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Cowpeas as growth substrate do not support the production of aflatoxin by *Aspergillus* sp.

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Abstract

A number of 21 *Aspergillus* sp. strains isolated from cowpeas from Benin (Africa) were characterized by RAPD methodology. Seven of these strains grouped with *A. flavus* in the dendrogram generated with the RAPD data. Only three were able to produce aflatoxin in significant amounts. Twelve other isolates grouped with *A. parasiticus*. All of these strains except 3 produced aflatoxin. Two additional strains neither fit with the *A. flavus* group, nor the *A. parasiticus* group according to their RAPD pattern. Both did not produce aflatoxin in measurable amounts.
Generally the aflatoxin positive strains produced high amounts of aflatoxin after growth on YES medium. However after growth on cowpea based medium aflatoxin biosynthesis was strongly ceased, albeit the growth of the colony was only partly reduced. This was true for media made either with the whole cowpea seed or with cowpea seed without seed coat. Interestingly when the cowpea medium was heat sterilized the fungus was able to produce high amounts of aflatoxin. This, however, was not the case after the use of gamma irradiation as sterilization method for the medium. The expression of the *nor*-1 gene, which is one of the early genes involved in aflatoxin biosynthesis, was significantly repressed after growth on gamma irradiated cowpea medium in contrast to YES medium.

Keywords: cowpea, aflatoxin, inhibitory substances, RAPD

Introduction

The contamination of food with mycotoxins has drawn world wide attention due to their negative impact on human health. Aflatoxins are secondary metabolites of fungi with strong carcinogenic potential (1). The most important aflatoxin producers are *A. flavus* and *A. parasiticus*. Amongst others, tropical crops like maize, groundnuts and cottonseeds show a great susceptibility to fungal infections and contamination by aflatoxins (2, 3, 4). Recent country wide studies were conducted to determine the natural infection of cowpeas (*Vigna unguiculata* (L.) Walp.) by toxigenic fungi and mycotoxin contamination in Benin, West Africa (Houssou *et al.*, submitted). These studies revealed that cowpeas are obviously less susceptible to aflatoxin contamination compared to other crops, as no or only very low amounts of aflatoxin could be found on these commodities. On the other hand fungi of the *Aspergillus* group, *e.g.* possible aflatoxin producers, were predominantly isolated from analysed cowpea samples. These results pinpoint to the possibility that obviously cowpeas do not inhibit the growth of *Aspergillus* but the production of aflatoxin. The lower susceptibility of cowpeas against aflatoxin contamination might be attributed to the presence of some substances like tannins that have antifungal properties as stipulated by Digrak *et al.,* (5). Tannin is found in cowpea seed and is mainly concentrated in the seed coat (6, 7) suggesting that the seed coat should play a vital role in preventing toxin production by fungi that infects cowpea.

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Alternatively cowpeas as substrate *per se* may not facilitate the production of aflatoxin, due to its nutritional composition. Thus the inhibitory role of cowpeas or its seed coat on the growth of fungi and mycotoxin production needs to be clarified. The present study was conducted to determine the effect of cowpeas and their constituents as growth substrate on the biosynthesis of aflatoxin by *A. parasiticus* at the phenotypical and at the molecular level.

Materials and Methods

Isolation of the strains

The strains belonging to the *Aspergillus* group were identified from representative cowpea samples from Benin. The procedure used for isolation of the fungi was as follows: from each sample 25 seeds were randomly chosen and the surface disinfected with 1% NaOCl after which they were plated (5 seeds per plate) onto dichloran 18% glycerol agar (DG 18) and incubated at 30 °C for 7 days. A total of 21 isolates belonging to *Aspergillus* (designated as AF strains 1-21) were purified for molecular characterization at the Institute of Safety and Quality of Fruits and Vegetables in the Max Rubner Institute, Federal Research Institute for Nutrition and Food (Karlsruhe, Germany).

Growth condition and aflatoxin production of the isolates

The growth was carried out by inoculating an equal amount of fungal spores for each isolate on solid YES medium (20 g/l yeast extract [Merck Darmstadt, Germany], 150 g/l sucrose and 20 g/l agar). Three point inoculations were applied for each isolate and incubated at 30 °C for 14 days.

