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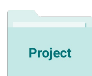

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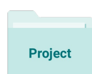

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SCOPOLETIN IN CASSAVA PRODUCTS AS AN INHIBITOR OF AFLATOXIN PRODUCTION

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ABSTRACT

The scopoletin level in a cassava variety from Benin, BEN 86052, and the compounds effect on the growth of an *Aspergillus flavus* isolate from cassava chips were investigated. The influence on the biosynthesis of aflatoxins was also investigated. Scopoletin was quantified using high performance liquid chromatography. An *in vitro* test was used to evaluate the inhibitory effects of cassava flour extracts and pure scopoletin on growth and biosynthesis. The *A. flavus* isolate was genetically characterized to be an aflatoxin producer using polymerase chain reaction. Scopoletin was found in roots and chips with a level ranging from 4.9 to 242.5 mg/kg dry weight. Scopoletin induction was noticed after a peeling and drying process (6 days). Aflatoxin production by a strain of *A. flavus* holding the cluster *Nor1*, *Omt1* and *OmtB* responsible for aflatoxin biosynthesis was inhibited by scopoletin and cassava flour at lower concentrations (0.024 mM). Scopoletin could be used on other commodities susceptible to mycotoxin contamination.

PRACTICAL APPLICATIONS

Scopoletin accumulated in cassava variety used for chips production. Scopoletin inhibited the production of aflatoxin by toxigenic *Aspergillus flavus*. The use of this compound on other crops susceptible to fungi infection and aflatoxin contamination could be investigated to reduce food spoilage and mycotoxin contamination.

INTRODUCTION

Scopoletin is a phenolic coumarin and an important member of the group of phytoalexins isolated from many plants (Tal and Robeson 1986). Phytoalexins are low molecular weight compounds that are biosynthesized by plants *de novo* following exposure to microorganisms (Edwards *et al.* 1997). Several low molecular compounds found in plants apparently have a function of defense (Wink 2003). While some are constitutive defense compounds, others thus are directly formed as a response of many plants to stress (drought, salt, cold), wound, viral infection or invasion by bacterial or fungal pathogens (Broekaert *et al.* 1997; Lay and Anderson 2005). The induced metabolites include antifungal phytoalexins, which may be of a variety of structural types including sesquiterpenes, isoflavonoids, stilbenes, polyacetylene and phenolic coumarins, but all share a number of characteristics (Edwards *et al.* 1997). All are synthesized *de novo* and rapidly accumulate at the site of infection up to millimolar concentrations, whereby they act as nonspecific biocides (Lay and Anderson 2005).

Accumulation of scopoletin in plants has been correlated with resistance to microbial attack and reaction to other stress such as mechanical injury and dehydration (Tanaka *et al.* 1983). Scopoletin often seems to be the most important product rising in concentration in the infected plant as

compared with other related coumarins and coumarin glycosides such as scopolin, esculetin and esculin and with other classes of compounds (Uritani 1999; Buschmann *et al.* 2000; Giesemann *et al.* 2008).

Aflatoxins are the most important mycotoxins in food with regard to occurrence, toxicity and impact on human health and trade. Aflatoxins are primarily produced by strains of *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* (Wilson *et al.* 2002), *Aspergillus pseudotamarii* (Ito *et al.* 2001) and *Aspergillus bombycis* (Peterson *et al.* 2001).

A. flavus populations are genetically diverse. Isolates vary considerably in their ability to produce aflatoxins and colonize plants. They generally can be grouped into two sclerotial morphotypes, L strains and S strains, the latter strains also named *A. flavus* var. parvisclerotigenus (Saito and Tsuruta 1993). L strain isolates produce abundant conidiospores and sclerotia that are usually larger than 400 μm in diameter, whereas S strain isolates produce fewer conidiospores and numerous sclerotia that are usually smaller. The S strain isolates typically produce higher amounts of aflatoxins than the L strain isolates on the same media. The aflatoxigenic trait of the S strain isolates seems very stable. Aflatoxin biosynthesis is controlled by a cluster of 25 genes among the simultaneous presence of *aflD*, *aflO* and *aflP* (Scherm *et al.* 2005). All of the *Aspergillus* species mentioned are found in the soil (Wilson *et al.* 2002). The four major aflatoxins commonly isolated from foods and feeds are aflatoxins B_1, B_2, G_1, and G_2. The presence of significant levels of aflatoxins has been reported in groundnut, cashew and maize together with other crops.

