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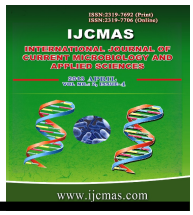

Original Research Article

Mycotoxin- Producing Potential of Moulds isolated from Traditional Cheese Wagashi produced in Benin

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K e y w o r d s

Mycotoxins; aflatoxinB_2; citrinin; moulds; Cheese wagashi; Benin.

A B S T R A C T

Sixteen strains of moulds isolated from traditional cheese wagashi produced in Benin have been screened for their ability to produce mycotoxins in Sucrose/Magnesium sulphate/ potassium (Kalium) nitrate/ Yeast extract (SMKY) broth media by thin layer chromatography. Griseofulvin was use as standard to determine retention factor value and to identify secondary metabolites produced. All strains were previously cultivated in Malt Extract Agar. Qualitative ability of mycotoxin production of these strains was performed by fluorescence emission under ultra violet light at 365 nm after extraction of metabolites with chloroform. Results obtained showed that *A. flavus* produced aflatoxin B_2 whereas *Penicillium citrinum* strain produced citrinin. *Aspergillus versicolor, A. ochraceus, A. tamarii, A niger, A terreus, A. nidulans, A. carbonarius, A. fisheri, A. candidus, A. wentii, P. griseofulvum, P. islandicum, F. poae* and *F. verticillioides* had also produced mycotoxins which were not confirmed because of the lack of reference data . In sum, all strains of moulds investigated are toxinogenic. These secondary metabolites detected and above all aflatoxin B_2 and citrinin could be produced inside cheese wagashi. Their presence in wagashi must be investigated in order to evaluate health hazardous for cheese wagashi consumers.

Introduction

Fungal contamination of food products is a chronic problem in developing countries and results in a decline in quality, quantity, nutrient content and monetary value. According to an investigation, pathogenic fungi alone cause a nearly 20% reduction in the yield of major food and cash crops (Tian *et al*., 2011; Prakash *et al*., 2012). Traditional cheese wagashi processed in Benin is an important food which can constitute a substitute of meat and eggs.

However, this foodstuff is susceptible to attack by various microorganisms such as *Aspergillus*, *Penicillium* and *Fusarium* species because of the warm, humid climate in this country (Sessou *et al*., 2012). Apart from their potential to cause yield losses and food decay, many fungal species represent a serious risk for consumers because of their production of dangerous secondary metabolites such as mycotoxins.

Mycotoxins are a group of structurally diverse secondary metabolites produced by various fungal species. These toxic compounds can contaminate foodstuffs, crops or human foods. The ingestion of these contaminated materials may be pathogenic in animals and humans as they may lead to serious health problems, such as liver, kidney or nervous system damage, immunosuppression and carcinogenesis. They comprise a group of several hundreds of chemically different toxic compounds (Zohra and Fawzia, 2013). Thus, cheese contaminated with these toxinogenic fungi is a major concern which has to receive attention due to their probable deleterious effect through their toxic metabolite on human health as well as their importance in international food trade (Soubra *et al*., 2009; Al-Rahmah *et al*., 2011). The survey of toxinogenic moulds in foodstuffs may have thus great interest in order to develop measures to their control and to avoid or minimize public health hazards.

In our previous work, 29 fungal strains were isolated in traditional cheese wagashi (Sessou *et al*., 2012) among which *Aspergillus candidus, A. carbonarius, A. fischeri, A flavus, A. nidulans, A. ochraceus, A. tamarii, A. terreus, A. versicolor, A. wentii, Penicllium griseofulvum, P. islandicum, P. citrinum, Fusarium poae* and *Fusarium verticillioides* were classified based on literature data as most fatal fungi through their production of very toxic metabolites (Pitt and Hocking, 2009). The examination of ability of these strains to produce toxic secondary metabolites may be studied in order to predict possible mycotoxins which may be encountered in traditional cheese wagashi. The objective of this study was to confirm that these moulds species classified as toxinogenic are able to produce mycotoxins in perspective to investigate the presence of metabolites detected in cheese wagashi and to propose alternative methods to their control.

Materials and Methods

The strains studied were previously identified based on their morphological and microscopic characteristics according to methods of Pitt and Hocking (2009). They were constituted of *Aspergillus candidus, A. carbonarius, A. fischeri, A flavus, A. nidulans, A. ochraceus, A. tamarii, A. terreus, A. versicolor, A. wentii, Penicllium griseofulvum, P. islandicum, P. citrinum, Fusarium poae* and *Fusarium verticillioides*.

