Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Characterization of *Aspergillus flavus* strains from Brazilian Brazil nuts and cashew by RAPD and ribosomal DNA analysis

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Keywords:
Aspergillus flavus, detection, internal amplification control, mycotoxins, PCR, RAPD, rDNA ITS.

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2007/0244: received 16 February 2008, revised 12 March 2008 and accepted 13 March 2008

doi:10.1111/j.1472-765X.2008.02377.x

Abstract

Aims: The aim of this study was to determine the genetic variability in *Aspergillus flavus* populations from Brazil nut and cashew and develop a polymerase chain reaction (PCR) detection method.
Methods and Results: Chomatography analysis of 48 isolates identified 36 as aflatoxigenic (75%). One hundred and forty-one DNA bands were generated with 11 random amplified polymorphic DNA (RAPD) primers and analysed via unweighted pair group analysis, using arithmetic means (UPGMA). Isolates grouped according to host, with differentiation of those from *A. occidentale* also according to geographical origin. *Aspergillus flavus*-specific PCR primers ASPITSF2 and ASPITSR3 were designed from ribosomal DNA internal transcribed spacers (ITS 1 and 2), and an internal amplification control was developed, to prevent false negative results. Specificity to only *A. flavus* was confirmed against DNA from additional aspergilli and other fungi.
Conclusions: RAPD-based characterization differentiated isolates according to plant host. The PCR primer pair developed showed specificity to *A. flavus*, with a detection limit of 10 fg.
Significance and Impact of the Study: Genetic variability observed in *A. flavus* isolates from two Brazilian agroecosystems suggested reproductive isolation. The PCR detection method developed for *A. flavus* represents progress towards multiplex PCR detection of aflatoxigenic and nonaflatoxigenic strains in Hazard Analysis Critical Control Point systems.

Introduction

Aflatoxins are carcinogenic natural substances. The fungi *Aspergillus flavus* Link, *A. parasiticus* Speare, *A. tammarii* Kita and *A. nomius* Kurtzman, Horn & Hesseltime all produce these secondary metabolites, most commonly with *A. flavus* producing aflatoxins B_1 (AFB_1) and B_2, (AFB_2) and *A. parasiticus* producing aflatoxins B_1, B_2, G_1 (AFG_1) and G_2 (AFG_2). Whilst most strains of *A. parasiticus* produce aflatoxins, only 40–50% of *A. flavus* strains are estimated to be aflatoxigenic (Geisen 1998).

Cashew (*Anarcardium occidentale* L.) and Brazil nut (*Bertholletia excelsa* Humb. & Bompl.) are important crops in Brazil. Mycotoxigenic fungi have been reported in cashew (Pitt and Hocking 1991; Pitt *et al.* 1993), with AFG_2 detected in kernels (Freire *et al.* 1999). *Aspergillus flavus* is dominant on Brazil nuts, (Freire *et al.* 2000; Arrus *et al.* 2005), with AFB_1, AFB_2 and AFG_2 reported in excess of 2·25 ppm (Castrillon and Purchio 1988).

Hazard Analysis Critical Control Point (HACCP) systems control risks of contamination of food products with pathogenic micro-organisms and chemical toxins

(Codex Alimentarius, 2003). Tools developed for detecting aflatoxigenic aspergilli (i.e. biological hazards) would enable identification of critical control points (CCPs) in the field, storage or transport. The need for detection methods for mycotoxigenic fungi in Brazil is also driven by cost and limited access to methods for mycotoxin detection (Salay 2003).

Identification of aflatoxigenic fungi can be conducted using specialized media for aflatoxin induction (e.g. Lin and Dianese 1976). Limitations exist, however, with insufficient sensitivity and misidentification of compounds during visual determination under UV radiation. Agar plug methods, by contrast, visualize mycotoxins in colony material via thin layer chromatography (TLC) (e.g. Singh *et al.* 1991). PCR detection methods are also under development for aflatoxigenic fungi, with primers targeting regions such as aflatoxin biosynthetic pathway genes and ribosomal DNA (rDNA). Multiplex PCR has been described (e.g. Chen *et al.* 2002), although problems have been reported, with amplification of homologues in non-producing fungi, and primer nonspecificity (Shapiro *et al.* 1996). RAPD PCR also offers potential for detection of aflatoxigenic fungi. RAPD SCAR (sequence characterized amplified region) markers can be designed from monomorphic amplicons, for detection across populations. This approach has been applied for ochratoxin A-producing aspergilli and penicillia (Niessen *et al.* 2005). RAPDs also have potential in fingerprinting for both detection of aflatoxigenic fungi at CCPs and traceback investigations of contamination incidents.