Reports on contamination levels in Africa have been partially reviewed by Wagacha and Muthomi (2008). Data collected on aflatoxin risk are, however still, rather spotty and isolated. When it comes to the crops of world importance such as maize, groundnut, cassava, cassava (*Manihot esculenta* Crantz) findings are most puzzling. Thus, in a study conducted in Tanzania, Muzanila *et al.* (2000) found no aflatoxin contamination in cassava flour from Tanzanian villages. Similarly, work done in Ghana by Wareing *et al.* (2001) showed that more than 40% of Kokonte (dried cassava products) samples were infected by *Aspergillus* spp.; however, it showed no aflatoxin contamination. Moreover, work done in Nigeria showed no aflatoxin in all marketed cassava chips samples (Jimoh and Kolapo 2008). Recently, Gnonlonfin *et al.* (2008) also showed that fully processed cassava chips samples stored by farmers in the villages in Benin were not contaminated by aflatoxins even though *A. flavus* was present. Scopoletin has been reported to be the most prominent compound accumulating in the tuberous roots of cassava during postharvest deterioration (Buschmann *et al.* 2000).

The aim of the present study was to investigate whether this compound (phytoalexin) suppresses the growth of *A. flavus* and/or the aflatoxin production.

MATERIALS AND METHODS

Plant Material

Manihot esculenta Crantz stalk from the variety named BEN 86052 were grown at the North Agricultural Research Center located at Ina, Institute of Agricultural Research, Ministry of Agriculture, Livestock and Fishery, Benin and the root tubers harvested after 6 months. Root tubers of this variety known to produce high amount of scopoletin during postharvest deterioration (Gnonlonfin *et al.* 2011) were used.

Plant Material Processing and Storage

Fresh cassava tuberous roots were processed into chips as described by Gnonlonfin *et al.* (2011). Shortly, fresh cassava roots were peeled, cut into slices (thickness 2 cm) and sun dried for 6 days. The temperature and relative humidity ranges during sun drying were 35–40.6C and 16.3–52.9%, respectively. Dried cassava chips were stored in a woven polyethylene bags. Approximately, 3 kg of chips were sampled and immediately taken to the laboratory for scopoletin analysis and the *in vitro* test described below.

Gene Characterization

The entire previously mentioned 18 *A. flavus* isolates gene were further characterized by extracting the DNA and perform a polymerase chain reaction for genes identification using the method described by Melo *et al.* (2006). Genes amplification and discrimination between aflatoxin producers and nonproducers were by using the method described by Rodrigues *et al.* (2007). Gene markers *aflD*, *aflO*, *aflP* (Sigma-Aldrich Chemie GmbH, Gillingham, Dorset, U.K.) were used and their specifications are shown in Table 1.

Microorganism and Chemicals

A collection of cassava chips were made across Benin. *Aspergillus* genus was predominant when analyzing the fungal flora. These were identified to species, and *A. flavus* was the most frequently isolated. A total of 18 such isolates were obtained and characterized as described in the previous section. Among the isolates, six were positive, i.e., aflatoxin producers when cultured on coconut agar medium (CAM). One such aflatoxin producing strain was chosen for the further use in *in vitro* test. Pure scopoletin was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). All other chemicals were analytical or high performance liquid chromatography (HPLC) grade.

TABLE 1. GENE, PRIMER PAIR AND PCR PRODUCT LENGTH (BP)

Gene	Primer pair	Primers sequences (5′ → 3′)	PCR product length (bp)
aflD	Nor1-F	ACGGATCACTTAGCCAGCAC	990
	Nor1-R	CTACCAGGGGAGTTGAGATCC	
aflO	OmtB(F)-F	GCCTTGACATGGAAACCATC	1,333
	OmtB(F)-R	CCAAGATGGCCTGCTCTTTA	
aflP	Omt1-F	GCCTTGCAAACACACTTTCA	1,490
	Omt1-R	AGTTGTTGAACGCCCCAGT	

PCR, polymerase chain reaction; BP, base pair.

Characterization of Aflatoxin Producers

CAM was prepared based on the method described by Davis *et al.* (1987). One colony of each 18 *A. flavus* strains was cultured on the surface of the prepared medium, and the plates were incubated for 7 days at 25C and photoperiodicity of 12 h. After incubation, the plates were observed for aflatoxin production by observing the presence or absence of a fluorescence ring in the agar surrounding the colony under ultraviolet (UV) light at wavelength of 365 nm (Rodrigues *et al.* 2007). The results were scored as positive or negative.