Each strain representing the fungal species of *Aspergillus*, *Penicillium* and *Fusarium* were tested to determine their ability to produce mycotoxins. The *in vitro* detection of their secondary metabolite was performed by coupled methods of Singh *et al.*, (1991); Shukla *et al.*, (2012); Mishra *et al.*, (2012); and Zohra and Fawzia (2013), using Thin Layer Chromatography (TLC). A fungal disc of 5mm diameter from seven days old culture of mould grown on Malt Extract Agar (MEA) medium was aseptically inoculated on 25 ml of the SMKY broth medium

(sucrose 200 g; MgSO4. 7H2O, 0.5 g; KNO3, 0.3 g and yeast extract, 7 g; 1 L distilled water) in each 100 ml flask. The flasks were incubated at 28±2◦C for 10 days. After incubation, content of each flask was filtered through Whatmann filter paper No. 1 and then extracted with 20 mL chloroform in a separating funnel. The extract was evaporated to dryness on water bath and redissolved in 1 mL of chloroform. Fifty micro liter chloroform extract was spotted on TLC plates (TLC Silica gel 60 F_{254}, Merck, Germany) with a standard Griseofulvin (Sigma, G4753-5G; 010M0537 Product of China, MSDS available SL 10243, EC. 204-767-4,WGK.3) and developed in the solvents system comprising TEF (Toluene/ ethylacetate/Formic acid, 5:4:1 v/v/v) and CAP (Chloroform/ acetone/ 2-propanol, 85:15:20, v/v/v). The plate was air dried and observed under long wave (365 nm) UV and then spray with agent sp1 (ANIS: 0.5% p-anisaldehyde in methanol/acetic acid/concentrate sulphuric acid (17:2:1 v/v/v). After spraying, the spot of each metabolite was air dried and observed under long wave (365 nm) UV light in a dark cabinet. The retention factors (RF_{CAP} and RF_{TEF}) of the individual spots on TLC were calculated and compared with that of Griseofulvin (Rf_g TEF: 1.00; Rf_g CAP: 1.00) and the colour of each spot was compared with those of standard mycotoxins to aid in the identification of mycotoxins presented.

Results and Discussion

Mycotoxins are known to induce serious health threats such as liver, kidney or nervous system damage, immuno-suppression and carcinogenesis in animals and human. Therefore, the presence of toxinogenic fungi and mycotoxins in foods especially cheese wagashi presents a potential hazard to human health (Al-Rahmah *et al*., 2011). In the present study, sixteen strains of moulds isolated from cheese wagashi were investigated for their ability to produce mycotoxins.

The results obtained from this study are presented in table 1 and showed that all the strains investigated were mycotoxins producers. *Aspergillus flavus* specie has shown aflatoxinogenic B2 potential whereas *Penicillium citrinum* showed the production of citrinin. The others species produced unknown metabolites which could not be confirmed because of the lack of reference data. The retention factors values determined for these metabolites after thin layer chromatography assay and the colors of spots of the extracted metabolites observed at 365 nm UV are shown in figures 1 and 2. Observation of these spots showed that the majority of the metabolites detected had blue fluorescence color, a characteristic color for most aflatoxins. The others colors are yellow green fluorescence (metabolite 8), blue green fluorescence (metabolite 16) and purple (metabolites 9 and 12). Aflatoxin B_2 was blue fluorescence color whereas citrinin color was yellow green fluorescence.

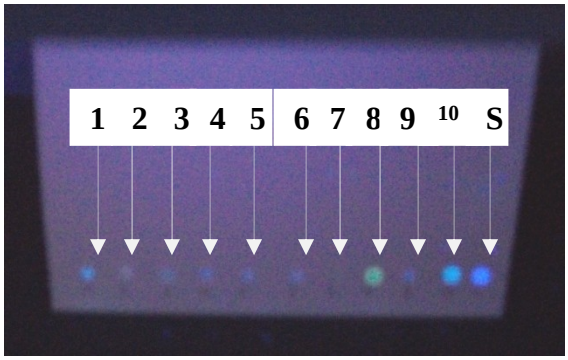
In this work, *Aspergillus nidulans* and *Aspergillus versicolor* producers of sterigmatocystin, *Aspergillus ochraceus*, *Aspergillus carbonarius*, *Aspergillus niger* producers of ochratoxin, *Fusarium verticllioides* and *Fusarium poae* respectively producers of fumonisins and T-2 toxin, *Aspergillus versicolor* and *Aspergillus tamarii* shown as sterigmatocystin and cyclopiazonic acid producers didn't produce their respective metabolite as reported elsewhere in

