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Full Length Research Paper

Palm kernel agar: An alternative culture medium for rapid detection of aflatoxins in agricultural commodities

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The feasibility of using palm kernel agar (PKA) as an alternative culture medium to desiccated coconut agar (DCA), the conventional medium for the recovery of aflatoxigenic fungi from mixed cultures and the detection of aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities was assessed. The medium recovered aflatoxigenic fungi in 48 h from mixed cultures and agricultural commodities in 58 h as compared to 62 h obtained for the two treatments on desiccated coconut medium. Aflatoxigenic fungi were detected in all the agricultural commodities except for onions with maize having the highest value of 13.18% (w/w) followed by melon (10.97), yam flour (10.23) and groundnut (8.52) as against 11.48, 10.0, 6.92 and 8.52% (w/w) obtained for DCA. All aflatoxigenic strains produced a characteristic yellow pigmentation on a pink background and blue or blue green fluorescence of palm kernel agar Under long wave UV light (366nm) as against the white background of DCA, which often interferes with fluorescence with corresponding yield of aflatoxins. This shows that the medium is able to efficiently detect aflatoxin production through direct visual observation of fluorescence. Palm kernel agar (PKA) can therefore be routinely used as an alternative culture medium for screening aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities since it is faster and has a unique pink background for easy identification.

Key words: Aflatoxins, aflatoxigenic moulds, mixed cultures, and desiccated coconut agar, palm kernel agar.

INTRODUCTION

The problem of food and feed contamination with aflatoxigenic fungi and elaboration of toxins has received great deal of attention during the last three decades. The frequent incidence of these toxins in agricultural commodities has a potential negative impact on the economies of the affected regions especially in the developing countries. This is because harvest and post harvest techniques adequate for the prevention of mould growth are seldom practiced coupled with inadequate storage facilities (Halliday, 1965).

Aflatoxins are highly toxic and carcinogenic compounds that have been implicated as causative agents in human hepatic carcinogenesis (Massey et al., 1995). They are produced by species of *Aspergillus flavus*, *A.* p *arasiticus and A. nomius* which is phenotypically similar to A. *flavus*, but with a distinctive bullet shaped sclerotia. Other species that produce aflatoxins in minute quantities include *A. peudotamarii*, *A. bonbysis* and *A. ochraceoro-seuss*. However, not all strains are able to produce aflatoxins and this has led to the development of various methods to screen for their aflatoxin producing abilities (Fente et al., 2001). In addition, it has been found that it

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is easier (especially in laboratories that lack facilities for chemical determination of aflatoxins) and more economical to first identify aflatoxigenic fungi in contaminated materials and aflatoxins by fluorescence of agar than to chemically test for aflatoxins. Desiccated coconut agar (DCA), the conventional medium used for this purpose (Davis et al., 1987) is difficult to come by in Nigeria, as desiccated coconut is not readily available. Furthermore, information on recent studies in media formulation using locally available materials are scanty (Adesemoye and Adedire, 2005). Such studies will however continue for some time to come especially in developing countries like Nigeria, where research in microbiology is hindered by high cost and scarcity of culture media (Poopathi et al., 2002) . Recently, a new and simple diagnostic medium (PKA) was formulated in our laboratory using palm kernel (Atanda et al., 2005). 20% (v/v) palm kernel extract was incorporated into agar medium and aflatoxigenic fungi detected *in vivo* by the production of a characteristic yellow pigmentation and a blue or blue-green fluorescence against a pink background. This is in contrast to the white background of the conventional DCA agar. Further tests with toxigenic isolates from various sources showed that pigmentation, fluorescence and aflatoxin production were complimentary (Atanda et al., 2005).

