European Journal of Plant Pathology (2005) 112: 283–287
DOI 10.1007/s10658-004-4888-8

Short communication

Relationships between aflatoxin production and sclerotia formation among isolates of *Aspergillus* section *Flavi* from the Mississippi Delta

H.K. Abbas[1], M.A. Weaver[2], R.M. Zablotowicz[2], B.W. Horn[3] and W.T. Shier[4]
[1]*USDA-ARS, CG&PRU, P.O. Box 345 Stoneville, MS 38776, USA (Fax: +1 6626865218; E-mail: habbas@ars.usda.gov);* [2]*USDA-ARS, SWSRU, Stoneville, MS 38776, USA;* [3]*USDA-ARS, NPRL, Dawson, GA 31742, USA;* [4]*College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA*

Accepted 10 October 2004

Key words: Aflatoxins, sclerotia, *Aspergillus flavus, A. parasiticus, A. nomius,* maize, peanut, rice, soil

Abstract

Aspergillus section *Flavi* isolates, predominately *A. flavus*, from different crops and soils differed significantly in production of aflatoxin and sclerotia. About 50% of the isolates from corn, soil and peanut produced large sclerotia, while only 20% of the rice isolates produced large sclerotia. There was a higher frequency of small sclerotia-producing isolates from rice compared to the other sources and isolates that did not produce sclerotia were significantly less likely to be toxigenic than strains that produced large sclerotia.

Aflatoxin contamination is a serious food safety problem throughout the world (CAST, 2003). Several *Aspergillus* species produce aflatoxins (B1, B2, G1 and G2) including *A. flavus*, *A. parasiticus* and *A. nomius* (Hesseltine et al., 1970; Scott, 1987). These fungi are found in food and feed commodities, as well as air-borne dust, soil, plants and insects (Horn and Dorner, 1998; Wicklow et al., 1998; Klich, 2002a). In the southern United States, there are chronic problems with aflatoxin in corn, peanuts and cottonseed (Payne, 1992; Robens and Cardwell, 2003). However, rice is relatively free of aflatoxin contamination, although *Aspergillus* species can often be isolated from it (Abbas et al., 2001).

Among *Aspergillus* isolates there is great variation in aflatoxin production especially within the most common aflatoxin-producing species, *A. flavus*. The importance of aflatoxin in food and feeds, coupled with the large variation in toxigenicity has prompted considerable interest in the intraspecific variability within the species. Subsequently, investigations have been carried out on vegetative compatibility (Papa, 1986; Horn and Green, 1995; Novas and Cabral, 2002), sclerotial production (Hessletine et al., 1970; Saito et al., 1986) and DNA-based profiles (Moody and Tyler, 1990; Keller et al., 1992; Tran-Dinh et al., 1999) and into the relationship between these characters and aflatoxin production. Others have described geographical differences in *A. flavus* and differences in isolates from different ecological niches (Klich and Pitt, 1988; Cotty and Cardwell, 1999). Analysis of restriction fragment length polymorphism patterns of 269 *A. flavus* isolates from maize kernels, soil, insects and air demonstrated a high level of genetic diversity with no clear relationship between subpopulations and source (Wicklow et al., 1998).

The phenotypic variation within *A. flavus* allows the species to be subdivided into groups based on morphology. One such characteristic is sclerotial size, the large strain (L) having sclerotia >400 μm in diameter and the small strain (S) with sclerotia <400 μm (Horn, 2003). The S strain was first recognized by Hesseltine et al. (1970) and was formally described as a variety

(*A. flavus* var. *parvisclerotigenus*) by Saito and Tsuruta (1993). The type culture of *A. flavus* var. *parvisclerotigenus* produces only B aflatoxins, but other S strain isolates produce both B and G aflatoxins (Hesseltine et al., 1970; Geiser et al., 2000; Novas and Cabral, 2002). Geiser et al. (1998 and 2000) stated that the molecular character of *A. flavus* is nonmonophyletic and is separable into two genetic groups that cannot be readily distinguished morphologically. Group I consists of both L and S strains that produce only B aflatoxins and group Π comprises S strains that produce B or B + G aflatoxins.

