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Evaluation of Seed Borne Mycoflora of Stored Cocoa Beans in South-West, Nigeria

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ABSTRACT

Healthy and infected stored cocoa beans were obtained from three randomly selected cocoa stores in each of the five cocoa producing states of Oyo, Ondo, Osun, Ekiti and Ogun in South-West, Nigeria. Mycoflora associated with the beans were isolated using three different techniques. A total number of eleven storage moulds: *Aspergillus flavus*, *A. ochraceous*, *A. versicolor*, *Rhizopus* sp., *Pythium* sp., *Fusarium solani*, *A. terreus*, *A. niger*, *A. fumigatus*, *Lasiodiplodia theobromae* and *Penicillium digitatum* were isolated from both healthy and infected beans samples. Findings from this study have revealed that while members of the *Aspergillus* spp. are the dominant storage fungi of cocoa beans in South-West, Nigeria, the direct plating technique can be relied upon for effective microbial isolation. Observation of good agricultural and handling practices is therefore necessary to reduce the storage mould population on the beans and thus limit the risk of contamination.

Key words: Healthy, infected, mycoflora, cocoa beans, contamination

INTRODUCTION

Theobroma cacao is a major non-oil foreign exchange earner in Nigeria. It is produced in fourteen (14) states of the country namely: Ondo, Cross River, Osun, Oyo, Ekiti, Ogun, Edo, Kogi, Akwa Ibom, Delta, Abia, Kwara, Adamawa and Taraba. Over 98% of the product is however exported and this provides the means of livelihood and employment to over five million (5,000,000) people (CRIN., 2008; MRP., 2012).

Cocoa beans are of commercial, nutritional and medical importance to man. Wholesome cocoa beans can be processed into different products like chocolate, cocoa beverages, cocoa butter, etc., which are of immense benefits to the body systems of consumers. Cocoa is a very important ingredient in cakes, biscuits, child-foods, ice-creams and sweets. Cocoa beans, originating as seeds in fruit pods of the tree *T. cacao*, are also sources of cocoa powder (Sanchez-Hervas *et al*., 2008).

Since, neither storage nor processing conditions of cocoa are strictly controlled in the tropical countries, fungi contamination of cocoa beans is possible at many critical points in the cocoa production chain (Magan and Aldred, 2005). The beans are susceptible to fungal spoilage during and after fermentation. Fungal species belonging to the genera *Aspergillus, Mucor, Penicillium* and *Rhizopus* have been observed on mishandled or improperly dried fermented beans (Sanchez-Hervas *et al*., 2008).

The development of moulds (fungi) on cocoa beans during storage has constituted a major challenge to cocoa production in the south-western part of Nigeria and in virtually all the fourteen cocoa producing states of the nation. Just like is the case for all plant pathogens, the continuous presence of these organisms would most expectedly cause some alterations in the physical and biochemical properties of the stored beans, thereby reducing their overall qualities, marketing values and overall acceptability in the world market. In addition, some storage moulds have been found to be able to produce toxic substances, which, if taken in with the infected plant materials or their by-products, may pose a threat to life (Fagbohun *et al*., 2011). This work is therefore aimed at isolating and identifying the specific storage moulds associated with both healthy and infected cocoa beans in the south-western region of Nigeria.

MATERIALS AND METHODS

Sample collection: Samples of infected and healthy cocoa beans were obtained in the dry season (November-January) from three randomly selected cocoa stores in each of the five cocoa producing states of Ondo, Oyo, Osun, Ekiti and Ogun in South-West, Nigeria. Seed mycoflora of the randomly picked cocoa beans were then isolated using the direct (agar) plating, dilution plating and standard blotter paper methods.

Direct plating: Healthy and infected stored cocoa beans obtained from each of the cocoa store locations were cut into small pieces with the aid of a sterile knife. The samples were then surface sterilized in 2% sodium hypochlorite for 2 min and rinsed twice in sterile distilled water. The surface sterilized beans samples were blotted with the aid of sterile Whatman No.1 filter papers and inoculated into already prepared Potato Dextrose Agar (PDA) Petri-plates. The plates were incubated at ambient temperature for 4 days and later sub-cultured to obtain pure cultures of the moulds.