The objectives of this study were to characterize *A. flavus* from *A. occidentale* and *B. excelsa* via RAPD and analysis of rDNA internal transcribed spacers (ITS) regions, and identify candidate sequences for PCR-based detection. This paper describes both an analysis of genetic variability in *A. flavus* on these hosts, and the first PCR-based detection method for *A. flavus* to include an internal amplification control (IAC). Specific primers are suitable for inclusion in PCR multiplex methods, applicable for HACCP systems.

Materials and methods

Origin, identification and preservation of isolates

Forty-eight isolates of *A. flavus* from *A. occidentale* and *B. excelsa* were isolated into culture according to Freire *et al.* (2000) (Table 1). Identifications to species level were made according to Singh *et al.* (1991), and isolates preserved on Czapek Yeast Autolysate (CYA) slopes (Pitt and Hocking 1997) and on sterile filter paper at 4°C.

Aflatoxin detection

Aflatoxin production was analysed in strains after 7 days growth at 30°C on Yeast Extract Sucrose (YES) (Pitt and Hocking 1997) and CYA. Mycelial plugs were wetted with chloroform/methanol (2:1) and placed in contact with a TLC plate (Silica gel 60; Merck, Darmstadt, Germany) for 5 s. Aflatoxin standards AFB_1, AFB_2, AFG_1 and AFG_2 (Sigma, St Louis, MO, USA) were included for quantification. Spots were dried and plates developed in an acetone:chloroform (10:90 v/v) mobile phase. Spots were visualized under UV light (366 nm) and toxins scored on a presence/absence basis, after growth on either of the media.

DNA extraction

Cultures were grown in 150 ml of CYA liquid media for 72 h at 25°C, with agitation at 120 rev min^{-1}. Mycelia were washed with sterile distilled water, harvested by vacuum filtration and freeze dried. Extraction of DNA was conducted according to Raeder and Broda (1985).

Ribosomal DNA amplification

Amplification of the ITS1, ITS2 and 5·8*s* rDNA gene of the nuclear ribosomal RNA gene cluster was conducted using primers ITS5 and ITS4 (White *et al.* 1990). PCR amplifications contained 0·4 μmol l^{-1} of primer, 200 μmol l^{-1} dNTPs, 1·5 mmol l^{-1} $MgCl_2$, 1·0 U *Taq* DNA polymerase (5 U μl^{-1}) (Invitrogen, São Paulo, Brazil), and 20 ng of DNA. Temperature cycling was performed on a PCT-100 thermocycler (MJ Research, Waltham, MA, USA) as follows: Initial denaturation at 95°C for 4 min, 40 cycles of denaturation at 92°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min, and a final extension at 72°C for 5 min.

DNA sequencing and primer design

Twenty nanogram of PCR product were sequenced on an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA). rDNA ITS sequences were also included for 43 *Aspergillus* species in Genbank, together with 19 species across 14 fungal genera commonly associated with *B. excelsa* and *A. occidentale* in Brazil (Freire *et al.* 1999, 2000). Sequence alignment was conducted using ClustalW (Thompson *et al.* 1994) and primers designed with Primer3 (Rozen and Skaletsky 2000). Sequences were deposited in GenBank under accession numbers EF409767–EF409814.

