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Influence of environmental conditions of atoxigenic *Aspergillus flavus*HFB1 on biocontrol of patulin produced by a novel apple contaminant isolate, *A. terreus*HAP1, in vivo and in vitro

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ABSTRACT

Patulin (PAT) is a mycotoxin produced in food and feed stuff by several filamentous fungi causing sever hazard for the human being. A novel fruit contaminant strain, *A. terreus*HAP1, was isolated from Egyptian apples. Screening study showed that *A. terreus*HAP1 produced a significant amount of PAT in its culture broth. Three atoxigenic strains of *A. flavus*, isolated from Egyptian fruits, were tested as biocontrol agents against *A. terreus*HAP1. The results showed that *A. flavus*HFB1 was the most potential agent for controlling of pathogen growth. The biocontrol activity of this strain was tested for reduction of patulin produced by *A. terreus*HAP1 in culture medium and in apple fruits under different environmental conditions. The maximum percentage of mycotoxin reduction was recorded when 1×10^6 spores of pathogen was co-cultured with 1×10^8 spores of antagonist in Potato Dexstrose Broth (PDB) medium adjusted at pH 5 and incubated at 30 °C for 15 days. In vivo study, *A. flavus*HFB1 reduced 62.43 ± 4.34% and 58.53 ± 4.6 of PAT in Egyptian and golden apples, respectively, whereas the rotting lesion increased in Egyptian apples faster than the other cultivars. The maximum reduction of patulin accumulation without apples decaying was detected when the Egyptian apples were pre-treated with 1×10^8 spores of *A. flavus*HFB1 at 36 h earlier their infection with *A. terreus*HAP1 and stored at 15 °C for 20 days. The fruits totally decayed after 15 days of storage at 30 °C whereas they decayed totally after 55 days of storage at 15 °C.

1. Introduction

Patulin (PAT) (4-hydroxy-4H-furo[3,2-*c*] pyran-2(6H)-one) is polyketide secondary metabolite produced by various pathogenic fungi. It is one of the most important mycotoxins that causes major issue for food leading to serious health hazard for humans such as nausea, lung congestion, convulsions and epithelial cell degeneration (Sant'Ana et al., 2008). *Penicillium* spp. especially *P. expansum* is the most commonly genus responsible for patulin production in culture media and in contaminated fruits (Frisvad et al., 2004). Other fungal genera including *Aspergillus, Byssochlamys, Gymnoascus* and *Paecilomyces* have been recorded as patulin producer species (Moake et al., 2005). In addition to *P. expansum*, other species such as *A. terreus*, *A. clavatus*, *A. giganteus*, *A.* and *Byssochlamys nivea* can produce patulin in culture filtrate and in contaminated food (Reddy and Reddy, 1988; Varga et al., 2007; Houbraken et al., 2006; Artigot et al., 2009).

Several fruits such as apples, peaches, pears, grapes and apricots are exposed to contamination with mycotoxigenic fungi either pre-harvest or post-harvest. These fruits and their derived products are naturally contaminant with PAT (Sant'Ana et al., 2008). *A. terreus* is considered one of the most common fungi which infects different types of food and fruits. It has the capability of producing various types of mycotoxins including patulin, citrinin and terretonin (Reddy and Reddy, 1988; Li et al., 2005). These mycotoxins can contaminate the human by direct or indirect consumption of contaminant foods and food products (CAST, 2003). Patulin is most frequently found as a natural contaminant of apples and derived products. It has also been detected in mouldy parts of various fruits such as pears, apricots, peaches and grapes (Cunha et al., 2014).

Control of fungal contamination is a critical means of avoiding the risks of mycotoxins. Elimination of PAT from food and feed stuff has gained profound attention. The use of chemical and physical methods for PAT detoxification have many disadvantages including low efficiency, highly cost, and different changes in the chemical and physical properties of food and food products (Sant'Ana et al., 2008). Control of patulin by these ways has been increasingly ineffective because of the presence of resistant strains to antifungal compounds and stability of patulin during thermal processing. Biological control of pathogenic

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fungi using different species of microorganisms has been used as an alternative method (Cazorla and Mercado-Blanco, 2016). The use of beneficial species of bacteria, yeasts and filamentous fungi as biocontrol agents against pathogenic fungi are considered a hopeful project to substitute chemical treatment (Chulze et al., 2015). Biocontrol of various plant pathogens using fungi is an exciting and rapidly developing research area. Some of the obvious advantages of using fungi in biocontrol is their ubiquitous nature and genetic diversity. The latter characteristic can provide a number of biocontrol agent from single species of fungi. Many *Aspergillus* species including *A. flavus*, *A. white*, *A. parasiticus* and *A. niger* were reported early to be able to degrade mycotoxins (Wu et al., 2009). The utilization of atoxigenic strains of *A. flavus* as biological controls to eliminate mycotoxin contamination has been performed from two decades (Dorner, 2004; Dorner et al., 2010). It has been successfully used for elimination of different mycotoxins in both laboratory and field trials (Atehnkeng et al., 2008). Yin et al. (2008) screened more than 30 non-toxigenic strains of *A. flavus* for reduction of *Aspergillus* populations by up to 99% in the agriculture soil. Atehnkeng et al. (2008) showed that non-toxigenic strains of *A. flavus* reduce aflatoxin concentrations in both laboratory and field trials by 70–99%. The application of nontoxigenic strains depends on their competitively exclusion of toxigenic strains in the same niche and competition for foodstuff substrates (Jane et al., 2012). Therefore, the biocontrol strains should be predominant in the same environments which are susceptible to be infected by the toxigenic strains (Yin et al., 2008).

