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# Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa

C. Probst [a], R. Bandyopadhyay [b], P.J. Cotty [a,c,*]

[a] The University of Arizona, School of Plant Sciences, Tucson 85721, USA
[b] International Institute of Tropical Agriculture (IITA), PMB 5320, Ibadan, Nigeria
[c] USDA-ARS, The University of Arizona, School of Plant Sciences, Tucson 85721, USA

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ABSTRACT

Crops frequently contaminated by aflatoxins are important sources of revenue and daily nourishment in many portions of sub-Saharan Africa. In recent years, reports have associated aflatoxins with diminished human health and export opportunities in many African Nations. Aflatoxins are highly carcinogenic metabolites mainly produced by members of *Aspergillus* sect. *Flavi*. The current study examined aflatoxin-producing fungi associated with maize grain intended for human consumption in 18 sub-Saharan African countries. 4469 *Aspergillus* sect. *Flavi* isolates were obtained from 339 samples. The majority (75%) of isolates belonged to the L strain morphotype of *A. flavus*. Minor percentages were *A. tamarii* (6%), *A. parasiticus* (1%), and isolates with S strain morphology (3%). No *A. bombycis* or *A. nomius* isolates were detected. Phylogenetic analyses of partial sequences of the nitrate reductase gene (*niaD*, 1.3 kb) and the aflatoxin pathway transcription factor gene (*aflR*, 1.7 kb) were used to verify isolate assignments into species and lineages. Phylogenetics resolved S strain isolates producing only B aflatoxins into two lineages fully supported by sizes of deletions in the gene region spanning the aflatoxin biosynthesis genes *cypA* (aflU) and *norB* (aflF). One lineage was the *A. flavus* S strain with either 0.9 or 1.5 kb deletions. The second lineage, recently described from Kenya, has a 2.2 kb deletion. Taxa with S strain morphology differed in distribution with strain $S_{BG}$ limited to West Africa and both *A. minisclerotigenes* and the new lineage from Kenya in Central and East Africa. African *A. flavus* L strain isolates formed a single clade with L strain isolates from other continents. The sampled maize frequently tested positive for aflatoxins (65%), fumonisins (81%), and deoxynivalenol (40%) indicating the presence of fungi capable of producing the respective toxins. Percentage of samples exceeding US limits for total aflatoxins (regulatory limit), fumonisins (advisory limit), and deoxynivalenol (advisory limit) were 47%, 49%, 4%, respectively.



## 1. Introduction

Maize is a staple grown and eaten throughout Africa (McCann, 2005). Mycotoxin contamination of maize increases health risks for both humans and domestic animals (Wild et al., 1991; Peers et al., 1976) and frequently diminishes crop value (Khlangwiset et al., 2011). Aflatoxins are highly toxic with aflatoxin $B_1$ listed by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (International Agency for Research on Cancer, 2002). The risks of human aflatoxicosis is high in countries where strict aflatoxin regulations are not in force and crops are consumed without monitoring (Shephard, 2003; Williams et al., 2004). Reduced growth in children (Gong et al., 2004, 2008), impaired immune systems (Turner et al., 2003), and severe liver damage (Turner et al., 2000) are consequences of dietary aflatoxin exposure. Fumonisins, first reported from moldy maize in South Africa (Syndenham et al., 1990), compromise human (e.g. neural tube defects in newborn children) and animal health by interfering with sphingolipid, phospholipid and fatty acid metabolism. Fumonisin B1 is possibly carcinogenic to humans and was classified by IARC as a Group 2B carcinogen (International Agency for Research on Cancer, 2002). Like fumonisins, deoxynivalenol (DON), also known as Vomitoxin, was first reported from moldy maize grain from southern Africa (Vesonder et al., 1973). Exposure to DON, a type B trichothecene, can cause typical symptoms of food-borne illness including vomiting, diarrhea and dizziness in both animals and humans (Pestka and Smolinski, 2005; Pestka, 2007). DON was implicated in one of the largest outbreaks of human mycotoxicosis in India in 1987 affecting 50,000 people (Bath et al., 1989).