Scopoletin Extraction and Analysis

Scopoletin extraction was done based on the method described by Buschmann *et al.* (2000). Cassava flour was produced from the sampled chips by grounding using a laboratory mortar. Then, 1 g of cassava flour was homogenized in 10 mL of ethanol (Sigma-Aldrich, St. Louis, MO) by means of blender. The extract was filtered through a fluted filter paper (Whatman, no. 1, Voigt Global Distribution Inc, Lawrence, KS), evaporated to an end volume of 3 mL and stored until use at −18C.

The compound analysis was with autosampler HPLC (Waters, model L-2200, Hitachi High Technologies America Inc., Pleasanton, CA) using a modified method of Buschmann *et al.* (2000) as described by Gnonlonfin *et al.* (2011).

Preparation of Culture Media for *In Vitro* Test

Cassava flour used for scopoletin extraction as described under the "Scopoletin Extraction and Analysis" section was also used to prepare two different cassava flour enriched CAM for the *in vitro* test. This was done by adding 10 g and 20 g of cassava flour to 100 mL of autoclaved CAM. Each media then contains an equivalent scopoletin concentration of 0.3 μg/mL (0.024 mM) and 0.5 μg/mL (0.039 mM). In addition, the test was done by adding ethanolic cassava flour extract giving rise to a resulting concentration of 1.2 μg/mL (0.092 mM).

In Vitro Test for *A. Flavus* Development and Aflatoxin Production

The selected aflatoxin producing *A. flavus* strain was cultured on CAM and on the different enriched CAM culture media in Petri dishes using standard method. The Petri dishes were incubated and observations were done under UV light as described under the "Characterization of Aflatoxin Producers" section. After incubation, the *A. flavus* colony diameter was measured using the method described by Pitt and Hocking (1999). The controls were

(1) CAM + *A. flavus* and
(2) CAM + *A. flavus* + ethanol (not shown in Table 2).

TABLE 2. SCOPOLETIN EFFECT ON *ASPERGILLUS FLAVUS* GROWTH AND AFLATOXIN PRODUCTION

		Aspect of *A. flavus* positive strain	
Culture media	Scopoletin concentration (mM)	Colony diameter (D)	UV observation (aflatoxin production)
CAM (control)	–	Normal growth (4.0 cm)[a]	Fluorescence (+)
CAM + pure scopoletin standard	2	Growth (3.0 cm)[a]	No fluorescence (–)
CAM + ethanolic extract of cassava flour	0.096	Growth (3.5 cm)[a]	No fluorescene (–)
CFCAM (10% cassava flour)*	0.024	Growth (3.4 cm)[a]	No fluorescence (–)
CFCAM (20% cassava flour)‡	0.039	Growth (3.5 cm)[a]	No fluorescence (–)

* and ‡ corresponding to 0.3 μg/mL and 0.5 μg/mL of scopoletin, respectively.

Values in the same column followed by the same letter are not significantly different. Least significant difference at $P = 0.05$; fluorescence (+), aflatoxin production; no fluorescence (–), no aflatoxin production.

CAM, coconut agar medium; CFCAM, cassava flour enriched coconut agar medium; UV, ultraviolet.

Statistical Analysis

SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL) was used for statistical analyses.

Analysis of variance and Student Newman Keuls test were used to compare the means of *A. flavus* colonies diameter. The parameters were independent.

RESULTS

Molecular Characterization

The molecular characterization of the 18 isolates of *A. flavus* showed that six isolates had all the three genes of the aflatoxin cluster *aflD*, *aflO* and *aflP* coding for *Nor1*, *OmtB* and *Omt1*, respectively (Fig. 1).

Scopoletin Identification and Quantification

Scopoletin was quantitated in the freshly harvested roots and in flour samples made from the processed chips. An example of a chromatogram is showing the scopoletin peak (Fig. 2). As seen earlier (Gnonlonfin *et al.* 2011), significant scopoletin levels were found in this cassava variety (BEN 86052) at harvest and after sun drying for 6 days as chips with a mean level of 4.9 mg/kg dry weight and 242.5 mg/kg dry weight, respectively. Spiking true cassava flour sample with pure scopoletin showed the recovery of the method used to be 95% with a linearity of $R^2 = 0.986,4$ in the concentration range of 0.01 mg/mL–0.1 mg/mL.