Table 1 Supporting information for examined isolates of *Aspergillus flavus*

Isolate number	Brazilian locality	Host	Aflatoxin (on YES media)	Aflatoxin (on CYA media)
UCB001	Amazonia	B	ND	AFB_1
UCB002	Amazonia	B	ND	ND
UCB003	Amazonia	B	ND	ND
UCB004	Amazonia	B	AFB_1	AFB_1
UCB005	Amazonia	B	ND	ND
UCB006	Amazonia	B	ND	AFB_1
UCB007	Amazonia	B	ND	ND
UCB008	Amazonia	B	ND	ND
UCB009	Beribe County, Ceará	A	AFB_1	AFB_1
UCB010	Beribe County, Ceará	A	ND	ND
UCB011	Beribe County, Ceará	A	AFB_1	AFB_1
UCB012	Beribe County, Ceará	A	AFB_1	AFB_1
UCB013	Beribe County, Ceará	A	AFB_1	AFB_1
UCB014	Beribe County, Ceará	A	AFB_1	AFB_1
UCB015	Beribe County, Ceará	A	AFB_1	AFB_1
UCB016	Beribe County, Ceará	A	AFB_1	AFB_1
UCB017	Beribe County, Ceará	A	AFB_1	AFB_1
UCB018	São Raimundo Nonato, Piauí	A	ND	ND
UCB019	São Raimundo Nonato, Piauí	A	AFB_1	ND
UCB020	São Raimundo Nonato, Piauí	A	AFB_1	AFB_1
UCB021	São Raimundo Nonato, Piauí	A	AFB_1	AFB_1
UCB022	São Raimundo Nonato, Piauí	A	AFB_1	AFB_1
UCB023	São Raimundo Nonato, Piauí	A	AFB_1	AFB_1
UCB024	São Raimundo Nonato, Piauí	A	AFB_1	ND
UCB025	São Raimundo Nonato, Piauí	A	AFB_1	ND
UCB026	São Raimundo Nonato, Piauí	A	AFB_1	ND
UCB027	São Raimundo Nonato, Piaui	A	AFB_1	AFB_1
UCB028	São Raimundo Nonato, Piaui	A	AFB_1	ND
UCB029	Amazonia	B	AFB_1	AFB_1
UCB030	Amazonia	B	AFB_1	ND
UCB031	Amazonia	B	AFB_1	ND
UCB032	Amazonia	B	AFB_1	AFB_1
UCB033	Amazonia	B	ND	AFB_1
UCB034	Amazonia	B	AFB_1	AFB_1
UCB035	Amazonia	B	AFB_1	AFB_1
UCB036	Amazonia	B	ND	ND
UCB037	Amazonia	B	AFB_1	AFB_1
UCB038	Amazonia	B	ND	AFB_1
UCB039	Amazonia	B	ND	ND
UCB040	Amazonia	B	AFB_1	AFB_1
UCB041	Amazonia	B	AFB_1	ND
UCB042	Amazonia	B	AFB_1	AFB_1
UCB043	Amazonia	B	AFB_1	ND
UCB044	Amazonia	B	ND	ND
UCB045	Amazonia	B	ND	ND
UCB046	Amazonia	B	AFB_1	AFB_1
UCB047	Amazonia	B	AFB_1	AFB_1
UCB048	Amazonia	B	AFB_1	AFB_1

ND, not detectable; B, *Bertholletia excelsa*; A, *Anarcardium occidentale*.

Determination of primer specificity and detection limit

Amplification conditions for the specific primer pair developed (ASPITSF2 and ASPITSR3) were as described above, using an annealing temperature of 60°C. Primers were tested for specificity against DNA from *Aspergillus awamore*, *Aspergillus fumigatus* and *Aspergillus niger*, as well as isolates of *Cladosporium cladosporioides*, *Fusarium solani f.* sp. *glycines*, *Fusarium solani*, *Penicillium citrinum* and *Trichoderma harzianum*. Isolates of

F. solani were obtained from the Plant Pathology Department, Universidade de Brasilia (CMUnB). Detection limit was assessed on diluted *A. flavus* DNA. All experiments were conducted in duplicate, with an IAC (see below) in each sample and a separate negative control lacking template DNA included with PCR amplifications.

Internal amplification control development

The specific PCR product amplified for *A. flavus* with primers ASPITSF2 and ASPITSR3 was cloned into the vector pGEMTeasy (Promega, Madison, WI, USA), and plasmid DNA isolated according to standard protocols. An IAC was constructed by splicing an internal fragment from the cloned amplicon. Digestion was performed with *Sma* I (Invitrogen), at base pair position 36, to linearize the plasmid construct. A second digestion was performed at position 147 with *Cla* I (Invitrogen), excising a 111-bp fragment. Following agarose gel electrophoresis, the large plasmid DNA fragment (3301 bp) was extracted using a Geneclean® II kit (Qbiogene, Irvine, CA, USA), and *Cla* I 5′ overhangs filled using DNA polymerase I, large (Klenow) fragment (Invitrogen). Blunt-ended fragments were ligated using T4 DNA Ligase (Promega) to produce a ligate 111 bp smaller than the original specific amplicon. Following cloning, the recombinant strain was stored as a glycerine culture at −80°C. Plasmid DNA was isolated, and at optimal concentration, used as an IAC template in specific PCR reactions.

