International Journal of Food Microbiology 289 (2019) 145–153

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterization of *Aspergilli* from dried red chilies (*Capsicum* spp.): Insights into the etiology of aflatoxin contamination

Pummi Singh[a], Peter J. Cotty[a,b,*]

[a] School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA
[b] USDA-ARS, 416 W Congress St, First Floor, Tucson, AZ 85701, USA

ARTICLE INFO

Keywords:
Dried red chili
Aspergillus
Aflatoxin
A. cicutus
A. occiafricanus

ABSTRACT

Aflatoxins are toxic carcinogens produced by several species of *Aspergillus* section *Flavi*, with some aflatoxin producers associated with specific crops. Red chilies (*Capsicum* spp.) are grown in warm regions that also favor aflatoxin-producers. Aflatoxins in red chilies may result in serious health concerns and severe economic losses. The current study sought to gain insight on causal agents of aflatoxin contamination in red chilies. Naturally contaminated chilies from markets in Nigeria (n = 55) and the United States (US) (n = 169) were examined. The *A. flavus* L strain was the predominant member of *Aspergillus* section *Flavi* (84%) in chilies. Highly toxigenic fungi with S strain morphology were also detected in chilies from both countries (11%), followed by *A. tamarii* (4.6%) and *A. parasiticus* (0.4%). Fungi with L morphology produced significantly lower quantities of aflatoxins (mean = 43 μg g^{-1}) compared to S morphology fungi (mean = 667 μg g^{-1}; $p < 0.01$) in liquid fermentation. Eighty-one percent of S morphology fungi from chilies in US markets produced only B aflatoxins, whereas 20%, all imported from Nigeria, produced both B and G aflatoxins; all S morphology fungi from Nigerian chilies produced both B and G aflatoxins. Multi-gene phylogenetic analyses of partial gene sequences for nitrate reductase (*niaD*, 2.1 kb) and the aflatoxin pathway transcription factor (*aflR*, 1.9 kb) resolved *Aspergilli* recovered from chilies into five highly supported distinct clades: 1) *A. parasiticus*; 2) *A. flavus* with either L or S morphology; 3) *A. minisclerotigenes*; 4) previously reported unnamed taxon S_{BG} erected as *A. occiafricanus* sp. nov., and 5) a novel taxon erected as *A. cicutus* sp. nov. *Aspergillus cicutus* and *A. occiafricanus* produced the highest concentrations of total aflatoxins in chilies, whereas *A. flavus* L strains produced the least. The results suggest etiology of aflatoxin contamination of chili is complex and may vary with region. Knowledge of causal agents of aflatoxin contamination of chilies will be helpful in developing mitigation strategies to prevent human exposure.

1. Introduction

Aflatoxins are hepatocarcinogenic metabolites produced by several *Aspergilli*, which frequently contaminate food and feed crops including maize, groundnut, cottonseed, spices, and tree nuts (Doster et al., 2014; Kachapulula et al., 2017). Of the four major aflatoxins, B_1, B_2, G_1 and G_2, aflatoxin B_1 is the most toxic, and is carcinogenic to both humans and animals (IARC, 2002). Most developed nations stringently enforce aflatoxin regulatory limits within food and feed (e.g. US regulates aflatoxins at 20 μg/kg total aflatoxins in human food) resulting in significant economic losses to growers (Robens and Cardwell, 2003; van Egmond et al., 2007). Across regions of the globe where regulations are either lacking or are not strictly enforced, aflatoxin contamination exposes humans and animals to severe health risks. Sub-lethal concentrations are associated with stunted development (Khlangwiset et al., 2011), immune suppression (Turner et al., 2003), and liver cancer (Liu and Wu, 2010), whereas high levels can cause liver cirrhosis followed by rapid death (CDC, 2004).