The relative distribution of the L and S strains in agricultural commodities and soil, and the quantity and type of aflatoxins produced by isolates from various sources has not yet been investigated. This study sought to answer such questions and shed light on the patterns of aflatoxin production and distribution in the soil compared to crops grown on that soil. The aflatoxin producing potential of 517 isolates of *A. flavus* from the Mississippi River Delta region was characterized. This large collection was also evaluated for sclerotial production to characterize any relationships between aflatoxin production, source of isolate and sclerotial type.

Surface soils (0–5 cm) and commodities were collected from commercial and experimental sites under various crop management practices in Mississippi and Arkansas (Table 1). No soils were collected from rice or peanut sites, and only isolates from soils previously cropped in corn were assessed in this study. Soils were diluted in agar solution (2 g l^{-1}) and plated on modified dichloronitroaniline rose bengal (MDRB) agar (Horn and Dorner, 1998). Corn samples (10 ears per site) were collected (Abbas et al., 1988). Depending on sample size, 50 or 100 kernels or seeds from each crop were surface sterilized by soaking in 2.5% sodium hypochlorite for 1 min, rinsed three times in sterile tap water, and transferred to MDRB agar. When possible, 3–5 discrete colonies were selected from each sample from MDRB plates and cultured on potato dextrose rose bengal media (PDA, Difco Laboratories, Detroit, MI). Cultures were maintained on homemade Czapak slants (Horn et al., 1996) and silica gel (Windels et al., 1988). Most of the isolates (99%) were *A. flavus* and the remainder comprised *A. nomius* (2 isolates) and *A. parasiticus* (2 isolates).

Aflatoxigenic potential was analyzed from fungal biomass obtained from 5 day-old PDA cultures with commercially available ELISA kits (''Vertox'', Neogen Corp., Lansing, MI) Abbas et al., 2004). All experiments were carried out at least two times. Aflatoxin recovered from aqueous methanol extracts from fungal biomass represented 58–92% of the total aflatoxin produced in the cultures (data not shown). In atoxigenic isolates no aflatoxin was recovered from the biomass or the medium.

Identification of *Aspergillus* species was based on the morphological criteria of Klich (2002b). To assay for sclerotial production, isolates were transferred to homemade Czapek agar slants and incubated for 2 weeks at 30 °C in darkness (Horn et al., 1996; Horn and Dorner, 1998). S- (<400 μm) and L- (>400 μm) selerotial type of each culture was confirmed by measuring the size of the sclerotia. Briefly, a sample of one hundred sclerotia from each culture was evaluated by measuring sclerotial size using a reticle in a 10× Nikon Stereo Microscope (Nikon Inc., Garden City, NY, USA) connected to SSM-2010, Trinitron Colour Monitor (Sony Corporation, Japan).

Each isolate was identified by source, sclerotial type and the level of aflatoxin production in culture. Isolates were categorized as 'none' if less than

Table 1. Sources of isolates used in present study

Source	Number of isolates	Year	Location
Soil	224	2000, 2001	16 sites in MS[1]
Corn	183	2000, 2001	4 sites in MS[2]
Rice	67	2000	AR (86 samples)[3]
		2001	4 sites in MS (16 samples)
Peanut	43	2002	4 sites in MS[4]
Total	517		

[1] Collected by authors from 12 commercial production sites and 4 experimental sites (Abbas et al., 2004).
[2] Collected by authors from 2 commercial production sites and 2 experimental sites.
[3] Unpolished rice grain samples (n = 86) were obtained from Rick D. Cartwright, Cotton Branch Experimental Station, University of Arkansas, Little Rock, Arkansas. 16 additional unpolished rice samples collected by authors (Abbas et al., 2001).
[4] Provided by Hector Portillo, BASF, Greenville, Mississippi and J. Ethridge, Valent USA Corp., Mid-South Agricultural Research Centre, Greenville, Mississippi.

20 ppb aflatoxin was detected, 'low' if aflatoxin levels were between 20 and 10,000 ppb and 'high' for isolates that produced greater than 10,000 ppb in culture. Differences in aflatoxin production among isolates when sorted by source or by sclerotial type were evaluated for significance by a Chi-Squared test. Similarly, a Chi-Squared test evaluated the distribution of sclerotial types among sources.