RAPD-based characterization

Following screening of 10-mer primers (Operon Technologies, Alameda, CA, USA), 11 primers were selected, based upon number of observable bands and reproducibility: OPA04 (5′-AATCGGGCTG-3′), OPB10 (5′-CTGCTGGGAC-3′), OPD10 (5′-GGTCTACACC-3′), OPD20 (5′-ACCCGGTCAC-3′), OPF10 (5′-GGCTGCAGAA-3′), OPF13 (5′-GGAAGCTTGG-3′), OPK20 (5′-GTGTCGCGAG-3′), OPO20 (5′-ACACACGCTG-3′), OPQ20 (5′-TCGCCCAGTC-3′), OPT20 (5′-GACCAATGCC-3′) and OPV10 (5′-GGACCTGGTG-3′). Amplification conditions were as described for rDNA analysis, using additional $MgCl_2$ (2·5 mmol l^{-1}), 1 mg ml^{-1} BSA, and a primer annealing temperature of 36°C. Experiments were repeated twice, with only reproducible bands scored for analyses. Combined data were analysed. Phenetic relationships were generated from combined binary data using MVSP v3.1 (Kovach 1999). Similarities were determined with Jaccard's coefficient, and visualized following UPGMA clustering.

Results

Aflatoxin detection

Chromatography data revealed 36 aflatoxigenic strains producing detectable levels of aflatoxin AFB1 (75%), from both *B. excelsa* and *A. occidentale* (Table 1), with YES media inducing detectable levels in a greater number of strains.

RAPD-based characterization

Phenetic analysis of combined RAPD data (141 amplified bands) showed each isolate to be genetically distinct (Fig. 1). Five main clusters grouped broadly according to host of origin (*B. excelsa*-derived isolates in clusters 1, 2 and 5; *A. occidentale*-derived isolates in clusters 3 and 4). Three exceptions were observed, with one isolate from *B. excelsa* (UCB046) grouping with others from *A. occidentale,* and two from *A. occidentale* remaining ungrouped (UCB026 and UCB028). Clusters 3 and 4 also separated isolates from *A. occidentale* according to geographical origin. RAPD SCAR markers could not be identified, as no correlation was observed between RAPD cluster and aflatoxin detection.

Ribosomal DNA sequencing and specific primer development

Inter-species variability was observed in ITS1 and ITS2 regions, with greater variability noted in the ITS1 region. Two sequences were identified for primer design, with ASPITSF2 (5′-GCCCGCCATTCATGG-3′) targeting within the ITS1 region and specific for *A. flavus*, and ASPITSR3 (5′-CCTACAGAGCGGGTGACAAA-3′) targeting within the ITS2 and specific at the genus level. The expected PCR product size was 397 bp.

Primer specificity and limit of detection

Primers ASPITSF2 and ASPITSR3 amplified a PCR product of the predicted size from target DNA from only *A. flavus*, when testing against DNA from other members of the genus and fungi associated with *B. excelsa* and *A. occidentale* (Fig. 2a,b). When using only fungal DNA template, an amplification detection limit of *c.* 10 fg was observed (Fig. 2c). In development of IACs, simultaneous amplification of distinct PCR products flanked by the same primer pair can cause inhibition or enhanced amplification of either product, according to their molar ratio. In this study, an IAC concentration of 10 pg was identified as optimum for simultaneous amplification of a 286-bp IAC and the 397 bp specific *A. flavus* amplicon.