Structures of fungal communities associated with crops heavily influence the severity of aflatoxin contamination (Cotty et al., 2008; Grubisha and Cotty, 2010). The contamination process is complex and starts in the field where crops first become infected by *Aspergilli* that reside in the soil and on decaying plant residues. Plant stress (e.g. physiological stress, insect damage) and an environment conducive to fungal growth (e.g. temperatures above 28 °C) increase susceptibility of crops to infection (Cotty et al., 1994, 2008). Contamination continues after crop maturation when the crop is exposed to warm temperature and

* Corresponding author at: The University of Arizona, School of Plant Sciences, Tucson 85721, USA. Tel.: +1 520 626 675; fax: +1 520 626 5944.
*E-mail address:* pjcotty@email.arizona.edu (P.J. Cotty).

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humidity, both in the field and during storage (Cotty et al., 1994, 2008). Aflatoxin levels may increase and new infections become established until crops are ultimately consumed (Cotty et al., 1994). In order to estimate risks associated with communities of aflatoxin-producing fungi resident on maize both aflatoxin-producing potentials and frequencies of occurrence need to be considered (Cotty et al., 2008; Probst et al., 2010).

Most fungi capable of producing aflatoxins belong to *Aspergillus* sect. *Flavi*. Members of this section vary widely in many characteristics including aflatoxin production and ability to infect and decay crops (Cotty, 1997; Horn, 2007). *A. flavus* is the most common causal agent of contamination (Klich, 2007). The species can be delineated into two major morphotypes, the L strain (produces copious amounts of conidia and relatively few, large sclerotia, average diameter >400 μm) and the S strain (produces few conidia and copious amounts of small sclerotia, average diameter <400 μm) (Cotty, 1989). On average, isolates of the L strain morphotype produce less aflatoxin than isolates of the S strain morphotype (Cotty, 1989; Probst et al., 2010). Atoxigenic (absence of aflatoxin production) isolates of the L strain morphotype are frequently isolated from crops and soils (Atehnkeng et al., 2008; Cotty, 1997). Increased frequencies of atoxigenics reduce the average aflatoxin-producing potential of fungal communities and, as a result, the aflatoxin content of crops (Cotty et al., 2008).

Molecular phylogenetics has increased the number of described species that produce aflatoxins (Feibelman et al., 1998; Ito et al., 2001; Peterson et al., 2001; Ehrlich et al., 2002; Pildain et al., 2008). However, the importance of newly described species to crop contamination remains unclear. Since recent outbreaks of acute human aflatoxicosis in Kenya (CDC, 2004), aflatoxin contamination of food crops in Africa has received increased attention (Integrated Regional Information Networks, 2010; Probst et al., 2010; Atser, 2011). Contamination events resulting in severe aflatoxicoses in Kenya have been attributed to a recently described fungal lineage with S strain morphology within section *Flavi* (Probst et al., 2007). This lineage is distinguished by a 2.2 kb deletion in the aflatoxin biosynthesis gene *cypA*. Aflatoxin-producing fungi with similar morphologies include the S strain of *A. flavus*, the aflatoxin B and G producing species *A. minisclerotigenes*, and an unnamed *Aspergillus* species common in West Africa (commonly referred to as strain $S_{BG}$) (Donner et al., 2010; Probst et al., 2012). Knowledge of the distribution of these aflatoxin producers in sub-Saharan Africa is limited (Cotty and Cardwell, 1999; Cardwell and Cotty, 2002; Kaaya and Kyamuhangire, 2006; Bandyopadhyay et al., 2007; Atehnkeng et al., 2008; Probst et al., 2010) and detailed comparisons among African nations on any single crop are lacking. Even though fungi with S strain morphology are associated with the most deadly known episodes of aflatoxicosis, such taxa have only been sporadically described in Africa and distributions across Africa are largely unexplored. The current study provides insights into both distributions of aflatoxin-producing fungi across the continent and causal agents of aflatoxin contamination of maize of potential regional importance.

The study objectives were: i) to characterize *Aspergillus* sect. *Flavi* communities on maize from countries in East, West and Southern sub-Saharan Africa, ii) to assess relationships of the identified fungi to described aflatoxin-producers, iii) to define distributions of aflatoxin-producing fungi with S strain morphology on maize across sub-Saharan Africa, and iv) to relate characteristics of *Aspergillus* communities to concentrations of aflatoxins in the examined maize. In addition, other mycotoxins (fumonisins and deoxynivalenol) common in maize and of concern for human health were quantified to assess importance of aflatoxins in relation to other mycotoxins.