For aflatoxin production an equal amount of the fungal mycelium grown for 5 days on YES solid medium was transferred into 1.5 ml micro reaction tubes and 500 µl of chloroform were added. The fungal mycelia were extracted for 30 min at room temperature on a rotary shaker, the mycelia were discarded and the chloroform extract was evaporated to dryness in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA). The residues were redissolved in 100 µl methanol and 20 µl were spotted onto TLC plates (Silica gel 60, Merck, Darmstadt, Germany). As mobile phase, toluol-ethylacetate-lactic acid (50:3:4 v/v/v) was used.

Quantitative determination of aflatoxin by HPLC

Detection and quantitative determination of aflatoxin from fungal colonies were performed by HPLC. For this purpose 200 mg of the fungal colony was extracted under shaking conditions in 500 µl chloroform at room temperature for 30 min. The residue was discarded and the chloroform was evaporated to dryness in a vacuum concentrator (Savant Instruments, Farmingdale, USA). The residue was redissolved in 500 µl methanol and subjected to HPLC analysis (20 µl) in a Pharmacia HPLC apparatus LKB 2150 (Pharmacia, Uppsala, Sweden). A nucleosil 100-5 C18 CCV 250/4 column was used for separation (Macherey and Nagel, Düren, Germany) at a flow rate of 1 ml/min with acetonitril:water:acetic acid (40:60:1, [v:v:v]). The peak was determined with a fluorescence detector (Shimazu RF551, Düsseldorf, Germany).

Fungal DNA extraction from pure culture

The isolation of DNA from pure fungal strains was performed according to a modified method originally described by Yelton *et al.* (8). For this purpose the strains were grown for 7 days under shaking conditions (180 rpm) in YES broth. The mycelia were harvested by filtration, transferred to a mortar, frozen in liquid nitrogen and ground to a powder. The powder was resuspended in 7 ml 50 mM lysis buffer (pH 8.5) vortexed and incubated for 15 min at 65 °C in a water bath. After centrifugation at 13.000 rpm and 4 °C for 20 min, 9 ml of the supernatant were transferred into a new centrifuge tube and 1 ml of sodium acetate (4 M) was added. This solution was placed on ice for 1 h and centrifuged for 20 min at 13.000 rpm and 4 °C. After centrifugation the supernatant was transferred into a fresh tube. The solution was precipitated by the addition of 2.5 volumes of absolute ethanol. After precipitation the DNA was purified using the DNeasy Plant Mini kit as suggested by the manufacturer (Quiagen, Hilden, Germany). The DNA concentration was measured with a spectrophotometer (Pharmacia Gene Quant, RNA/DNA Calculator).

RAPD (randomly amplified polymorphic DNA) characterization

The chromosomal DNA of the isolated strains was diluted to 2 µg/ml and used as template for

the RAPD-PCR reaction. PCRs were performed in a total volume of 50 µl containing 5 µl of genomic DNA, 1.5 µl of random primer (Aristo 1: 5- CAG GAC GAA CTC GAT GGA TAC- 3), 8 µl of dNTPs (Perkin–Elmer, Massachusetts, USA), 0.5 µl of Taq DNA polymerase, 5 µl of buffer provided by the manufacturer (Genaxxon, Biberach, Germany) and 30 µl of sterile distilled water. PCR reactions were performed in a Mastercycler 5330 plus (Bio-RAD laboratories, Hercules, California) which was set to the following conditions: 1x: 95 °C, 2 min 30 s; 45 x: 95 °C for 60 s; 36 °C for 1 min; 72 °C for 4 min and 1x: 4 min at 72 °C. The amplified DNA products were separated by gel electrophoresis in 1 % (w/v) agarose (Serva, Heidelberg, Germany) in 10x TBE buffer according to Sambrook *et al.* (9). Gels were stained in a 1.0 µg/ml ethidium bromide solution, visualised under UV light (366 nm) and photographed on a gel documentation system (Gel Doc 2000, Bio-Rad laboratories). As DNA marker a 100 bp DNA ladder (MBI Fermentas, St. Leon-Rot, Germany) was used as size control on the gel. Pure DNA extracted from aflatoxin producing strains of *A. flavus* and *A. parasiticus* were used as controls. A dendrogram was constructed from the RAPD profile obtained by using the Bionumerics fingerprint analysis software package (Applied Maths NV, Sint-Martens-Latem, Belgium).