Dilution plating: Both the healthy and mouldy (infected) cocoa beans were surface sterilized in 2% sodium hypochlorite and blot-dried. The beans were ground with the aid of a sterile mortar and pestle. One gram of each of the samples was serially diluted with 9 mL sterile water in sets of test tubes. The first (10^{-1}) and third (10^{-3}) dilutions of each of the samples were pour-plated with molten but cooled (45°C) PDA already sterilized at 121°C and 1.1 kg cm^{-2} for 15 min. The inoculated plates were incubated at ambient temperature for 4-7 days and pure cultures were obtained.

Standard blotter paper method: Pairs of sterile white blotter papers of 8.5 cm diameter were soaked with distilled water and placed in pre-sterilized Petri-plates of 8.5 cm diameter. The cocoa beans samples (healthy and infected) were surface sterilized with 2% sodium hypochlorite after which, three beans of each of the test samples were placed at equal distance on the moist blotter inside a Petri-plate. The plates were incubated at ambient temperature for 7 days. On the seventh day of incubation, the various fungal growths observed were thoroughly examined under a stereoscopic microscope. Sub-cultures were made to obtain pure cultures of the observed fungi colonies.

Identification/characterization and enumeration: The cultural and microscopic characteristics of each of the fungi isolates obtained at each of the isolation instances described

above were noted for proper identification with the aid of a compound light microscope and Barnett and Hunter (1998) fungi identification book. Percentages of occurrence and populations of the mycoflora were also determined.

Statistical analysis: Results obtained from the experiment were subjected to Analysis of Variance (ANOVA) using Fisher's Least Significant Difference (LSD) test at 5% level of probability and with the aid of Statistical Analysis System (SAS) 9.1 statistical package.

RESULTS

Pure cultures of eleven storage fungi isolates: *Aspergillus flavus*, *A. ochraceous*, *A. versicolor*, *Rhizopus* sp., *Pythium* sp., *Fusarium solani*, *A. terreus*, *A. niger*, *A. fumigatus*, *Lasiodiplodia theobromae* and *Penicillium digitatum* were obtained from healthy as well as infected stored cocoa beans samples across the south-western region of Nigeria. The percent occurrence of the moulds isolated from each of the five south-western states of Oyo, Ondo, Osun, Ekiti and Ogun are as shown in Table 1. The significantly highest occurrence of *Aspergillus flavus*, 48.62% ($p \leq 0.05$) was observed on infected beans obtained from Oyo State. This was closely followed by those of the infected and healthy samples from Ekiti (44.81%) and Ondo (34.66%), respectively. The lowest occurrence of the organism (8.52%) was however observed on the infected samples from Osun State.

Infected cocoa beans samples obtained from Osun State had significantly highest occurrence ($p \leq 0.05$) of *A. fumigatus* (6.89%). This was followed by Oyo (infected), Osun (healthy) and Ekiti (infected) samples with 3.24, 1.85 and 1.19% respective percent occurrence. The fungus was not observed on Ondo and Ogun (healthy and infected), Oyo (healthy) and Ekiti (healthy) samples. The frequency of occurrence of *A. ochraceous* observed on healthy and infected samples from Oyo and Ondo States as well as those of healthy samples obtained from Osun and Ekiti ranged between 4.09-11.22%. The significantly highest frequency of occurrence (44.94%) was however recorded for the infected Ogun State samples (Table 1).

Infected samples from Ondo State gave significantly highest occurrence (30.53%) of *A. versicolor* ($p \leq 0.05$). This was followed by the infected samples from Ogun (22.74%), Osun (7.41%) and

Table 1: Percentage of storage moulds occurrence in healthy and infected cocoa beans across South-West, Nigeria