The large number of isolates included in this study permitted a comprehensive investigation of the relationships between aflatoxin and isolate source and sclerotial production. The toxigenic potential of isolates and the distribution of sclerotial types differed according to source (Figures 1 and 2). Also, isolates from different sources significantly differed by sclerotial type (Figure 2). Taken together, this would reject a hypothesis that the *A. flavus* population is randomly distributed. Instead, it would seem that in the small geographic area studied, different *A. flavus* populations are preferentially isolated from particular niches. It is often assumed that the primary inoculum for *A. flavus* infection of corn comes from conidia produced by overwintering sclerotia and mycelia in the soil (Payne, 1992). This is not consistent with the observation that among Mississippi Delta *Aspergillus* isolates, the relative frequency of aflatoxin production among corn isolates is different than the frequency of aflatoxin production among soil isolates (Figure 1). Corn had a smaller proportion of high aflatoxin-producing isolates than the soil isolates with no corn isolates producing small sclerotia. This might suggest that either there are other significant sources of primary inoculum or that the virulence of the *A. flavus* isolates on corn differs with toxigenicity and sclerotial types. Furthermore, it is possible that many of the low aflatoxin-producing isolates on corn do not overwinter efficiently in soil. The distribution of aflatoxin-producing isolates might be related to the ecological role of aflatoxin production (Ehrlich, 1987); however, the real role of aflatoxin in the ecological competence has not been fully elucidated.

Overall, *A. flavus* recovered from rice had the lowest incidence of atoxigenic isolates, and the lowest percentage of sclerotial producers. About 25% of unpolished rice collected from Arkansas and Mississippi contained 20–270 ppb aflatoxin (Abbas et al., 2001). In a three-year study of rice from Texas, no aflatoxin was found in one year, but in the other two years about 16% of rice samples contained 2–282 ppb aflatoxin (Schroedder and Boller, 1973). Furthermore, only 20–58% of *Aspergillus* isolates from the Texas rice samples produced aflatoxin *in vitro*. It would be appropriate for future studies to assess the characteristics of

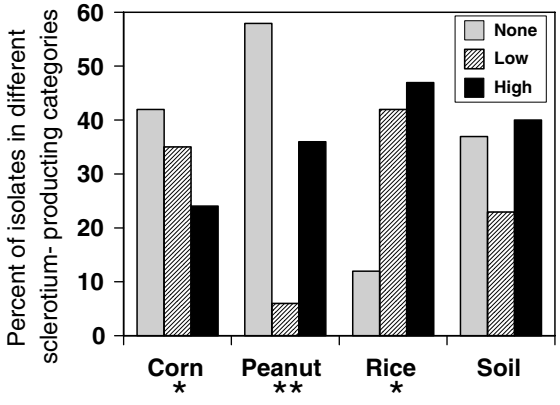

Figure 1. Aflatoxin production by *Aspergillus* isolates, as determined with ELISA and separated by source. Isolates evaluated include: corn = 183; peanut = 43; rice = 67; and soil = 224. *Source is significantly different from the overall distribution at the 0.05 level. **Source is significantly different from the overall distribution at the 0.001 level. None includes isolates producing less than 20 ppb. Low includes isolates producing between 20 and 10,000 ppb. High includes isolates producing 10,000 ppb or more.

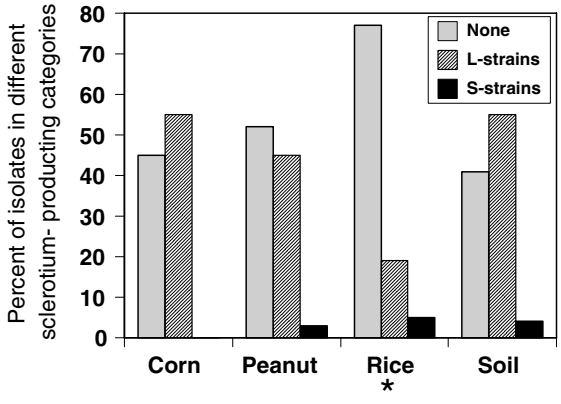

Figure 2. Sclerotial production by *Aspergillus* isolates on Czapek agar medium, separated by source. Isolates evaluated included: corn = 183; peanut = 43; rice = 67; and soil = 224. * Source is significantly different from the overall distribution at the 0.05 level.

