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Identification and Lipase-producing Abilities of Moulds Isolated from Ivorian Raw Cocoa Beans

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Abstract: A total of 100 samples of cocoa beans characterized by high Free Fatty Acids content were collected and analysed for numeration, identification and lipase-producing abilities of filamentous fungi. Analysis revealed the occurrence of *Absidia corymbifera*, *Rhizopus oryzea* and *Penicilium chrysogenum* from over 80% of the samples. *Aspergillus tubingensis, A. flavus and A. tamarii* were isolated from 40-50% of the samples. The predominant moulds were *R. oryzea* and *A. corymbifera*. *R. oryzea* grew abundantly on Czapeck-dox agar enriched with oil substrates and had greatest lipase-producing abilities. FFA accumulation in raw cocoa beans could be attributed mainly to the presence and the action of *R. oryzae* and *A. corymbifera*.

Keywords: Raw Cocoa beans, moulds, *Rhizopus oryzea, Absidia corymbifera*, *Penicilium chrysogenum, Aspergillus tubingensis, A. flavus, A. tamarii*, lipase-producing abilities.

INTRODUCTION

Nowadays, one of the most widespread problems in advanced technological countries is food quality and safety. Cocoa is a very important ingredient in several kinds of foods, such as cakes, biscuits, child-foods, ice-creams and sweet. Cocoa beans are actually seed from the fruit of *Theobroma cacao* tree. Cocoa bean is an important source of cocoa powder and of food worldwide and constitutes an inexpensive fat source from Africa, Central and South America. Côte d'Ivoire is the world's leading exporter of cocoa beans. Cocoa butter was currently reported to be the main vegetable fat used in the chocolate manufactures because of its rheological, textural and chemical characteristics such as triglycerides fatty acids composition[38]. The general opinion is that higher free fatty acids (FFA) content must be considered as cocoa beans value-reducing factor. The FFA content is a cocoa beans important quality parameter therefore; the directive 73/241/EEC[11] limits the concentration of FFA in cocoa nibs to 1.75%. In all warm and wet countries, weather and agronomic conditions are favourable for fungi growth and consequently food quality deterioration. In addition, storage and processing conditions of cocoa in the countries are not very safe and mycotoxinogenic fungi contamination may be possible at many critical points of the cocoa production chain. Generally, poor post-harvest management can lead to rapid deterioration in stored foodstuffs and feeds quality and initiation of fungal activity[22]. Post harvest crops fungal spoilage results in significant economic losses. Additionally, if the spoiling fungi are toxigenic or pathogenic, they could pose a health risk for the consumers. Some of these moulds could produce mycotoxins while grown on crops[34]

Fungal contaminants are also responsible for substantial effects in stored foodstuffs including discolouration, contribute to losses in nutritional value, produce off-odours, deterioration in baking and milling quality, contamination with mycotoxins and severely increase of FFA content [6, 22]. It is important to isolate and identify fungal contaminants in cocoa beans because some moulds can grow and produce mycotoxins on these commoditities while certain moulds can cause infections or allergies. It was suggested that FFA accumulation in ferment dried cocoa beans would be linked to the action of lipases secreted by moulds that contaminated the product if poorly stored[29,39]. It was demonstrated that high and increasing FFA contents which were frequently and recurrently observed in Ivorian stored cocoa beans were due to fungal action[15]. Several moulds species belonging to *Aspergillus*, *Penicillium* and *Mucor* genera

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have ever been isolated from commercial cocoa beans since long time[30]. But their role and their action in the production of FFA were unknown. The main targets of this paper were (1) to investigate the current fungal profiles of Ivorian cocoa beans, (2) to study their lipase-producing abilities and their growth on media enriched with oil substrates.

MATERIALS AND METHODS

Cocoa Samples: A total of 100 samples (approximately 1 kg each) of different types characterized by FFA content were collected during the first 6 months of 2003 from Ivorian cocoa producing chain (from farmer to exporter levels at Abidjan harbour). The traders were questioned on where from was the cocoa beans batch. The moisture content was determined by oven-drying at 105°C to constant weight. Assessment of free fatty acids content in cocoa butter.