In Vitro Test for *A. Flavus* Development and Aflatoxin Production

No inhibition of neither growth nor aflatoxin production was seen for ethanol alone. Strikingly, however, our investigation of *A. flavus* growth and aflatoxin production has shown normal growth of the fungus but total inhibition of aflatoxin production on CAM added either cassava flour or extract. Adding pure scopoletin to the CAM gave the same result. Full inhibition (cassava flour) was seen at least down to the lowest concentration of 0.3 μg/mL tested (Table 2).

DISCUSSION

Our investigation of *A. flavus* isolates from cassava chips showed that two third of the isolates did not possess all the genes needed for the biosynthesis of aflatoxins, while one third of the isolates did possess the genes, i.e., *aflD*, *aflO* and *aflP*, cluster genes previously shown to be present in aflatoxin-producing strains as compared with aflatoxin nonproducing strains of *A. flavus* (Scherm *et al.* 2005).

Data from the scopoletin analysis showed its presence in the cassava variety BEN 86052 in Benin. The concentration increased strongly upon drying after harvest (Gnonlonfin *et al.* 2011). This investigation of harvested cassava root tissues thus confirmed the results described by other authors (Tanaka *et al.* 1983; Buschmann *et al.* 2000). These earlier investigations showed an early increase (after 6 days of drying) because of scopoletin accumulation in the cell walls of the xylem vessels and in the parenchymatic cells (Buschmann *et al.* 2000). This increase was interpreted as a direct response to wounding (during processing into chips) and a defensive response to the invasion of microorganisms (Buschmann *et al.* 2000).

Different biological activities of scopoletin have been described including antifungal activity (Rodriguez *et al.* 2000). However, this is the first time that scopoletin is investigated both concerning its activity against toxigenic *A. flavus* growth and on aflatoxin production (Table 2). Pure scopoletin, ethanolic root extract (in which scopoletin is soluble) and cassava flour CAM inhibited the production of aflatoxin, pointing to this compound as a candidate for the seen inhibition; maybe together with other compounds in presence.

Coumarins, in general, show inhibitory effects on enzymes and interactions with DNA (Zobel 1997; Ojala *et al.* 1999). The normal growth combined with the absence of fluorescence and hence aflatoxin production (Table 2) is in agreement with previous results showing presence of *A. flavus* in aflatoxin negative cassava chips samples collected in Benin (Gnonlonfin *et al.* 2008; unpublished observations,

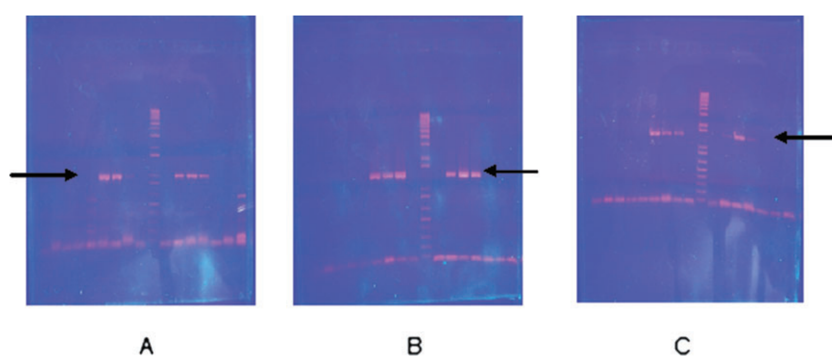

FIG. 1. AMPLIFIED DNA FRAGMENTS DETECTED ON AGAROSE GEL (INDICATED BY THE ARROWS) IN THE AFLATOXIN-PRODUCING STRAINS OF *ASPERGILLUS FLAVUS* GROWN FOR 5 DAYS AT 25C IN AFLATOXIN-INDUCING MEDIUM. THE MOLECULAR WEIGHT (BP) OF SPECIFIC TRANSCRIPTS IS INDICATED. (A) *Nor1* (990 BP), (B) *OmtB* (1,333 BP) AND (C) *Omt1* (1,490 BP)