Preparation of cowpea medium

For the preparation of cowpea medium 50 g of cowpea seed flour, either prepared from dehulled or non-dehulled cowpeas and 20 g of agar were added to 1 l of water. This suspension was sterilized either by autoclaving at 121 °C for 15 min and poured into petri dishes. Alternatively 50 g of cowpeas were sterilized by electron beam irradiation at 30 kGy at room temperature and added to autoclaved agar solution (20 g/L). Clean maroon cowpea variety samples previously checked to be free of aflatoxin were used along the study. YES solid medium (20 g/L yeast extract [Merck Darmstadt, Germany], 150 g/L sucrose and 20 g/L agar) was used as positive control as it is a strong aflatoxin supporting medium.

RNA extraction and Real-Time PCR analysis

Total RNA isolation and subsequent Real-Time PCR were performed according to the method described by Mayer *et al.* (10). The copy number of the *nor-1* gene transcripts was determined. The RNA of selected strains was isolated from pure cultures grown 7 days on solid YES medium and cowpea based medium. The total RNA of the strains was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). An amount of 0.5-1.0 g of the mycelium was ground in a mortar in the presence of liquid nitrogen. About 250 µg of the mycelial powder was used for isolation of total RNA. The mycelium was resuspended in 750 µl buffer RLC plus 7.5 µl β-mercaptoethanol in RNase free micro reaction tubes. About 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) were added. The samples were mixed thoroughly and incubated for 3 min at 56 °C in a water bath. All further procedures were essentially the same as suggested by the manufacturer of the kit. For cDNA synthesis, 12 µl of the DNase I treated total RNA were used along with the Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany). The reaction mixture was composed essentially as described by the manufacturer and incubated at 37 °C for 1 h. The cDNA was directly used for Real-Time PCR according to Mayer *et al.* (10). All experiments were repeated twice.

Results

Aflatoxin production ability of the isolates

Twelve strains among the 21 strains of the *Aspergillus* isolated from cowpea produced detectable levels of aflatoxin. The remaining 9 strains did not show any detectable amounts of aflatoxin after 14 days incubation in YES medium (Figure 1). Twelve of the 21 strains grouped together after RAPD analysis with a strain of *A. parasiticus*. Nine of these 12 strains were able to produce aflatoxin. Seven strains grouped together with a strain of *A. flavus*. Three of these seven strains were able to produce aflatoxin.

It was observed that 7 out of 11 of the aflatoxigenic strains were isolated on cowpea samples from southern agro-ecological zones of Benin, while many of the nonaflatoxigenic strains (6 out 10) were identified from cowpea samples from the two northern agro-ecological zones of Benin.