Table.1 Strains with characteristics of their secondary metabolite produced

Number of strain	Name of strain	Rf_g TEF	RF_g CAP	Colour	Suspected metabolite name
1.	*A. candidus*	0.32	0.48	Blue fluorescence	Unknown
2.	*A. carbonarius*	1.23	0.86	Blue fluorescence	Unknown
3.	*A. fisheri*	0.76	1.12	Blue fluorescence	Unknown
4.	*A. tamarii*	0.41	0.85	Blue fluorescence	Unknown
5.	*A. nidulans*	ND	1.00	Blue fluorescence	Unknown
6.	*A. niger*	ND	0.94	Blue fluorescence	Unknown
7.	*A. ochraceus*	1.28	0.40	Blue green fluorescence	Unknown
8.	*A. flavus*	0.39	0.85	Blue fluorescence	Aflatoxin B2
9.	*A. terreus*	1.12	0.15	Blue fluorescence	Unknown
10.	*A. versicolor*	1.14	1.37	Purple	Unknown
11.	*A. wentii*	0.45	0.77	Blue fluorescence	Unknown
12.	*F. poae*	ND	0.96	Blue fluorescence	Unknown
13.	*F. verticillioides*	ND	1.00	Blue fluorescence	Unknown
14.	*P. citrinum*	1.12	0.28	Yellow green fluorescence	Citrinin
15.	*P. griseofulvum*	1.15	0.85	Blue fluorescence	Unknown
16.	*P. islandicum*	ND	0.92	Purple	Unknown

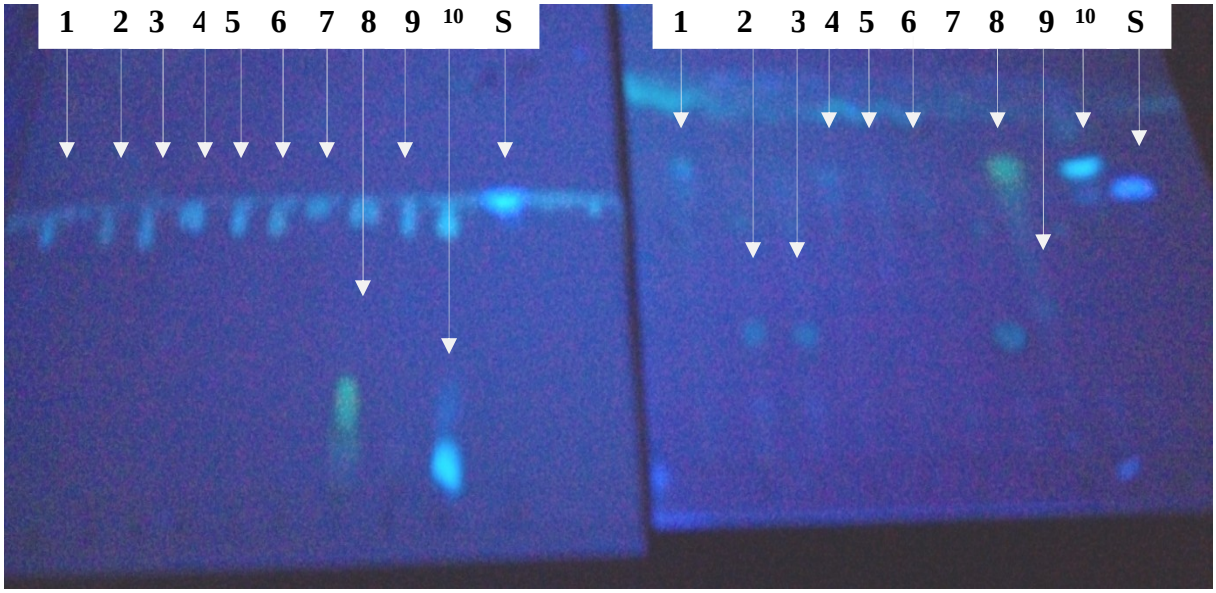
Key: Rf_g TEF: retention factor value determined for metabolite based on standard griseofulvin retention factor after elution in solvent TEF.
Rf_g CAP: retention factor value determined for metabolite based on standard griseofulvin retention factor after elution in solvent CAP.

Figure.1 Photography of spots of secondary metabolites extracted from strains observed at UV 365 nm without their elution in solvent of TLC.



1, 2, 3, 4, 5, 6, 7, 8, 9, 10 are respectively metabolites of *P. griseofulvum, A. tamarii, A. flavus, A. niger, F. poae, F. verticillioides, A. nidulans, P. citrinum, P. islandicum* and *A. terreus;* S: standard griseofulvin.