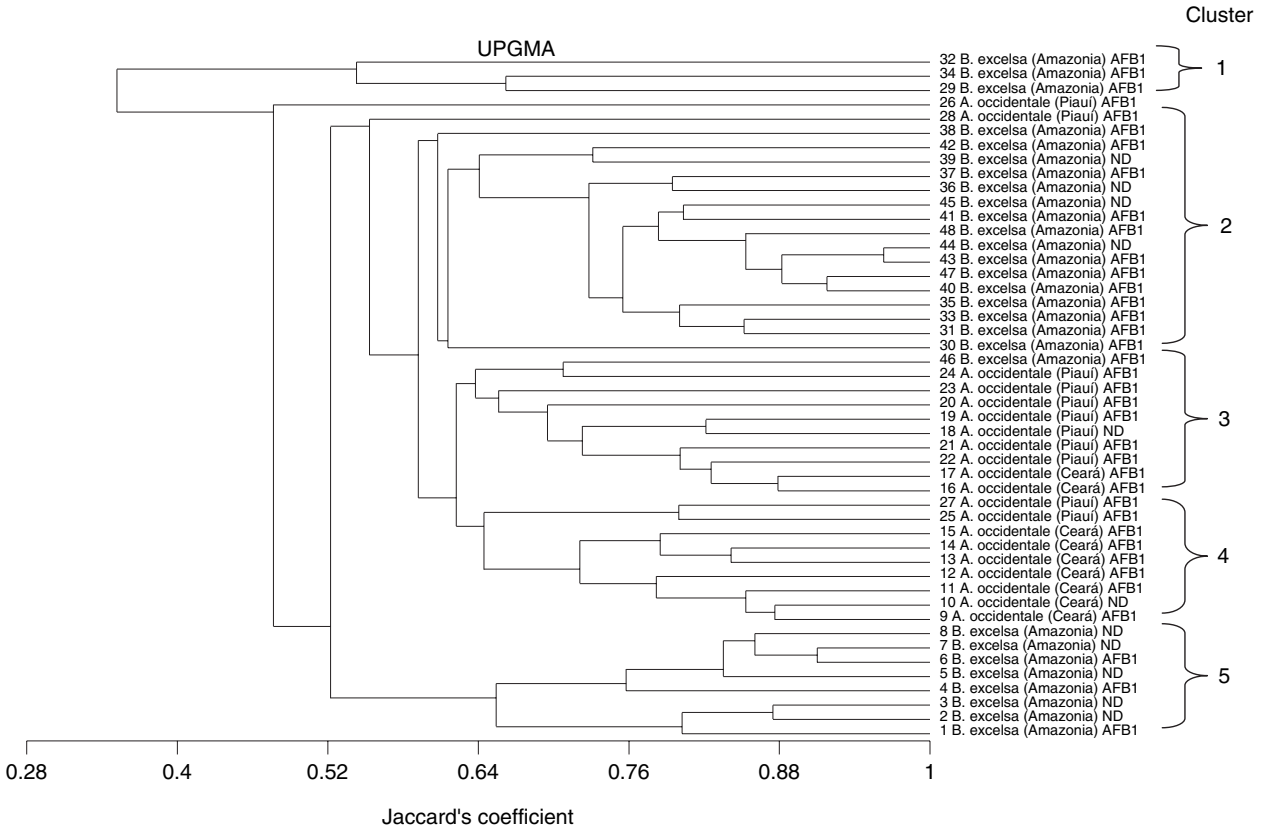

Figure 1 UPGMA phenogram showing genetic relationships between *Aspergillus flavus* isolates from *Anarcardium occidentale* and *Bertholletia excelsa*, based on combined RAPD fingerprints. *Aspergillus flavus* isolates are divided into host of origin clusters.

Successful co-amplification of PCR products was observed when tested across all 48 *A. flavus* isolates, with specificity to only *A. flavus* depicted in Fig. 2b. A detection limit of 1 pg of *A. flavus* DNA was reached, when incorporating 10 fg of IAC plasmid DNA (data not shown).

Discussion

Despite the importance of *A. flavus*, its population biology remains poorly understood. Analysis of RAPD data revealed intraspecific groupings correlating with plant host. This may indicate either a different origin of isolates on each host, or reproductive isolation, with selective pressure conserving genes involved in host specificity. Localized intraspecific variability in soil-borne *A. flavus* has also been reported by Griffin *et al.* (2001). Our RAPD data also distinguished based upon geographical origin in the case of isolates from *A. occidentale*. RAPD fingerprinting of isolates of the human pathogen *A. fumigatus* has been applied in traceback investigations (e.g. Khan *et al.* 1998). Given such applications, RAPDs offers potential in HACCP for monitoring *A. flavus*. Although we did not observe correlation between RAPDs and aflatoxin detection, as also reported in Brazilian isolates from corn and soil (Lourenço *et al.* 2007), potential exists in development of RAPD SCAR approaches for aflatoxigenic strain detection.

Interestingly, TLC analyses showed most isolates of *A. flavus* from *A. occidentale* producing detectable levels of AFB_1. Previously, only aflatoxin G_2 had been reported in *A. occidentale* in Brazil (Freire *et al.* 1999).