	Occurrence (%)										
State and samples	AF	AFM	AO	AV	AT	AN	PD	RS	PS	LT	FS
Oyo											
Healthy	8.57^{c}	0.00^{b}	7.62^{b}	0.00^{c}	0.00^{b}	25.11^{a}	3.03^{a}	0.00^{b}	13.33^{a}	12.73^{a}	29.61^{a}
Infected	48.62^{a}	3.24^{b}	11.22^{b}	0.00^{c}	0.00^{b}	7.97^{bc}	0.00^{a}	12.24^{ab}	6.67^{a}	0.00^{a}	10.05^{abc}
Ondo											
Healthy	34.66^{abc}	0.00^{b}	9.07^{b}	5.06^{bc}	1.28^{ab}	1.59^{bc}	32.02^{a}	2.56^{b}	5.79^{a}	0.00^{a}	7.95^{bc}
Infected	12.87^{c}	0.00^{b}	4.09^{b}	30.53^{a}	7.50^{ab}	3.92^{bc}	8.31^{a}	24.26^{a}	3.92^{a}	3.45^{a}	1.15^{c}
Osun											
Healthy	9.79^{c}	1.85^{b}	9.52^{b}	0.00^{c}	13.49^{a}	0.00^{c}	25.40^{a}	0.00^{b}	4.23^{a}	9.26^{a}	26.45^{ab}
Infected	8.52^{c}	6.89^{a}	25.19^{ab}	7.41^{bc}	8.74^{ab}	0.00^{c}	5.56^{a}	15.93^{ab}	5.56^{a}	9.33^{a}	6.89^{bc}
Ekiti											
Healthy	13.65^{c}	0.00^{b}	4.44^{b}	0.00^{c}	2.22^{ab}	20.16^{ab}	38.10^{a}	15.87^{ab}	0.00^{a}	1.11^{a}	4.44^{c}
Infected	44.81^{ab}	1.19^{b}	29.94^{ab}	3.57^{c}	0.00^{b}	3.18^{bc}	0.00^{a}	5.95^{ab}	8.67^{a}	0.00^{a}	2.69^{c}
Ogun											
Healthy	21.09^{abc}	0.00^{b}	18.33^{ab}	0.00^{c}	0.00^{b}	2.56^{bc}	0.00^{a}	19.55^{ab}	0.00^{a}	0.00^{a}	5.13^{c}
Infected	16.55^{bc}	0.00^{b}	44.94^{a}	22.74^{ab}	7.59^{ab}	2.89^{bc}	1.03^{a}	0.51^{b}	0.00^{a}	3.75^{a}	0.00^{c}

Means followed by the same superscript letter along the column are not significantly different at $p \leq 0.05$, according to Fisher's LSD test, AF: *Aspergillus flavus*, AFM: *A. fumigatus*, AO: *A. ochraceous*, AV: *A. versicolor*, AT: *A. terreus*, AN: *A. niger*, PD: *Penicillium digitatum*, RS: *Rhizopus* sp., PS: *Pythium* sp., LT: *Lasiodiplodia theobromae*, FS: *Fusarium solani*

Ekiti (3.57%), which were significantly different ($p \leq 0.05$) from one another. Healthy and infected samples obtained from Oyo State as well as healthy samples from Osun, Ekiti and Ogun State did not indicate the presence of the organism. The highest frequency of occurrence of *A. terreus* (13.49%) was observed on healthy cocoa beans samples obtained from Osun State. This was followed by those observed on infected samples from the same state (8.74%). The latter was however not significantly different ($p \leq 0.05$) from a lower frequency of the organism (7.50%) as observed on infected Ondo State samples. Healthy and infected Oyo State samples as well as Ekiti (infected) and Ogun (healthy) samples showed no presence of the organism.

Aspergillus niger had highest frequency of occurrence, 25.11% in healthy samples from Oyo State, followed by those obtained from healthy Ekiti samples (20.16%). Osun State cocoa beans samples did not indicate the presence of the mould. While, *P. digitatum* and *Rhizopus* sp. had the highest frequencies of occurrence, 33.02 and 24.26% on healthy and infected Ondo State samples, respectively, there were no traces of the former in Oyo (infected), Ekiti (infected) and Ogun (healthy) samples. *Rhizopus* sp. was not found in healthy samples obtained from Oyo and Osun States (Table 1).