This paper reports the use of palm kernel agar (PKA) as an alternative culture medium to desiccated coconut agar (DCA), for the recovery of aflatoxigenic fungi from mixed cultures and the detection of aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities

MATERIALS AND METHODS

Agricultural commodities

All samples were purchased from local markets in Nigeria in sterile polyethyene bags and used immediately or stored at 4ºC overnight after which they were analysed. Chilli and coriander seed were purchased from India and stored at 4ºC before shipment to Nigeria. Samples were not frozen or chilled during the 24 h transit time. All experiments were replicated thrice to reduce variability except in the quantification of aflatoxins where duplicate determinations were carried out

Chemicals

Aflatoxin B_1 and G_1 standards were obtained from Sigma Aldrich Company. Other chemicals from Sigma include benzene, chloroform and acetone and were analar grades.

Test organisms

Twenty three fungi cultures out of which 12 were aflatoxigenic were obtained from the National Chemical Laboratory, Pune, India, Microbial Type Culture Collection, Chandigarh, India and the American Type Culture Collection, USA. They were stored in the Culture Collection Centre of Food Microbiology Department, Central Food Technological Research Institute (CFTRI), Mysore, India and used for this study. The fungi were cultivated on Czapek dox agar at 30ºC in darkness for 7 days and stock cultures maintained and renewed bimonthly on same agar at 4ºC. The cultures had been screened for production of pigments and fluorescence on PKA and DCA (Atanda et al., 2005). *Aspergillus parasiticus* CFR223, which had the brightest fluorescence because it produced aflatoxins B_1, B_2, G_1 and G_2 was used as the reference isolate.

Media formulation

The basal medium (PKA) and DCA were prepared according to Atanda et al. (2005) and Davis et al. (1987), respectively**.**

Recovery of aflatoxigenic moulds from mixed cultures

A loophole of spores from all the test organisms was mixed together to form a spore suspension that was diluted ten fold and 0.1 ml of the spore suspension spread plated on potato dextrose agar (PDA), a general purpose agar, DCA and PKA. Plates were incubated at 30ºC for 120 h in the incubator. Aflatoxigenic moulds were detected in the mixed cultures by yellow pigmentation and fluorescence of agar under UV light (365 nm). The time of production of pigments and the appearance of media fluorescence were assayed. In addition, the total number of isolates recovered from PDA, PKA and DCA were enumerated and the relative percentage of toxigenic isolates calculated by simple proportion.

Detection of aflatoxigenic moulds from common foods and poultry feeds

For each commodity, a total of 10 samples were analysed. Each sample was diluted ten fold and 0.1 ml portions spread plated on PDA, PKA and DCA media to which 0.01% chloramphenicol had been added separately to inhibit bacterial growth. Plates were incubated at 30ºC for 120 h and aflatoxigenic moulds detected as stated above. The time of production of pigments and the appearance of media fluorescence were also assayed. In addition, the total number of isolates recovered from PDA, PKA and DCA were enumerated and the relative percentage of toxigenic isolates calculated.

Extraction and quantification of aflatoxins

Since pigmentation, fluorescence of agar and aflatoxin production were complimentary on palm kernel agar (Atanda et al., 2005), all isolates showing pigmentation and positive fluorescence on PKA and DCA were extracted according to the Pon's (1980) method as modified by Rati et al. (1987) and developed in a benzene: glacial acetic acid : methanol (18:1:1, v/v/v) solvent system and air dried. Aflatoxins were identified on the basis of co-migration with aflatoxin standards and their characteristic fluorescent colour under long wave UV light (365 nm). The spots that gave minimum fluorescence were noted and the quantity of aflatoxin calculated according to Coomes et al. (1965) method.

Statistical analyses

The data obtained were analysed by ANOVA using SPSS statistical version 10.1 (Anon, 2002).

Table 1. Aflatoxigenic moulds[1] recovered from mixed cultures by basal medium.