Kumeda and Asao (1996) utilized rDNA ITS variability for classification of *Aspergillus* section *flavi* into four groups, namely *A. flavus/A. oryzae*, *A. parasiticus/A. sojae*, *A. tamarii* and *A. nomius*. A two-step nested PCR detection system using ITS-derived primers has been used for clinical isolates of *A. fumigatus*, *A. niger* and *A. flavus* (Sugita *et al.* 2004). To date, PCR methods for mycotoxigenic fungi have not included IAC systems (Paterson 2007). Analysis of rDNA ITS regions in our isolates enabled the design of a species- and genus-specific primer from ITS1 and ITS2 regions, respectively. Together with the developed IAC, this primer pair for *A. flavus* has potential for inclusion in multiplex PCR or RT-PCR methods (e.g. Scherm *et al.* 2005),

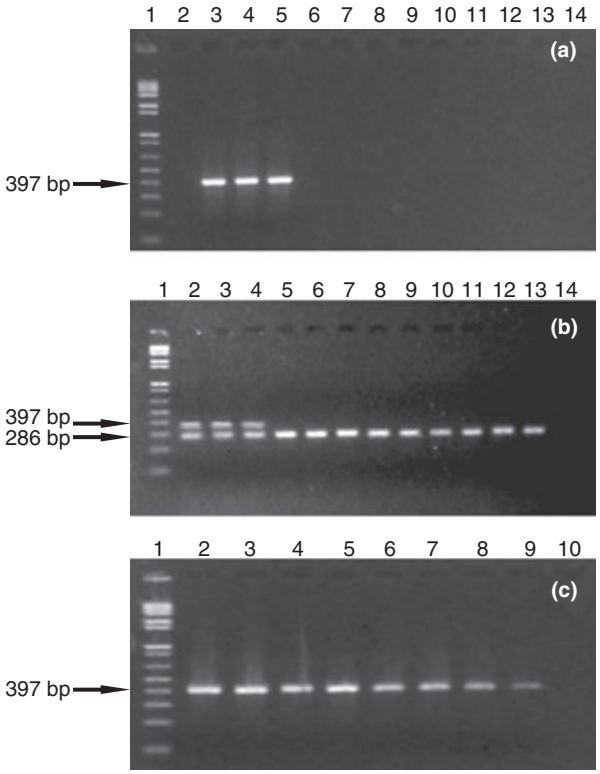

Figure 2 PCR amplification with primers ASPITSF2 and ASPITSR3 (a), specific PCR amplification plus an internal amplification control (b) and limit of detection with primers ASPITSF2 and ASPITSR3 (c). (a) 1: Low DNA Mass ladder (Invitrogen); 2: negative control; 3–5: *Aspergillus flavus* isolates UCB024, UCB045 and UCB060; 6 and 7: *Trichoderma harzianum* isolates 1 and 2; 8 and 9: *Aspergillus fumigatus* isolates 1 and 2; 10 and 11: *Aspergillus awamore* isolates 1 and 2; 12 and 13: *Fusarium solani* f. sp. *glycines* isolates CMUnB 1824 and 1848; 14: *F. solani* isolate CMUnB 1974. (b) 1: 1 Kb Plus DNA ladder (Invitrogen); 2–4: *A. flavus* isolates UCB036, UCB040 and UCB044; 5: *A. awamore* isolate 1; 6: *A. fumigatus* isolate 1; 7: *Aspergillus niger* isolate 1; 8 and 9: *F. solani* f. sp. *glycines* isolates CMUnB 1824 and 1848; 10: *F. solani* isolate CMUnB 1974; 11: *P. citrinum* isolate 1; 12: *T. harzianum* isolate 1; 13: *C. cladosporioides* isolate 1; 14: negative control. (c) 1: 1 Kb Plus DNA ladder (Invitrogen); 2–9: PCR products amplified with 20 ng, 5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg and 10 fg *A. flavus* DNA; 10: negative control.

to complement markers for aflatoxin biosynthetic pathway genes, such as *aflP* or *aflQ* (Paterson 2006). Nuclear rDNA regions are also attractive markers because of high copy number, facilitating PCR amplification. Indeed, the detection limit of our specific ITS product (10 fg) was more sensitive than obtained with primers targeting single copy genes, which typically detect down to only nanograms of template DNA. Uptake of a robust PCR-based system in HACCP is important in Brazil, given limited chromatographic analyses on products for internal markets.

Acknowledgement

Financial support was provided by the CNPq (Rhae-50·4601/2004-0), Prodetab (003-01/01) and FAP-DF (PAPPE-193·000·295/2004).

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