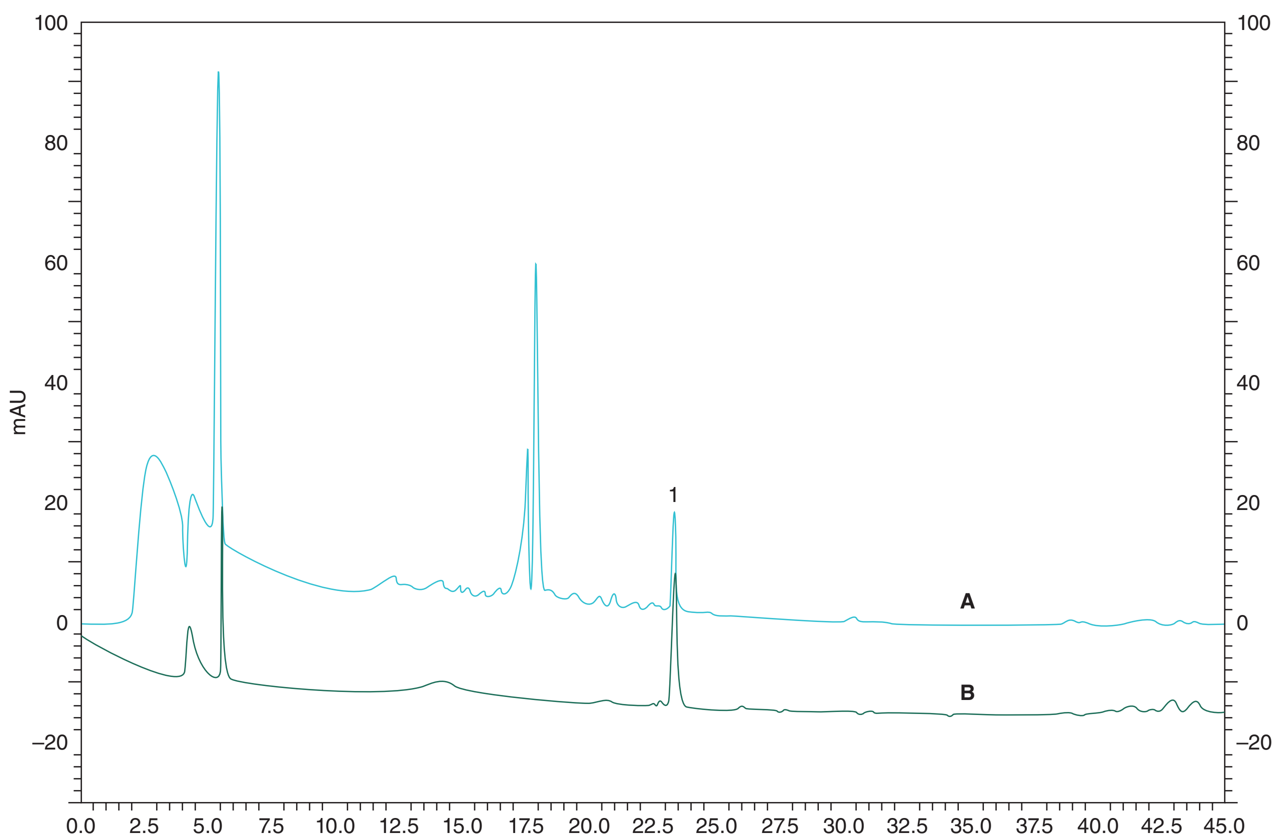

FIG. 2. LIQUID CHROMATOGRAMS OBTAINED DURING THE ANALYSIS. (A) CASSAVA FLOUR AND (B) PURE PURCHASED STANDARD (0.1 mg/mL). 1, SCOPOLETIN PEAK

2011) and with similar results from Tanzania (Muzanila *et al.* 2000), Ghana (Wareing *et al.* 2001) and Nigeria (Jimoh and Kolapo 2008). In spite of the finding of *A. flavus* in all these investigations, absence of aflatoxin can then be explained by an inhibition of aflatoxin synthesis.

The results presented here showed evidence of scopoletin accumulation in a cassava variety used for chips production in Benin in agreement with earlier reports by Buschmann *et al.* (2000), who also showed scopoletin to be dominant compound to accumulate. In addition, this compound shows an inhibitory effect on the production of aflatoxin by toxigenic *A. flavus*. The results could present an explanation to the absence of aflatoxin in *A. flavus*-infected cassava products earlier reported (Muzanila *et al.* 2000; Wareing *et al.* 2001; Gnonlonfin *et al.* 2008; Jimoh and Kolapo 2008).

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