Time (h)	PDA (Mould count)	PKA		DCA (Control)	
		Aflatoxigenic Mould	% No of Aflatoxigenic Mould	Aflatoxigenic Mould	% No of Aflatoxigenic Mould
48	3.32[a]	2.00[a]	4.76[a]	0.00[a]	0.00[a]
62	3.36[a]	2.30[b]	8.70[b]	2.30[d]	8.70[b]
72	3.36[a]	2.60[d]	17.39[d]	2.48[c]	13.04[c]
120	3.36[a]	2.57[c]	16.08[c]	2.00[b]	0.04[a]
SE_M	0.11	0.11	0.11	0.11	0.11

[1] Log_{10} cfu/ml

Mean values followed by different superscript within a column are significantly different using Duncans multiple range test ($P \leq 0.05$).

Table 2. Aflatoxigenic moulds[1] detected from agricultural commodities with basal medium.

Commodities	PDA (Mould count)	PKA		DCA (Control)	
		Aflatoxigenic Mould	% No of Aflatoxigenic Mould	Aflatoxigenic Mould	% No of Aflatoxigenic Mould
Chilli	6.80[d]	2.57[c]	0.01[a]	2.73[f]	0.01[a]
Sorghum	6.77[d]	2.29[c]	0.00[a]	2.35[c]	0.00[a]
Groundnut	4.10[c]	3.03[f]	8.52[f]	3.03[g]	8.52[f]
Coriander seed	6.40[d]	2.0b	0.0a	1.10[b]	0.04[a]
Yam flour	3.60[b]	2.61[c]	10.23[g]	2.44[c]	6.92[e]
Cassava flour	3.75[b]	2.45[d]	5.01[d]	2.17[d]	2.63[c]
Poultry feeds	6.26[d]	2.14[d]	0.01[a]	2.32[c]	0.01[a]
Maize	4.15[c]	3.27[g]	13.18[h]	3.21[b]	3.21[b]
Rice	3.20[b]	2.00[b]	6.31[e]	2.00[d]	6.31[e]
Beans	3.70[b]	2.31[c]	4.07[c]	2.29[e]	3.89[d]
Melon	3.21[b]	2.25[c]	10.97[h]	2.21[d]	10.00[g]
Onions	2.17[a]	0.00[a]	0.68[a]	0.00[a]	0.68[a]
Garri	4.0[c]	2.23[c]	1.70[b]	2.20[e]	1.59[b]
SE_M	±0.01	±0.01	±0.01	±0.01	±0.01

[1] log_{10} cfu/ml

Mean values followed by different superscript within a column are significantly different using Duncans multiple range test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Aflatoxigenic moulds were recovered from mixed cultures on PKA and DCA at 48 h and 62 h, respectively (Table 1). However, when a pure culture of A. parasiticus CFTR 223 was used, fluorescence was observed earlier at 44 h on PKA and 48 h on DCA (Atanda et al., 2005). The result confirms our earlier findings *in vitro*, that PKA is a faster medium for the detection of aflatoxigenic fungi. The delay in appearance of fluorescence when compared with pure isolates used *in vitro*, could be due to the competing microflora in the mixed cultures. Maximum intensity of fluorescence was observed in both media at 72 h.

Table 2 shows the counts of aflatoxigenic moulds recovered from some agricultural commodities majority of which our common foods by PKA and DCA. Aflatoxigenic moulds were detected in all food commodities except for onions with maize having the highest value 13.18% (w/w) followed by melon (10.97), yam flour (10.23) and groundnut (8.52) . Extracts of onions and welsh onions had been shown to be inhibitory against *A. flavus* and *A. parasiticus* (Sharma et al., 1979; Fan and Chen, 1999). Several workers had reported *A. parasiticus* and *A. flavus* in Nigerian maize and groundnut cakes (Aja et al., 1994; Akano and Atanda, 1990). It was also observed that PKA was faster than DCA in detecting aflatoxigenic moulds in the foods and poultry feeds with a time of 58 h (data not shown) as compared to 62 h of DCA. In addition, all aflatoxigenic moulds exhibited a characteristic yellow pigmentation and blue fluorescence (B_1) or blue green fluorescence (G_1) against a pink background on PKA. This unique characteristic was not observed on DCA whose white background often interferes with fluorescence. This characteristic can be used to presumptively screen large numbers of microbial isolates in fields and in commodity purchase stations. Maximum intensity of fluo-