## 2. Material and methods

### 2.1. Maize samples

Maize samples were collected in Burkina Faso (n = 50), Cameroon (n = 16), DR Congo (n = 22), Ethiopia (n = 81), Ghana (n = 7), Ivory Coast (n = 4), Kenya (n = 22), Malawi (n = 9), Mali (n = 7), Mozambique (n = 42), Rwanda (n = 16), Senegal (n = 20), Sierra-Leone (n = 17), Somalia (n = 21), Tanzania (n = 5), Uganda (n = 17), Zambia (n = 28) and Zimbabwe (n = 19) during 2006 and 2007 (Fig. 1). To ensure that grains were produced locally, maize grain was sampled from individual farmers' fields or small local markets. Generally, a 1 to 2 kg sample from a single location was obtained, mixed thoroughly and a 100 to 200 g subsample was imported to the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), at the University of Arizona, Tucson under permits issued by the USDA Animal and Plant Health Inspection Service (APHIS). Upon arrival, maize samples were weighed, analyzed for water content (HB43 Halogen Moisture Analyzer; Mettler Toledo, Columbus, OH, USA), dried to under 8% moisture in a forced air drying oven (40 °C), if necessary, and stored for up to 4 weeks at 4 °C prior to further analyses. The maize was finely ground in a laboratory hammer mill (IKA Labortechnik, Heitersheim, Germany), and fungal isolates were recovered as described below.

### 2.2. Fungal isolation and quantification

*Aspergillus* sect. *Flavi* colonies were obtained by dilution plate technique on modified Rose Bengal agar (Cotty, 1994). Ground maize (about 1 g) was suspended in 15-ml test tubes containing 5 ml sterile-distilled water and mixed by inverting for approximately 20 min. Aliquots of an appropriate dilution (100 μl per plate) of the resulting suspension were spread on modified Rose Bengal agar plates (n = 3) and incubated for 3 days at 31 °C in the dark. *Aspergillus* sect. *Flavi* colonies were microscopically identified and enumerated [Colony Forming Units (CFU)/g]. Up to 10 discrete colonies per isolation were aseptically transferred to 5/2-agar plates (5% V8-juice, 2% agar, pH 5.2), incubated unilluminated for 5 to 7 days at 31 °C, and identified as to species and, if applicable, strain on the basis of colony, conidia and sclerotia morphologies (Klich and Pitt, 1988; Cotty, 1989; Probst et al., 2012). Fungal isolations were conducted at least twice for each sample to yield a total of 15 to 20 isolates. Representative isolates for each morphotype from each country were subjected to DNA based phylogenetic analyses to verify assignments based on morphological criteria.

### 2.3. In vitro aflatoxin production

Ability to produce either no aflatoxins, B and G aflatoxins, or only B aflatoxins was determined on autoclaved maize grain for the same isolates subjected to phylogenetic analyses utilizing the aflatoxin production assay previously described (Probst and Cotty, 2012). Briefly, healthy, undamaged maize kernels (10 g/250 ml Erlenmeyer flask) were autoclaved for 60 min at 121 °C. Maize water content was determined with a HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH) after sterilization and adjusted to 25% with sterile, ultrapure water. Maize was inoculated with a conidia suspension of the tested isolate ($10^6$ conidia/ml). The quantity of conidia was determined by turbidity using a turbidity meter (Model 965-10; Orbeco-Hillige, Farmingdale, NY) and concentrations were calculated [Nephelometric turbidity unit (NTU) versus colony forming unit (CFU) curve (Y = 49,937X; X = NTU, Y = conidia per ml)]. Inoculated maize was incubated for 7 d at 31 °C in the dark. At the end of the incubation period, maize kernels were washed with 80% methanol (50 ml/10 g maize). The maize–methanol mixture was homogenized in a laboratory grade Waring Blender (seven-speed laboratory blender, Waring Laboratory, Torrington, CT) for 30 s on speed seven, filtered through Whatman No. 4 paper, and aflatoxins were visualized using thin-layer chromatography (TLC) and quantified with a scanning densitometer (TLC Scanner 3, Camag Scientific Inc., Wilmington, NC).