Figure. 2 Photography of spots of secondary metabolites extracted from strains observed at UV 365 nm after TLC assay in solvents CAP (left) and TEF (right)



1, 2, 3, 4, 5, 6, 7, 8, 9, 10 are respectively metabolites of *P. griseofulvum, A. tamarii, A. flavus, A. niger, F. poae, F. verticillioides, A. nidulans, P. citrinum, P. islandicum* and *A. terreus;* S: standard griseofulvin

literature. This fact could be explained by the fact that SMKY medium used is not very suitable for these metabolites production (Makun *et al*., 2011). Also, this fact may be linked to the fact that the extract solvent chloroform appropriate for aflatoxins extraction is not proper for ochratoxin, fumonisins, cyclopiazonic acid, patulin and sterigmatocystin. However, this study certifies that all the strains are mycotoxins producers (Makun *et al*., 2011).

The *in vitro* production of secondary metabolites especially citrinin and aflatoxin B_2 by *Penicillium citrinum* and *Aspergillus flavus* respectively could present a health hazards to wagashi consumers if once inside this cheese these toxinogenic strains investigated. Also, these toxinogenic strains investigated may lead to occurring in cheese wagashi of very toxic mycotoxins and therefore may be a potential risk of consumers. In fact, aflatoxins are both acutely and chronically toxic to man. They have long been known to produce four distinct effects: acute liver damage, liver cirrhosis, induction of tumors and teratogenic effects (Pitt and Hocking, 2009). More recent information indicates that the consequences of prolonged aflatoxin exposure are more widespread, including immunosupression and interference with protein uptake (Williams *et al*., 2004).

Citrinin is a significant hepato-nephrotoxin (Bao-jun *et al*., 2006). It is

heat sensitive and decomposes during heat treatment to form other complex compounds, such as citrinin H1 and citrinin H2, respectively with higher and weaker cytotoxicity than the original citrinin (EFSA, 2012). *Aspergillus nidulans* and *Aspergillus versicolor* have been reported to produce sterigmatocystin responsible for human liver cancer (Minervini *et al*., 2002) whereas *A. niger*, *Aspergillus ochraceus* and *Aspergillus carbonarius* produces ochratoxin A which is a probable human carcinogen (Hocking *et al*., 2006). *Aspergillus tamarii* produce cyclopiazonic acid (Pitt and Hocking, 2009) whereas *Fusarium poae* can indeed produce T-2 toxin which is one of the most toxic trichothecenesis, haematotoxic and immunosuppressive (Pitt and Hocking, 2009).

The major mycotoxin produced by *Fusarium verticillioides* is Fumonisin B1, the cause of liver cancer in human oesophageal cancer (Shephard *et al*., 2007). *Penicllium griseofulvum* produces four mycotoxins, patulin, cyclopiazonic acid, roquefortine C and griseofulvin (Samson and Frisvad, 2004). Patulin and cyclopiazonic acid are moderately toxic compounds (Pitt and Hocking, 2009). *Penicillium islandicum* produces at least four mycotoxins Cyclochlorotine, islanditoxin, luteoskyrin and erythroskyrin, unique to the species. Both Cyclochlorotine and islanditoxin compounds are very toxic. Cyclochlorotine caused liver cirrhosis, fibrosis and tumours (Uraguchi *et al*., 1972). Luteoskyrin and erythroskyrin are both liver and kidney toxins, though less acutely toxic than cyclochlorotine. Luteoskyrin is also carcinogenic (Pitt and Hocking, 2009).

Thus, cheese contaminated with these toxinogenic fungi is a major concern due to their deleterious effect on human health through their mycotoxins production (Soubra *et al*., 2009). In sum, the presence of these toxinogenic strains in cheese wagashi allows thinking the production of citrinin, aflatoxin and others toxic metabolites such as ochratoxin A, cyclopiazonic acid, patulin, sterigmatocystin and many more in cheese wagashi. The quantitative analysis of these toxins in cheese wagashi may be considered.

This study has demonstrated the ability of moulds isolated in cheese wagashi to produce mycotoxins in SMKY broth. Whereas *Aspergillus flavus* have shown aflatoxigenic B_2 potential, *Penicillium citrinum* produced citrinin and the other species produced other unknown secondary metabolites. Further studies are needed to be performed in order to identify these unknown metabolites. Inhibition of these toxinogenic species growths and their toxic metabolites production by plants extracts will be tested for the reduction of illness due to these secondary metabolites consumption in cheese wagashi.

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