Table 3. Natural occurrence of aflatoxins in some agricultural commodities

Commodity	Types of Aflatoxin (μg/g)*						
	Positive** sample	PKA			DCA		
		B1	G1	B1+G1	B1	G1	B1+G1
Chilli	1	2.2	1.8	4	2.1	1.4	3.5
Sorghum	2	2.8	1.4	4.2	2.8	1.25	4.05
Groundnut	6	4	2.3	6.3	4	2.24	6.24
Coriander seed	2	1	0	1	1	0.02	1.02
Yam flour	6	3.5	2.5	6	3.5	2.5	6
Cassava flour	6	2.2	1.8	4	2.15	1.6	3.75
Poultry feed	2	3	1	4	2.85	1	3.85
Maize	7	4.3	3.3	7.6	3	4.5	7.5
Rice	3	0.8	2	2.8	0.9	2	2.9
Beans	4	0.65	0	0.65	0.4	0	0.4
Melon	6	2.6	4	6.6	2.35	3.4	5.75
Onions	0	0	0	0	0	0	0
Gari	3	1.7	0	1.7	1.48	0	1.48

*Data are means of two replicates.
** Positive samples out of a lot of 10 samples/commodity.

rescence was observed at 72 h on both media and this agrees with the findings of Lin and Dianese (1976) for coconut medium.

Table 3 shows the natural occurrence of aflatoxins in agricultural commodities. Interestingly, aflatoxins were produced in all food commodities in which aflatoxigenic moulds were detected while no detectable level of aflatoxin was found in onions. This suggests that the medium is able to efficiently detect aflatoxin production through direct visual observation of blue fluorescence (B_1) or blue green fluorescence (G_1). A similar result was observed by Ordaz et al. (2002) on DCA. The result also showed that 70% (w/w) of maize sampled contained aflatoxins followed by melon, yam flour, cassava flour and groundnut each with 60% (w/w) levels of aflatoxins. This result is in agreement with the findings of Ibeh (1992), Kaushal (1990) and Halliday (1965). The concentration of aflatoxins found in maize (7.50 μ/g), melon (6.60 μ/g), yam flour (6.00 μ/g), cassava flour (4.00 μ/g) and groundnut (6.30 μ/g) were higher than the minimum human virtual safe dose (MHVSD) of 0.2ng/kg body weight/day (Oluwafemi, 2000). The finding of aflatoxins in doses above the acceptable levels in some of our common foods taken by a large segment of the population is of major health concern. A value of 3.85 μg/g was obtained in poultry feeds which are below the (FAO/WHO, 1990; 1992) limit of 5.0 μg/g. However Ibeh (1992) observed that rats and rabbits fed with feeds contaminated with 8.05 μg/g of aflatoxin B_1 for 14 days were unable to effect conception and had decreased body weight. They also had increased sperm abnormalities, decreased sperm mobility and viability and greater destruction of sperm cells. Prolonged intake of these contaminated materials can therefore constitute a health risk and significantly reduce net population growth rate. Similarly, Oluwafemi (2000) observed that aflatoxins had a negative impact on fertility.

The high incidence of aflatoxigenic moulds and aflatoxins in our foods and poultry feeds could be due to inadequate storage facilities, late harvesting of crops from farms and climatic conditions (high moisture content), which all favour fungal attack on foods. The processing methods in addition to the presence of fermentable carbohydrates in the foods also facilitate aflatoxin production by *Aspergillus* species (Patten, 1981). Proper handling methods, adequate storage facilities and planting of resistant varieties will help to reduce the high incidence of aflatoxins in our food commodities.

In conclusion, this study shows that palm kernel agar (PKA) can be routinely used as an alternative culture medium to desiccated coconut agar (DCA) for the recovery of aflatoxigenic fungi from mixed cultures, detection of aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities since it is faster and has unique pink background for easy identification.

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