Aspergillus flavus is the most frequently implicated causal agent of aflatoxin contamination of crops (Klich, 2007). The species can be divided into two major morphotypes known as the L and S strains. The S strain isolates produce copious amounts of small sclerotia (< 400 μm) and fewer conidia, whereas the L strain isolates produce sparse amounts of large sclerotia (> 400 μm) but abundant conidia (Cotty, 1989). The L strain produces variable quantities of aflatoxins, and isolates can either be atoxigenic or produce moderate to high levels of aflatoxins; however, the S strain of *A. flavus* and other members of section *Flavi* with S morphology are known to consistently produce high concentrations of aflatoxins (Cotty, 1989; Cotty and Cardwell, 1999; Probst et al., 2010). Aflatoxin contamination can start before harvest when crops are

* Corresponding author at: USDA-ARS, 416 W Congress St, First Floor, Tucson, AZ 85701, USA.
E-mail address: Peter.Cotty@ARS.USDA.GOV (P.J. Cotty).

https://doi.org/10.1016/j.ijfoodmicro.2018.08.025
Received 19 May 2018; Received in revised form 22 August 2018; Accepted 24 August 2018
Available online 27 August 2018
0168-1605/

infected by resident *Aspergilli* dispersed by various mechanisms. Crop infection is facilitated by plant stress (physical damage, by insects, drought) and warm temperatures (above 28 °C) (Cotty et al., 2008). Contamination continues after crop maturation in the presence of conducive conditions, both in the field, and post-harvest during storage, processing or transportation (Cotty et al., 2008; Marín et al., 2009). Since conidia of *Aspergilli* are air-borne and ubiquitous, new infections may occur during post-harvest stages, exacerbating contamination levels (Cotty et al., 1994).

Red chili (*Capsicum* spp.), a member of the nightshade family of Solanaceae, is a globally consumed spice. *Capsicum* was domesticated in America about 6000 years ago and has now spread into Asia, Africa and Europe (Perry et al., 2007). Chilies are mainly cultivated in warm regions that provide suitable conditions for crop infection by *Aspergillus* propagules and subsequent contamination with aflatoxins. India, China and Thailand produced most of the world's dried red chilies during the past decade (FAOSTAT, 2017). Post-harvest practices and conditions during growth render the spice susceptible to aflatoxin contamination. Contaminated chilies can result in loss of lucrative European and US markets where aflatoxins are stringently regulated (European Spice Association, 2004; Yu, 2012). Consumption of aflatoxin contaminated chilies has recently been associated with gall bladder cancer in Bolivia, Chile and Peru (Asai et al., 2014; Nogueira et al., 2015). Aflatoxin contamination is a problem in major chili producing regions (Reddy et al., 2001; Shamsuddin et al., 1995; Singh and Cotty, 2017), yet *Aspergillus* section *Flavi* associated with chilies and the etiology of chili contamination with aflatoxins have not been examined in detail. Structure of fungal communities associated with crops is an important determinant of the severity of aflatoxin contamination (Cotty et al., 2008; Probst et al., 2010), since higher incidences of aflatoxin-producers result in increased average aflatoxin-producing potentials of fungal communities, leading to overall high contamination (Cotty et al., 2008).

A previous study evaluated aflatoxin contamination of dried red chilies between the US and Nigeria, two markets differing in regulation enforcement (Singh and Cotty, 2017). Aflatoxin concentrations and fungal load were significantly higher in Nigerian chilies compared to those from US markets. The current study sought to understand communities of *Aspergillus* section *Flavi* associated with dried red chilies and to obtain insight into the etiology of contamination of dried red chilies. Our objectives were to (i) relate *Aspergillus* section *Flavi* in chilies from markets in the US and Nigeria to previously described members of section *Flavi*, and (ii) to determine the relative importance of each as etiologic agents of contamination. The acquired knowledge on aflatoxin-producing fungi associated with red chilies may be helpful in devising aflatoxin management strategies for the spice.

2. Materials and methods

2.1. Sampling

Dried red chili was collected from markets in the US and Nigeria, as reported previously (Singh and Cotty, 2017). Briefly, 169 chili samples, sealed in airtight packets were purchased in the US from retail markets including supermarkets and ethnic groceries, where chilies were kept at ambient room temperature throughout storage. The retail stores were sampled in Arizona (n = 64), California (n = 68), Minnesota, (n = 3), and New York (n = 34) during 2014–15 as representative locations for the US. US samples consisted of whole (n = 60, mean = 250 g), ground (n = 78, mean = 200 g) and crushed chili (n = 12, mean = 150 g), and paprika (n = 19, mean = 180 g). Fifty-eight percent of chili samples collected from US markets were imported (Singh and Cotty, 2017). Nations of origin for the imported chili samples are listed in Table 1S. Only whole red chilies were collected in Nigeria (n = 55, mean = 70 g), primarily from rural, small-scale markets in Kaduna (n = 50) and Lagos (n = 5) states during 2015–16. Nigerian samples were imported to the USDA-ARS laboratory in the School of Plant Sciences at the University of Arizona, Tucson, under permits issued by the USDA Animal and Plant Health Inspection Service (APHIS) within a week of purchase.