About 20 g of cocoa beans were frozen at -80°C in liquid nitrogen and then ground in a coffee grinder. Cocoa butter was extracted by Soxhlet method from about 10 g of cocoa powder homogenized in 350 ml ether during one night. After cocoa butter extraction during 8 hours solvent was removed by evaporation and cocoa butter was dried by oven-drying at 110°C during 4 hours. FFA content expressed as percentage oleic acid was measured using the method described previously [2]. About 5 g of cocoa butter was weighed (W_1) and dissolved in 50 ml ether/ethanol (1:1) mixing prior neutralised and containing thymol blue indicator. Fatty acids titration was carried out with 0.1N KOH to a khaki colour stable for 30 s end point. The volume of KOH solution used (V) was noted. FFA (% oleic acid) was calculated as follows:

$$\%FFA = \frac{(28.2 \times V \times N)}{W_1}$$

Isolation, Enumeration and Identification of Fungi Isolated from Cocoa Beans: The assessment of the level of mould contamination was done by weighting aseptically and transferring 10 g of cocoa beans from each sample in 90 ml of sterile peptone solution containing Tween 80 (0.05%)[32]. Samples were thoroughly homogenized and mixed in the stomacher[24] for 5 min to obtain 10^{-1} stock. Further 10-fold serial dilutions were prepared in sterile water up to 10^{-4}. For fungal counts duplicate 1 ml volumes of each sample were dispend in Petri dishes containing potato dextrose agar (PDA)[1,3] complemented with 100 $mg.l^{-1}$ chloramphenicol. After incubation for 3-5 days at 28°C, fungal colonies were counted the results were expressed in CFU g^{-1}. Then the results were also expressed as a percentage of infected samples (% infection). For isolation of fungi from cocoa beans the direct plating technique of cocoa beans on Dichloran 18% Glycerol agar (DG18) was used because of its water activity below 0.95. After incubation of Petri dishes for 5-7 days at 28°C, the isolates were sub cultured on Czapek-Dox agar medium for identification purposes[31, 35] and later identified[27,28]. Fungal isolates were sent to Leuven Catholic University, B-1348 Louvain-la-Neuve, Belgium) for to complete or to confirm the identity.

Assessment of Hyphal Growth Rate and Lipase-producing Ability of Strains Isolated: Firstly all strains isolated from cocoa beans were cultivated on PDA medium and incubated at 25°C during 5 days. Conidia were collected by scratching in 0.04% Tween 80 solution. Five µl of 10^6 conidia/ml suspension of each strain were put down in the centre of Petri dishes containing rhodamin B (RB) medium and incubated during 2-3 days at 28°C to determine its lipase-producing abilities through the visualisation of a bright pink fluorescence zone surrounding colonies under UV light (365 nm). RB medium contained 10 g peptone, 3 g yeast extract, 5 g NaCl, 30 g bacto agar (Difco), 5 g commercial rapeseed oil, 2% polyvinyl alcohol and 10 mg rhodamine B[20] . Czapeck-Dox agar medium containing 5% rapeseed oil or cocoa butter Petri dishes were incubated during 80 hours for the measurement of each strain's radial mycelial growth in two directions at right angles to each other. Measurements were recorded on alternate days during the growth until the Petri Plates were completely colonized. Growth rates, expressed as $mm.day^{-1}$, were calculated by linear regression of colony radius against time of each strain[20]. All experiments were made in triplicate.

RESULTS AND DISCUSSIONS

Isolation and Identification of Moulds: Table 1 shows mould counts in beans of different qualities collected from Ivorian cocoa chain. The contamination level (percent of infection) in cocoa samples differed according the characteristics and quality of cocoa beans. Mould counts reached maximum values of $5.8\pm1.1\times10^8$ and $4.3\pm0.9\times10^7$ $CFU.g^{-1}$ respectively in broken beans and clustered beans. Brown beans showed minimal mould counts values of $7.5\pm0.7\times10^2$ $CFU.g^{-1}$. Mycological analysis of all 100 cocoa beans samples revealed seven moulds strains belonging to five genera of fungal contaminants, *Aspergillus, Absidia, Rhizopus, Penicilium* and *Monilia*. Three strains belong

to *Aspergillus* genus. All identified fungi genera have already been recorded in cocoa beans samples[7, 10, 18,25,30] and in others stored foodstuffs such as sunflower beans[16], groundnuts[37], cereals[23] and coffee cherries[13]. There was a qualitative heterogeneous distribution of filamentous fungi in samples from cocoa beans analysed. All genera isolated in this experiment are typical storage species that are able to thrive at relatively low water activity. That allowed supposing post-harvest management has been conducted poorly by farmers and particularly cocoa drying systems have been certainly inefficient.