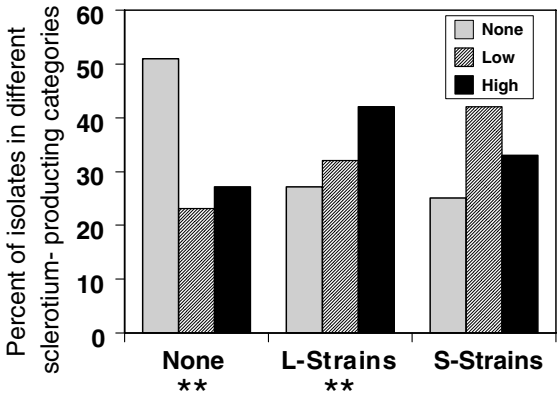

Figure 3. Relationship between aflatoxin and sclerotial production, isolates not producing sclerotia = 244; small sclerotial isolates = 12; and large sclerotial isolates = 261 evaluated. * Distribution of toxin production for sclerotial type is significantly different from the overall distribution at the 0.05 level. ** Distribution of toxin production for sclerotial type is significantly different from the overall distribution at the 0.001 level. None includes isolates producing less than 20 ppb. Low includes isolates producing between 20 and 10,000 ppb. High includes isolates producing 10,000 ppb or more.

Aspergillus Flavi section in rice soils. The management practices, especially flooding, during rice production may affect the microbial community (Reichardt et al., 2001); thus flooding may also have an impact on *Aspergillus* populations.

Previous reports based on the morphological properties of *A. flavus* isolates have grouped them into two classes of sclerotial sizes (Cotty, 1989; Orum et al., 1997). Horn (2003) designated *A. flavus* isolates that produce sclerotia with an average diameter of <400 μm as S strains (small sclerotial producers) and those that produce sclerotia with an average diameter of >400 μm as L strains (large sclerotial producers). In a 3300 km transect from Texas to Virginia, Horn and Dorner (1998) found that the S strain was most abundant only in a limited area of Texas and Louisiana, while the L strain was dominant from Mississippi to Virginia and suggested that the distribution was related to environmental factors.

In this study, overall about 50% of the isolates from corn, soil and peanut produced large sclerotia, while only 20% of the rice isolates produced large sclerotia (Figure 1). The overall distribution of sclerotial types among the rice isolates was significantly different than the overall distribution. There was a non-random relationship between the sclerotial type and the production of aflatoxin; that is, strains that did not produce sclerotia were significantly less likely to be toxigenic than strains that produced large sclerotia. The isolates producing the highest levels of aflatoxin were found with the highest abundance among the isolates with large sclerotia (Figure 3). This differs from the findings of Chang et al. (2001) who found an inverse relationship between aflatoxin and sclerotial production. It also differs from the results of a survey of 70 isolates from Arizona where all isolates produced sclerotia and S-strain isolates produced about ten times more aflatoxin, *in vitro*, than the L-strain isolates (Cotty, 1989).

Acknowledgements

We thank Bobbie Johnson and Patrick Fratesi, CG&PRU, USDA-ARS, Stoneville, MS, for their technical help in this project.

References

Abbas HK, Mirocha CJ, Meronuck RA, Pokorny JD, Gould SL and Kommedahl T (1988) Mycotoxins and *Fusarium* spp. associated with infected ears of corn in Minnesota. Applied and Environmental Microbiology 54: 1980–1933

Abbas HK, Zablotowicz RM, Cartwright RD, Xie W and Shier WT (2001) Occurrence of aflatoxins, fumonisins and the producing fungi in rice (*Oryzae sativa*). Phytopathology (Supplement) 91: S1

Abbas HK, Zablotowicz RM, Weaver, MA, Horn BW, Xie W and Shier WT (2004) Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. Canadian Journal of Microbiology 50: 193–199

CAST (Council for Agricultural Science and Technology) (2003) Mycotoxins: Risks in Plant, Animal and Human systems Task Force Report # 139. Ames, IA USA

Chang PK, Bennett JW and Cotty PJ (2001) Association of aflatoxin biosynthesis and sclerotial development in *Aspergillus parasiticus*. Mycopathologia 153: 41–48

Cotty PJ (1989) Virulence and cultural characteristic of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79: 808–814

Cotty PJ and Cardwell KF (1999) Divergence of West African and North American communities of *Aspergillus* section Flavi. Applied and Environmental Microbiology 65: 2264–2266

Ehrlich K (1987) Effect on aflatoxin production of competition between wild-type and mutant strains of *Aspergillus parasiticus*. Mycopathologia 97: 93–96