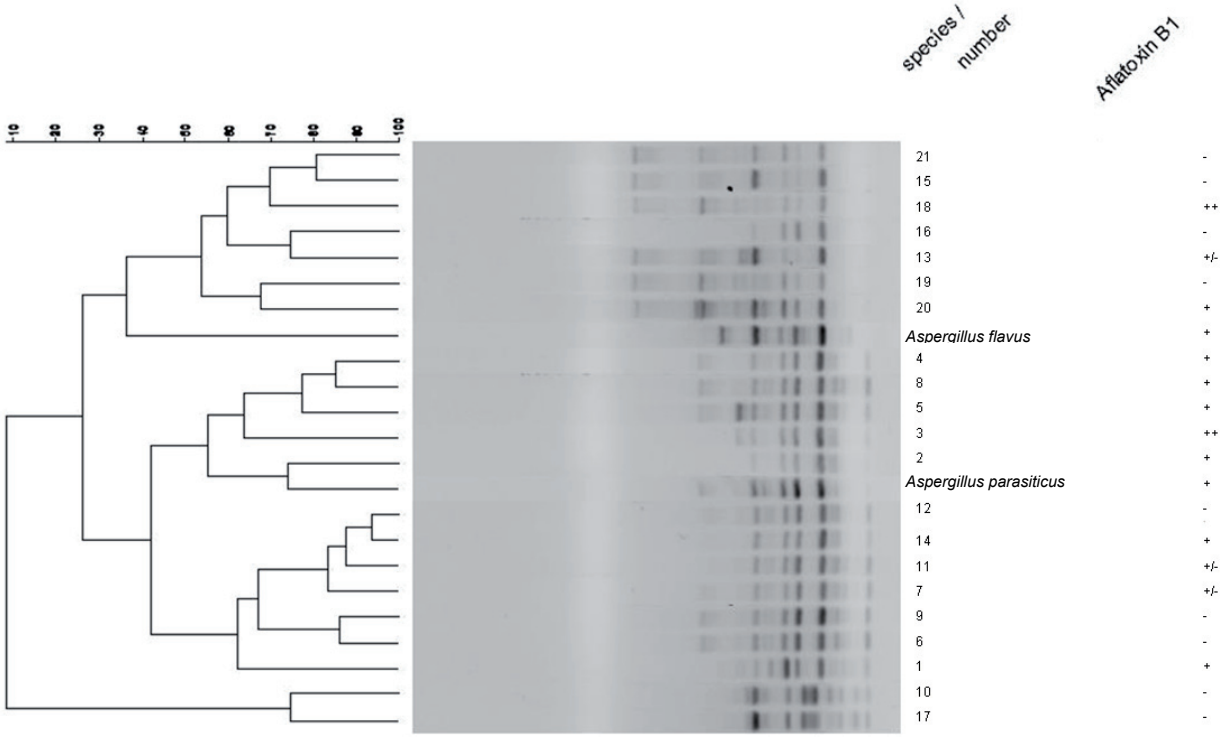

Figure 1. Dendrogram based on the RAPD profiles of the *Aspergilli* isolated from cowpeas.
One group clusters together with a strain of *A. flavus,* the other group with a strain of *A. parasiticus*.
The ability of the strains to produce aflatoxin is indicated

Influence of cowpea medium on the production of aflatoxin

To analyse the possibility if cowpeas as substrate inhibit the production of aflatoxin by *Aspergillus*, the strong aflatoxin producing strain AF-8, which was isolated from cowpeas, was grown on cowpea medium as a cowpea model system. In comparison growth and aflatoxin biosynthesis were tested on YES medium. Results showed that AF-8 was able to grow on all media over the incubation period but differences could be observed in the growth pattern. Normal growth was observed in YES medium whereas on cowpea based media the growth was slightly reduced but a high number of conidiospores were formed. This was true for both cowpea media, either prepared with the whole or with the dehulled cowpeas. A strong influence of the cowpea medium on aflatoxin AFB_1 biosynthesis could be observed (Figure 2). The aflatoxin producing strains showed high aflatoxin production after growth on YES medium, but only very low aflatoxin production after growth on cowpea medium. In Figure 2 aflatoxin production by the strain AF-8 on the different media is shown as an example. This was again true for cowpea medium made by using either the whole or the dehulled cowpea seeds (Figure 3). A difference in aflatoxin production, however, could be observed if the

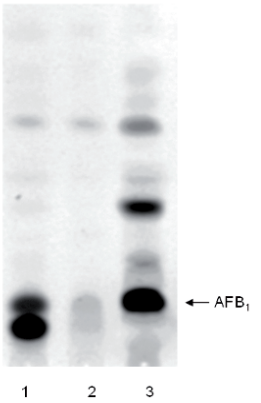

Figure 2. TLC analysis of aflatoxin production of the strain *Aspergillus* AF-8 isolated from cowpeas, grown on autoclaved cowpea medium (lane 1), irradiated cowpea medium (lane 2) and on YES medium (lane 3)