Identification and enumeration of fungi indicated *Absidia corymbifera* and *R. oryzea* were the most abundant and frequently encountered moulds followed by *Aspergillus sp*. in ours cocoa samples as described previously[17]. Three strains were recovered in all cocoa samples analysed whatever the quality and the FFA content of beans: *P. chrysogenum*, *R. oryzea* and *Monilia sp*. Only one strain as *Aspergillus flavus* isolated from samples analysed could produce mycotoxin such as aflatoxin[14,26]. As most identified moulds were post-harvest fungi spoilage[9], some fungi could contaminate the beans in post-harvest storage and marketing conditions while others such as *Monilia sp*. could start the spoilage from the field and proliferate after harvest when the main plant defences are reduced or eliminated[36]. *Monilia sp*. was lower isolated than others probably because it is considered as a field fungus and it would be proliferated after harvesting. Nearly all species of fungi recovered in the present work can be considered as common saprophytic soil organism and common food-born fungi.

Table 2 showed that poor quality cocoa beans samples (black, clustered and broken beans) were more infected by moulds than brown beans which presented best quality. Indeed, the lower fungi contamination level recorded in brown beans was probably due to the fact that these beans were compact, without damage injury neither visible default. Broken beans were severely infected by fungi because they probably provided a great available substrate for fungal proliferation. Mechanical damage of these cocoa beans would be also conducive to entry of spoilage fungi in insufficiently dried grain as previously showed in stored products[33]. The high level of contamination of black beans could be attributed to their probable origin from spoiled or rotten pods. Otherwise, field fungi invaded cocoa pods as they are developing on the plants in the field and before they are harvested can lead to the development of storage fungi in cocoa beans during post harvest storage and marketing conditions. Strong fungi contamination level recorded in clustered beans could be explained by their exposure to high moisture due to inadequate drying. The high moisture content could be attributed to the development of a crusty surface on the agglomerated beans, preventing evaporation of the water from beans which conducted to the high moisture content[5]. According to many studies the moisture content and temperature are the most important variables in determining development or growth of fungi. But the occurrence of several storage fungi species in raw cocoa beans can not be specifically explained because mould occurrence in agricultural commodities depends on a complex interaction of factors, such a temperature, moisture, endogenous fungal species, storage history and storage time[8,22].

Growth and Lipase-producing Abilities of Strains Isolated on Media Enriched with Fatty Substrates: As a first step, growth of different isolates on both Czapek-dox agar enriched with rapeseed oil or cocoa butter was rather variable (Figs. 1 & 2). All strains showed poor growth except *R. oryzea* which grew abundantly. The growth of *P. chrysogenum* on Czapek-dox agar enriched with oil is strongly lowest. This poor growth could be probably attributed to the quality of culture media. Available substrates containing in the culture media and perhaps incubation temperature were inappropriate factors for the good growth of *P. chrysogenum* in vitro. The rapid growth of *R. oryzea* would be due to its natural hyphal growth properties because it grew abundantly when it was sub cultured on Czapek-dox medium without oil substrate. All isolated strains in this experiment were examined for lipase production on RB medium. They showed pink fluorescence zone surrounding their colonies except *A. tubingensis* and *P. chrysogenum*. So *P. chrysogenum* and *A. tubingensis* presented poor lipase-producing abilities and their action caused no substantial spoilage relating to FFA accumulation in cocoa beans. Consequently they are saprophytic fungi in cocoa beans. That explained widely why their population were the lowest among all fungi isolated from cocoa beans (Table 1) and they contaminated less cocoa samples than others isolated fungi (Table 2).

The presence of characteristic bright pink fluorescence zone surrounding *Absidia corymbifera* and *R. oryzea* colonies under UV light (365 nm) when sub cultured on Czapeck-dox containing rapeseed oil or cocoa butter (5%) indicated that these moulds has used oil substrates as source of energy. The bright pink fluorescence zone expressed the occurrence of free fatty acids from break downing of triglycerides brought by adjunction of fatty substrates. That demonstrated the capacities for *Absidia corymbifera* and *R. oryzea* biomass to produce extra cellular lipases as previously

Table 1: Numeration and identification of total moulds (CFU.g^{-1}) and frequency of isolation of moulds from 100 cocoa beans samples characterized by their FFA contents (Mean of twenty samplings of each type of cocoa beans)

Cocoa beans samples	Quality of beans	Moulds count (CFU.g^{-1})	Main strains isolated
Cocoa beans with FFA<1.75%	Brown beans	$7.5\pm0.7\times10^{2}$ [a]	*Penicillium chrysogenum, Rhizopus oryzea, Aspergillus tamarii, Monilia sp.*
	Black beans	$5.4\pm0.9\times10^{4}$ [b]	
Cocoa beans with FFA>1.75%	Very black beans	$7.5\pm0.4\times10^{5}$ [c]	*Absidia corymbifera, Rhizopus oryzae, Aspergillus flavus, A. tubingensis, A. tamarii, Pénicillium chrysogenum*
	Clustered beans	$4.3\pm0.9\times10^{7}$ [d]	
	Broken beans	$5.8\pm1.1\times10^{8}$ [e]	

Table 2: Rate of cocoa beans samples infection by moulds and percentage occurrence of moulds isolated.