2.2. Fungal isolation and characterization

Whole chili samples were dried (forced air oven, (40 °C)) to below 8% moisture content and sealed in plastic bags; ground chili, crushed chili and paprika were sealed in plastic bags immediately after receipt. After bagging, samples were stored at room temperature. After drying, whole and crushed chili samples were finely ground in a laboratory mill (Retsch Grindomix GM200, Newtown, PA) for 30 s at 10,000 rpm prior to fungal isolation.

In a previous study (Singh and Cotty, 2017), fungi belonging to *Aspergillus* section *Flavi* were recovered from the above samples by dilution plate technique on modified rose Bengal agar (Cotty, 1994a). Fungi isolated in Singh and Cotty, 2017 were assigned to morphological groups (S morphology, *A. flavus* L morphotype, and *A. parasiticus*) using colony characteristics, sclerotia and spore morphology (Cotty, 1989; Klich and Pitt, 1988). Fungal isolations were performed at least twice from each chili sample. All fungal isolates were subjected to dilution plating on malt agar (1% malt, 2% agar, 1000 ml of water) followed by incubation at 31 °C for 48 h. At dilutions providing < 10 colonies per plate, discrete colonies were transferred to 5–2 agar (5% V-8 juice; 2% agar; pH 6.0) and incubated at 31 °C for 5–7 days in dark. Fungal isolates were stored as plugs of sporulating culture in sterile distilled water (2 ml) and used as working cultures for conducting aflatoxin and phylogenetic analyses.

2.3. Screen for aflatoxin producers

Aflatoxin production was evaluated for both L and S morphology isolates recovered from chilies. The L morphology fungi (n = 130) were randomly selected with at least one isolate from each chili sample positive for *Aspergillus* section *Flavi*. Fungi with S morphology (n = 75) were randomly selected from 30 Nigerian and 5 US chili samples from which S morphology fungi were recovered. At least 1 isolate from each of the 35 samples positive for S morphology fungi was included. Fungi were evaluated in a chemically-defined aflatoxin production liquid medium (Mateles and Adye, 1965) supplemented with 22.5 mM urea as the sole nitrogen source (Cotty and Cardwell, 1999; Probst et al., 2012). Fungal inoculum for each isolate was prepared as described previously (Probst et al., 2012). Erlenmeyer flasks containing 70 ml of the liquid medium were seeded with conidial suspensions (10^6 conidia ml^{-1}), covered with stoppers that allow gas exchange, and incubated with agitation in dark for 5 days (31 °C, 160 rpm). Fermentations were terminated by addition of acetone (70 ml acetone per 70 ml fermentation) and swirled to allow mixing. Cultures were allowed to sit for at least one hour to allow for lysis of fungal cells and release of aflatoxins contained in the mycelia. Acetone extracts were directly spotted onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) and separated adjacent to aflatoxin standards (Aflatoxin Mix Kit-M; Supelco, Bellefonte, PA). Plates were developed in a solution of ethyl ether-methanol-water (96:3:1), air-dried, and aflatoxins were visualized under 365-nm UV light. Total aflatoxins were quantified directly on TLC plates using a scanning densitometer (TLC Scanner 3, Camag Scientific Inc., Wilmington, N.C.). Filtrates initially negative for aflatoxins were partitioned twice with dichloromethane and concentrated prior to quantification (limit of detection 104 μg/kg mycelia) as previously described (Cardwell and Cotty, 2002). Mycelial mass from the fermentation was captured during vacuum filtration on Whatman No. 1 filter paper and dried (40 °C, 48 h) in a forced air oven. Aflatoxin concentrations were expressed as μg total aflatoxin per g mycelium.

2.4. DNA isolation and gene amplification

DNA extraction, PCR amplification and sequencing for fungi