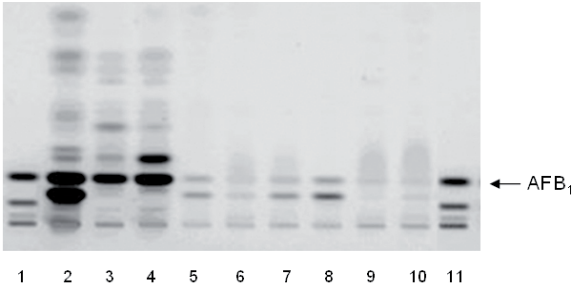

Figure 3. TLC analysis of the aflatoxin production by *Aspergillus* grown on different media.
Lane 1, aflatoxin standard; lane 2 to 4, YES medium; lanes 5 to 7, medium made from whole cowpeas; lanes 8 to 10, medium made from dehulled cowpeas; lane 11, aflatoxin standard. Lanes 2, 5 and 8, *A. parasiticus* BFE96p; lanes 3, 6 and 9, *Aspergillus* isolate AF-8 from cowpeas; lanes 4, 7 and 10, *Aspergillus* isolate AF-3 from cowpeas

strains grow on autoclaved (heat sterilized) or on gamma irradiated (non-heat sterilized) cowpea based medium. High aflatoxin production by the aflatoxigenic strain AF-8 could only be observed in autoclaved cowpea based medium (Figure 2, lane 1). In contrast there was a much reduced aflatoxin production (Figure 2, lane 2) in irradiated cowpea based medium. This suggested that heating (autoclaving) influenced significantly the cowpea medium characteristics and transformed it into a more favourable substrate for growth and aflatoxin production. These results suggest that either a heat labile aflatoxin inhibiting substance is destroyed by autoclaving, or a nutritional compound is made accessible to the fungus which supports toxin biosynthesis.

Aflatoxin production and nor-1 gene expression

To analyse the possibility that the observed antiaflatoxigenic effect of irradiated cowpea medium has an influence on aflatoxin biosynthesis at the molecular level, a *nor*-1 gene expression analysis has been carried out. After growth of strain AF-8 on YES or cowpea medium the amount of aflatoxin produced was determined by HPLC/TLC and in parallel the expression of the *nor*-1 gene was measured by Real Time PCR. As expected high levels of aflatoxin were produced on YES medium and very low levels on cowpea medium. The result of the subsequent Real Time PCR analysis of the expression of the *nor*-1 gene fits very well to the phenotypic toxin production. A low copy number of *nor*-1 transcripts were recorded in cowpea medium. In contrast a 45 times higher copy number of the *nor*-1 gene transcripts was detected for the same strain in YES medium (Table 1). This is paralleled by the phenotypical production of aflatoxin B_1 as determined by HPLC analysis. These results suggest that obviously non-heated cowpeas are not a suitable substrate for the activation of the aflatoxin biosynthesis genes and that the inhibitory activity of the cowpeas are exerted at the transcriptional level.

Table 1. Expression of the *nor*-1 gene in relation to aflatoxin biosynthesis on different media

Medium	*nor*-1 Gene expression (copy number)	Aflatoxin biosynthesis (ng/ml)
Cowpea	5838	19.79
YES	265650	861.31

Discussion

Although cowpeas generally are not contaminated by aflatoxins to a high level, the current study revealed that they can be infected by aflatoxin producing strains. Twenty-one potential aflatoxin producing *Aspergillus* strains have been isolated from different cowpea varieties. Twelve of the examined strains grouped together with *A. parasiticus* after RAPD analysis of which 9 were aflatoxin producers. On the other hand 7 of the isolated strains grouped together with *A. flavus* of which only 3 produced aflatoxin. Similar results were reported by Konietzny and Greiner (11) that indicate that almost all isolated *A. parasiticus* strains were capable of producing aflatoxin while only 40 to 50 % of the isolated *A. flavus* strains produced aflatoxin. The current study showed that cowpeas as substrate are not conducive for aflatoxin biosynthesis by *Aspergillus*, albeit they do not reduce the growth of *Aspergillus* to a large extent. It has been described that cowpeas contain substances like tannins concentrated in the seed coat (6) which have potential antifungal activity (5). Tannin is the main composition of the cowpea seed coat (6, 7). Moreover the antifungal activity of tannin was stipulated by Baba-Moussa *et al.* (12). In contrast to these previous reports indicating the antifungal activity of tannin, the present study showed that the cowpea seed coat, although rich in tannin, is not responsible for the inhibition of aflatoxin production after growth on cowpeas, because no difference on the antiaflatoxigenic activities of cowpeas could be found after growth on medium made from whole cowpeas or from dehulled cowpeas. This indicates that the seed coat is not responsible for this inhibiting effect.