Cocoa beans samples	% infection	% occurrence				
		Aspegillus sp.	*Absidia. corymbifera*	*R. oryzea*	*P. chrysogenum*	*Monilia*
Brown beans	6	80	nd	2	10	5
Black beans	60	90	95	65	53	nd
Very black beans	97	100	89	90	41	nd
Clustered beans	100	100	80	87	74	10
Broken beans	100	100	100	93	86	26

* which means: Occurence of alla isolated strains belonging to Aspergillus genus (A. flavus, A. tubingensis, A. tamarii). This description is under the table

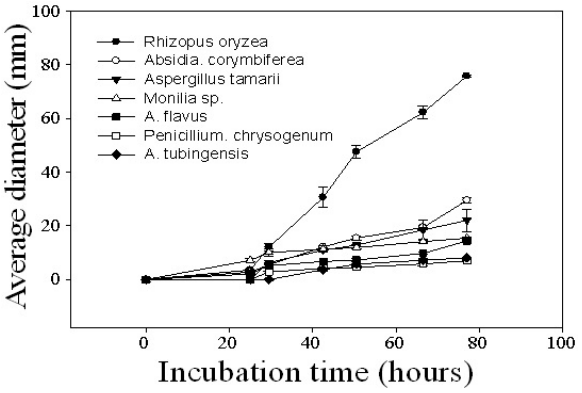

Fig. 1: Growth of moulds isolated from Ivorian cocoa beans on Czapeck-dox agar enriched with 5% rapeseed oil.

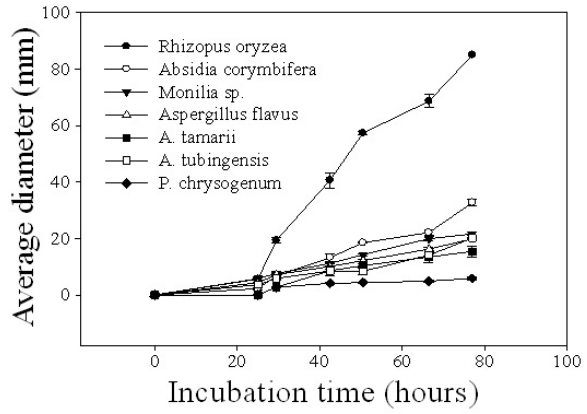

Fig 2: Growth of moulds isolated from Ivorian cocoa beans on Czapeck-dox agar enriched with 5% cocoa butter.

showed by several studies[12,17,19]. The action of these extra cellular lipases is to break down oil substrates triglycerides into glycerol and free fatty acids. The high contamination level of *R. oryzea* and *Absidia corymbifera* in ferment and dried cocoa beans whatever the quality of sample was probably linked to their great lipase-producing abilities. Indeed cocoa beans containing high butter content (57%) that constituted an excellent source of energy and best growth factor for fungi strains which are able to produce lipases. In order to reach this substrate and use it as source of carbon, *Absidia corymbifera* and *R. oryzea* produced extra cellular lipases which hydrolyze cocoa butter triglycerides such previously demonstrated in the case of palm fruits[19]. The massive invasion of cocoa beans by *R. oryzea* and *Absidia corymbifera* suppose that both species found great growth conditions in ferment and dried cocoa beans. They were followed by *A. tammarii*, *Monilia sp.* and *A. flavus*. *A flavus* group's lipase-producing abilities were previously demonstrated[4,21]. Although they are able to produce extra cellular lipases, the presence of fungi belonging to the *A. flavus* group among Ivorian raw cocoa beans fungal profile revealed the risk of aflatoxins formation[26] before manufacture and consumption of cocoa beans. Thus properties of fungal lipases and mycotoxin-producing abilities of fungi isolated from raw cocoa beans were not jointly elucidated in this study, the relatively high lipase-producing abilities exhibited by most moulds identified in cocoa beans showed good evidence to support their potential toxigenic abilities and to determine if correlations might exist between cocoa beans contamination level by each mould species and the FFA content.

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