hylocereus undatus (Haw.) fruit

For the *in vivo* assay, *H. undatus* (Haw.) fruit (35 d of growth after flowering) were collected in Marialva, Paraná, Brazil (23°46′35.51″S, 51°79′71.10″W), then selected, washed and sanitized with 1% sodium hypochlorite. Using a sterile scalpel, uniform cuts 2 mm deep and 5 mm wide were made in the epidermis of the fruit in the equatorial region. Conidia were collected in a sterile saline solution after 10 d of incubation. A quantity of 10 µl of a conidial suspension containing isolated *A. alternata* and *A. alternata* ATCC 46582 (5×10^4 conidia/mL) was then inoculated into each cut. The fruit were stored at 25 °C (± 3 °C) for 8 d, which is its estimated shelf life under natural conditions (Mizrahi and Nerd, 1999; Castro et al., 2017). Four fruit were used for each strain, including the control group (not inoculated), giving a total of 12 fruit. We followed the methodology used by Feng et al., (2011), with modifications. *A. alternata* strains caused visible deterioration and these sites were later used for *in vivo* extraction.

2.5. In vivo mycotoxin extraction

Contaminated *H. undatus* (Haw.) fruit with visible deterioration were submitted to the extraction method used by López et al. (2016), with modifications. A quantity of 2.5 g of the deteriorated fruit epidermis, in sections of 0.5 × 0.5 cm, were extracted using 10 mL of acetonitrile/water/formic acid (84:16:1, v/v/v). The mixtures were agitated for 1 h in a Shaker agitator at 25 °C and 185 rpm, then centrifuged for 10 min at 3000 rpm. A 6 mL quantity of the supernatant of the extracts was filtered using a 0.22 µm PVDF filter vial, with a 33 mm diameter for injection. Extraction was carried out under protection from light.

2.6. UHPLC-HRMS method

For *A. alternata* mycotoxin analysis, aliquots of the extracts were analyzed by UHPLC-HRMS using an Nexera X2 ultra-high performance liquid chromatography system. The system was equipped with 2 LC-30AD pumps and a Shimadzu XR-ODSIII (150 × 2 mm) column and maintained at 40 °C with a linear gradient elution, using water (0.1% formic acid) (A) and acetonitrile (0.1% formic acid) (B) as solvents, both of LC–MS grade purity. The chromatographic separation was performed in 20 min.

The gradient used was: 1 min., 95% solvent A and 5% solvent B; 10 min., 50% solvent A and 50% solvent B; 12 min., 5% solvent A and 95% solvent B; 13 min., 5% solvent A and 95% solvent B; 17 min., 95% solvent A and 5% solvent B; and 20 min., 95% solvent A and 5% solvent B. The flow was maintained at 0.20 mL min^{-1} throughout the entire chromatographic separation.

A Q-tof Impact II model (Bruker, Germany) mass spectrometer with an electrospray ionization source was used, in AutoMS/MS acquisition mode. The three most intense ions of each chromatographic peak were selected. The acquisition rate was 5 Hz (MS and MS/MS), and the tuning was in the *m/z* 70–1200 range. The mass spectra were collected using ESI in positive and negative-ion mode, with capillary voltage set at 4.50 kV, a source temperature of 200 °C, and an 8 L min^{-1} desolvation gas flow rate. The product ion scans (daughter scans) were performed using collision-induced dissociation (CID) from a collision energy ramp in the 15–40 eV range and 3.06 10^{-3} mBar collision gas pressure in the collision chamber.

The ion chromatogram and MS and MS/MS spectra were visualized using Data Analysis 4.3 software, then compared to the existing literature and analyzed using a free-access mass spectrometry database, such as the Human Metabolome Database (Wishart et al., 2013), and the LIPID Metabolites and Pathways Strategy (LIPID MAPS®), (Fahy et al., 2009).

3. Results

3.1. In vitro mycotoxin extraction

The production of secondary metabolites by the isolated *A. alternata* was greater than that of *A. alternata* ATCC 46582 in *in vitro*, identified by positive-ion mode analysis (Table 1). The AOH, AME, TEN, ATX I, ATX II and ATX III mycotoxins were identified for the isolated *A. alternata* strain in *H. undatus* (Haw.) fruit. For *A. alternata* ATCC 46,582 (Table 1), ATX I and ATX II were identified in positive-ion mode.

Secondary metabolites were identified and confirmed using criteria from literature and a free access mass spectrometry database, the Human Metabolome Database (HMDB). The presence of AME (Lau et al., 2003), TEN (López et al., 2016), ATX I (Kjer, 2009), and AOH (HMDB, ID: HMDB30831, Wishart et al., 2013) was confirmed.