The fact that the inhibition of aflatoxin production is only exerted after growth on irradiated cowpea medium, but not on heat sterilized cowpea medium suggests that cowpea may contain a heat labile inhibiting substance. However the possibility that the heating releases cowpea constituents, which are otherwise not accessible to the fungus and which support aflatoxin biosynthesis, cannot be ruled out yet.

Ye *et al.* (13) demonstrated the presence of α- and β- antifungal proteins in cowpea seed that have the ability to retard mycelial growth of fungi. In the current study a slight decrease in growth could also be observed which might be due to these antifungal proteins. Recently Rose

et al. (14) reported that vicilins (7S storage proteins) from cowpeas have also antifungal activities.

Taken together the results show that non-heated cowpeas are not a susceptible substrate for aflatoxin biosynthesis, albeit *A. flavus* and *A. parasiticus* can be isolated from this habitat.

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References

1 Eaton DL, Gallagher EP (1994) Mechanisms of aflatoxin carcinogenesis. Ann Rev Pharmacol Toxicol 34: 135-172
2 Hell K, Cardwell KF, Setamou M, Poehling HM (2000a) The influence of storage practices on aflatoxin contamination in maize in four agro-ecological zones of Benin, West Africa. J Stored Prod Res 36: 365-382
3 Hell K, Cardwell KF, Setamou M, Schulthess F (2000b) Influence of insect infestation on aflatoxin contamination of stored maize in four agroecological regions in Benin. Afr Entomol 6: 169-177
4 Bankole SA, Adebanjo A (2003) Mycotoxins in food in West Africa: current situation and possibilities of controlling. Afr J Biotechnol 2: 254-263
5 Digrak M, Hakki A, Ahmet I, Selim S (1999) Antibacterial and antifungal effects of various commercial plant extracts. Intern J Pharmacognosy 37: 216-220
6 Plahar WA, Annan NT, Nti CA (2001) Cultivar and processing effect on the pasting characteristics, tannin content and protein quality and digestibility of cowpea (*Vigna Unguiculata).* Food Research Institute (CSIR) Accra, Ghana. J Food Technol Afr 6: 50-55
7 Vincenzo L, Roberto T, Nunzia C, Angela C, Di DV, Vito L (2005) Seed coat tannins and bruchid resistance in stored cowpea seeds. J Sci Food Agri 85: 839-846
8 Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a *trp*C plasmid. Proc Natl Acad Sci 81: 1470-1474
9 Sambrook J, Russel DW (2001) Molecular Cloning. 3 Edn. Cold Spring Harbour Laboratory Press
10 Mayer Z, Färber P, Geisen R (2003) Monitoring the production of aflatoxin in wheat by measureing the concentration of *nor*-1 mRNA. Appl Environ Microbiol 69: 1154-1158
11 Konietzny U, Greiner R (2003) The application of PCR in the detection of mycotoxigenic fungi in foods. Braz J Microbiol 41: 1-28
12 Baba-Moussa F, Akpagana K, Bouchet P (1999) Antifungal activities of seven West African Combretaceae used in traditional medicine. J Ethnopharmacol 66: 335-338
13 Ye XY, Wang HX, Ng TB (2000) Structurally dissimilar proteins with antiviral and antifungal potency from cowpea (*Vigna unguiculata*) seeds. Life Sci 67: 3199-3207
14 Rose TL, Gomes VM, Cunha M Da, Fernandes KVS, Xavier-Filho J (2003) Effect of sugars on the association between cowpea vicilin (7S storage proteins) and fungal cells. Biocell 27: 173-179