This study aims to investigate the biocontrol efficacy of atoxigenic *A. flavus*HFB1 against PAT produced by *A. terreus*HAP1 in culture filtrate and in apple fruits. Also, it aims to investigate the most favorable environmental conditions for maximum inhibition of PAT produced by *A. flavus* in both culture medium and in apple fruits. This is the first report about biocontrol of patulin produced by *A. terreus* using atoxigenic *A. flavus*.

2. Material and methods

2.1. Chemicals and solvents

Potato Dextrose Agar (PDA), czapek yeast extract agar and yeast extract were obtained from Sigma Aldrich, Lyon, France. Patulin standards, methanol, ethyl acetate and sodium sulfate anhydrous were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were of HPLC grade. The water was double distilled with Millipore water purification system (Bedford, MA).

2.2. Fruit

The fruit of apples were collected, according to their maturity, homogeneous size, absence of injuries and color, from different local markets in Cairo stat, Egypt.

2.3. Fungal strains

The toxigenic fungal strains used in this study were isolated from different naturally infected, apple, peach, strawberry, bear fruits traded in Egypt. All strains were isolated and maintained on potato dextrose agar (PDA). The fungal isolates were morphologically identified according to Raper and Fennel (1965). Morphological identification of antagonist strain and patulin producer strain was confirmed by molecular analysis of 18S rRNA–28S rRNA (flanking the sequence of ITS1, 5.8S rRNA, and ITS2). The ITS1 and ITS2 and the inverting 5.8S coding rDNA were amplified by PCR using the ITS1 and ITS4 primers as recorded by White et al. (1990). The retrieved sequences of *A. terreus*HAP1 and the antagonists *A. flavus*HF-B1 were deposited in the GenBank under accession numbers, KU646829 and KY417158, respectively. Spore suspensions were prepared from 7-day-old cultures by flooding the fungal slants with sterile distilled water containing 0.05% (v/v) Tween 80. The concentrations of *A. terreus* spores were determined by hemocytometer and adjusted to 1×10^7 spores per ml.

2.4. Patulin estimation

Patulin was estimated qualitatively by TLC analyses. The dried crude extract was dissolved in absolute ethanol and loaded with reference standard patulin (PAT) solution on aluminum-backed silica gel 60 F254 plates. The chromatography was performed at room temperature in glass tanks using toluene/ethyl acetate/formic acid 5:4:1 (v/v/v) as developing system. Plates were then sprayed with a 2% phenylhydrazine hydrochloride solution dissolved in sterilized distilled water, heated at 120 °C for 15 min and visualized under 360 UV light. Patulin analysis was done by HPLC as described by MacDonald et al. (2000) with slight modifications. The HPLC system consisted of Waters Binary Pump Model 1525, Model Waters 1500 Rheodyne manual injector and Waters 2475 Multi Wavelength Fluorescence Detector (Waters Pacific Pte Ltd, Science Park Road, Singapore). The UV detector set at 276 nm was connected to a data integration system and a 250- × 4.6-mm internal diameter, 5 µm Supelco LC18 column. The mobile phase was acidified water/ acetonitrile 9:1 (v/v) with a flow rate of 1 ml/min and a total run of 15 min. Standard serial solutions of patulin in acidified water were injected and peak areas were determined to generate standard curve data for quantitative analyses.

2.5. Extraction and determination of aflatoxins (AFs)

Aflatoxins were extracted from *A. flavus* isolates according to the method described by El-Banna et al. (1987). Extraction was carried out using 20 ml of chloroform (twice with 10 ml media), and homogenization for 3 min in a separation funnel. The chloroform phase was filtered through filter paper Whatman No. 3 and concentrated to dryness under a nitrogen stream. The samples were resolved in 3 ml of chloroform and used for screening at Thin Layer chromatography (TLC) plate according to Haddon et al. (1971). The extracted sample was spotted side by side with the aliquots of standard aflatoxins B1, B2, G1 and G2 on a TLC plate precoated with silica gel GF245. The plate was developed in solvent system, chloroform-acetone (9:1 v/v). TLC plate was dried and then examined under 365 nm UV lamp. The sample spots were compared with that of the standard aflatoxins. The HPLC analysis, with mobile phase water: methanol: acetonitrile (240:120:40 v/v/v), was used to confirm the production of AFs. The fluorescence detector was operated at wavelength of 360 nm for excitation and 440 nm for emission. The amounts of AFs in samples were determined from the standard curve using peak area for quantitation

2.6. Antagonistic activity assay in vitro

The antagonistic activity of *A. flavus*HF-B1 was tested by Dual culture technique as previously described (Huang and Hoes, 1976) with slight modification. The pathogenic organism was grown with the antagonistic organisms on the same PDA plate. Place a 9 mm mycelial disc cut from the margin of the actively growing colonies of pathogenic culture (*A. terreus*HAP1) near the periphery on one side of the PDA plate. The antagonistic strains were streak on the other side of the same plate, at an angle of 180 °C from the pathogen. The percentage inhibition of mycelial growth was determined by the per formula of Dar et al. (2013) as following: % growth Inhibition = Growth of pathogenic - Growth of pathogenic organism in control plate organism in presence of test organism × 100 divided by Growth of pathogenic organism in control plate.

2.7. In vitro, influence of culture conditions on biocontrol activity

Spore suspensions of *A. flavus*HFB1 and *A. terreus*HAP1 were