Geiser DM, Dorner JW, Horn BW and Taylor JW (2000) The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. Fungal Genetics and Biology 31: 169–179

Geiser DM, Pitt JI and Taylor JW (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proceedings of the National Academy of Science, USA 95: 388–393

Hesseltine CW, Shotwell OT, Smith M, Ellis II, Vandegraft F and Shannon G (1970) Production of various aflatoxins by strains of the *Aspergillus flavus* series. In Proceedings of the First US–Japan Conference on Toxic Microorganisms and the US Department of the Interior, Washington, DC, USA

Horn BW (2003) Ecology and population biology of aflatoxigenic fungi in soil. Journal of Toxicology-Toxins Reviews 22: 355–383

Horn BW and Greene RL (1995) Vegetative compatibility within populations of *Aspergillus flavus, A. parasiticus* and *A. tamarii*, from a peanut field. Mycologia 87: 324–332

Horn BW, Greene RL, Sobolev VS, Dorner JW, Powell JH and Layton RC (1996) Association of morphology and mycotoxin production with vegetative compatibility groups in *A. flavus*, *A. parasiticus*, and *A. tamarii*. Mycologia 88: 574–587

Horn BW and Dorner JW (1998) Soil populations of *Aspergillus* species from section *Flavi* along a transect through peanut-growing regions of the United States. Mycologia 90: 767–776

Keller NP, Cleveland TE and Bhatnager D (1992) Variable electrophoretic karyotypes in *Aspergillus* section *Flavi*. Current Genetics 21: 371–375

Klich MA (2002a) Biogeography of *Aspergillus* species in soil and litter. Mycologia 94: 21–27

Klich MA (2002b) Identification of common *Aspergillus* species. Centralbureau voor Schimmelcultures, Utrecht, Netherlands, 116 pp

Klich MA and Pitt JI (1988) Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Transactions of the British Mycological Society 91: 99–108

Moody SF and Tyler BM (1990) Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* Group: *A. flavus*, *A. par asiticus* and *A. nominus*. Applied and Environmental Microbiology 56: 2453–2461

Novas MV and Cabral D (2002) Association of mycotoxin and sclerotia production with compatibility groups in *Aspergillus flavus* from peanut in Argentina. Plant Disease 86: 215–219

Orum TV, Bigelow DM, Nelson MR, Howell DR and Cotty PJ (1997) Spatial and temporal patterns of *Aspergillus flavus* strain composition and propagule density in Yuma County, Arizona, soils. Plant Disease 81: 911–916

Papa KE (1986) Heterokayron incompatibility in *Aspergillus flavus*. Mycologia 78: 98–101

Payne GA (1992) Aflatoxins in maize. Critical Review of Plant Sciences 10: 423–440

Reichardt W, Briones A, de Jesus R, and Padre B (2001) Microbial population shifts in experimental rice systems. Applied Soil Ecology 17: 151–163

Robens J and Cardwell K (2003) The cost of mycotoxin management to the USA: management of aflatoxins in the United States. Journal of Toxicology-Toxin Reviews 22: 143–156

Saito M and Tsuruta O (1993) A new variety of *Aspergillus flavus* from tropical soil in Thailand and its aflatoxin productivity. Proceedings of the Japanese Association of Mycotoxicology 37: 31–36

Saito M, Tsuruta O, Sinacha P, Kawasugi S, Manabe M and Buangsumon D (1986) Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. Proceedings of the Japanese Association of Mycotoxicology 24: 41–46

Scott PM (1987) Mycotoxins: Review. Journal of Association Official Analytical Chemists 70: 276–281

Schroedder HW and Boller RA (1973) Aflatoxin production of species and strains of the *Aspergillus flavus* group isolated from field crops. Applied Microbiology 25: 885–889

Tran-Dinh N, Pitt JI and Carter DA (1999) Molecular genotype analysis of natural toxigeneic and nontoxingenic isolates of *Aspergillus flavus* and *A. parasiticus*. Mycological Research 103: 1486–1490

Wicklow DT, Mcalpin CE and Platis CE (1998) Characterization of the *Aspergillus flavus* population within an Illinois maize field. Mycological Research 102: 263–268

Windels CE, Burns PM and Kommedahl T (1988) Five-year preservation of *Fusarium* species on silica gel and soil. Phytopathology 78: 107–109