The highest percent occurrences of *Pythium* sp. (13.33%), *L. theobromae* (12.73%) and *F. solani* (29.61%) were observed on healthy cocoa beans samples obtained from Oyo State. Infected samples from Ondo State had the least occurrence of *Pythium* sp. (3.92%), while the organism was conspicuously absent in healthy samples obtained from Ekiti and Ogun States. The lowest frequency of occurrence of *L. theobromae* (1.11%) was observed on the healthy beans from Ekiti State while Oyo (infected), Ondo (healthy), Ekiti (infected) as well as Ogun (healthy and infected) samples showed no presence of the organism. While, the lowest frequencies of occurrence of *F. solani* (5.13, 4.44, 2.96 and 1.15%) were noticed on Ogun (healthy), Ekiti (healthy), Ekiti (infected) and Ondo (infected) samples respectively, the organism was not observed only on infected Ogun State samples (Table 1).

Table 2 shows the average occurrence of fungi obtained from healthy and infected cocoa beans across south-western cocoa producing states of Nigeria. Out of the eleven fungi isolated, *Aspergillus flavus* had significantly highest frequency of occurrence, 26.56% ($p \leq 0.05$), followed by *A. ochraceous* (21.59%), *A. versicolor* (15.13%), *Rhizopus* sp. (12.26%), *Pythium* sp. (4.73%), *A. terreus* (4.15%), *Fusarium solani* (4.00%), *Lasiodiplodia theobromae* (3.37%), *A. niger* (3.15%), and *Penicillium digitatum* (2.86%), while *A. fumigatus* (2.19%) had the lowest occurrence. There was however no significant difference ($p \leq 0.05$) in the populations of *A. terreus*, *A. niger*, *P. digitatum*, *Pythium* sp., *L. theobromae* and *F. solani*. Similar number of fungi were isolated from

Table 2: Average percentage of moulds occurrence across South-West, Nigeria

	Occurrence (%)	
Moulds	Healthy	Infected
Aspergillus flavus	21.11^{a}	26.56^{a}
A. fumigates	0.57^{c}	2.19^{d}
A. ochraceous	17.68^{ab}	21.59^{ab}
A. versicolor	0.93^{c}	15.13^{abc}
A. terreus	2.38^{c}	4.15^{cd}
A. niger	7.30^{bc}	3.15^{cd}
Penicillium digitatum	12.85^{abc}	2.86^{cd}
Rhizopus sp.	10.310^{abc}	12.26^{bcd}
Pythium sp.	3.22^{c}	4.73^{cd}
Lasiodiplodia theobromae	6.914^{bc}	3.37^{cd}
Fusarium solani	16.73^{ab}	4.00^{cd}

Means followed by the same superscript letter along the column are not significantly different at $p \leq 0.05$ according to Fisher's LSD test

Table 3: Mould population (CFU g^{-1})

	Mould populations (CFU g^{-1})	
State	Healthy beans	Infected beans
Oyo	6.79^{bc}	30.21^{b}
Ondo	24.33^{a}	30.88^{b}
Osun	6.42^{bc}	15.08^{c}
Ekiti	3.92^{c}	36.75^{b}
Ogun	13.88^{b}	62.17^{a}

Means followed by the same superscript letter along the column are not significantly different at $p \leq 0.05$ according to Fisher's LSD test

Table 4: Mould population with respect to methods of isolation

	Mould populations (CFU g^{-1})	
Methods of isolation	Healthy beans	Infected beans
Direct plating	22.80^{a}	41.40^{a}
Dilution plating	7.00^{b}	59.40^{a}
Blotter paper	3.40^{b}	4.25^{b}

Means followed by the same superscript letter along the column are not significantly different at $p \leq 0.05$ according to Fisher's LSD test

healthy cocoa beans samples but with lower populations. *Aspergillus flavus* (21.11%) was found to be significantly dominating ($p \leq 0.05$) in healthy cocoa beans as well, followed by *A. ochraceous* (17.68%), *F. solani* (16.73%), *P. digitatum* (12.85%), while *A. fumigatus* (0.57%) had the least occurrence (Table 2 and 3).

The populations of moulds isolated from healthy and infected cocoa beans from across the five cocoa producing south-western states ranged between 3.92-24.33 and 15.08-62.17 CFU g^{-1}, respectively. The population of moulds obtained from healthy beans samples taken from Ondo State (24.33 CFU g^{-1}) was significantly the highest ($p \leq 0.05$), followed by that of Ogun State (13.88 CFU g^{-1}), while those obtained from Ekiti State had the lowest (3.92 CFU g^{-1}). Infected stored cocoa beans obtained from Ogun State had the highest mould population. This was significantly higher ($p \leq 0.05$) than those obtained from Ekiti, Ondo, and Oyo with 36.75, 30.88 and 30.21 CFU g^{-1}, respectively, which were, however not significantly different ($p \leq 0.05$) from one another (Table 3). Also, the population of fungi isolated from healthy cocoa beans across the south-western region with the aid of direct plating method was significantly higher ($p \leq 0.05$) than dilution plating and standard blotter paper methods (Table 4). Fungi population obtained from infected cocoa beans with the aid of the dilution plating method of isolation (59.40 CFU g^{-1}), although the highest, was not significantly different ($p \leq 0.05$) from the population isolated with the aid of the direct plating method (41.40 CFU g^{-1}), while the blotter paper method gave the least (4.25 CFU g^{-1}).

DISCUSSION

Cocoa beans, just like many other seeds are generally associated with different types and varieties of saprophytic or parasitic microorganisms such as fungi, bacteria, etc. The seeds are often exposed to microbial contamination right from the field, during processing and at storage (Fagbohun *et al*., 2011). Rathod *et al*. (2012) noted in his findings that the potential pathogens eventually perpetuate in seed lots on the advent of favourable conditions. It means that a seed may be considered healthy until the environmental condition becomes favourable to the potential pathogens residing in it. This therefore explains the reason why pathogens isolated in this study from infected cocoa beans were also found (though in smaller numbers) in the healthy ones.

The prominence of members of the *Aspergillus* spp as well as the occurrence of *L. theobromae*, *Fusarium* spp. and *Penicillium* spp. among the populations of moulds isolated from both healthy and infected stored cocoa beans in this study has been found to be in agreement with the isolation work carried out by Fagbohun *et al*. (2011) on infected cocoa beans in Ado Ekiti, Ise, Emure and Ikere towns of Ekiti State, Nigeria and that of Dharmaputra *et al*. (1999) conducted in Indonesia. Findings from this study however disagrees with the claims by Dharmaputra *et al*. (1999) that *A. niger* is among the most predominant cocoa beans storage moulds, as the organism has been observed to be among the least isolated from infected beans obtained across the south-western region of the country (Table 3), more so, since the samples were collected around the same (dry) season of the year. The disparity may however, be due to the influence of different geographical locations on the distribution of moulds on plant materials (Collado *et al*., 1999). The lowest storage mould populations observed in healthy and infected cocoa beans samples obtained from Ekiti and Osun States respectively may be due to the better observation of good agricultural and handling practices during fermentation, drying and storage which in turn restricted the access of the moulds (potential pathogens) to the beans and hence, minimizing risks of microbial food safety hazards (AMS., 2012).

While, evaluating the effectiveness of agar plate (direct plating), standard blotter paper and seed washate techniques of isolation in the management of seed borne mycoflora of groundnut, Rathod *et al*. (2012) concluded that out of the three methods, the agar plate method favoured the growth of fungi and gave the highest incidence than the other two. A similar research conducted by Panchal and Dhale (2011) on some sorghum seeds led to the isolation of higher population of saprophytic fungi when the agar plate method was used. Findings from this study agree with the discoveries of these scientists. The isolation of higher populations and species of fungi when direct plating method was used in this study can however be attributed to the external supply of nutrients obtainable from the PDA (Panchal and Dhale, 2011). Findings from this study have also shown that in addition to the fact that members of the *Aspergillus* spp. are the dominant storage fungi of cocoa beans in South-West, Nigeria, the direct plating method can be used for their effective isolation from healthy and infected cocoa beans, while the dilution plating technique is best used on infected beans. Observation of good agricultural and handling practices is therefore suggested to reduce the storage mould populations on the beans and thus, limit the risks of contamination that may expose consumers to life threatening health hazards.

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