

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.709.464>

Occurrence of Molds and Identification of Mycoflora Contaminating Millet and Sorghum Produced and Consumed in Benin

Noël Tovide^{1*}, Kifouli Adéoti¹, Pacôme A. Noumavo^{1,2}, Kamal Garba¹, Brice Ohin¹, Akim Soninhekon¹, Fidèle Tchobo³, Christophe Gandonou⁴, Fatiou Toukourou¹ and Farid Baba-Moussa¹

¹Laboratoire de Microbiologie et des Technologies Alimentaires, ²Laboratoire de Biologie et de Typage Moléculaire en Microbiologie, ³Laboratoire d'Étude et de Recherche en Chimie Appliquée, ⁴Laboratoire de Physiologie Végétale et d'Étude des Stress Environnementaux, Université d'Abomey-Calavi, Bénin

*Corresponding author:

ABSTRACT

The purpose of this work is to assess microbial contamination level of millet and sorghum in Benin and to identify the molds responsible for this contamination. Sampling (240 millet and 240 sorghum grain) was done in Beninese agro-ecological zones (I to V) where both cereals are produced. Fungi were isolated using Direct Plating method and characterised according to macroscopic and microscopic criteria. Grains microbial density was evaluated using the suspension-dilution method. The moisture content obtained per agro-ecological zone varies from 9.12% to 10.42% for millet and from 10.71% to 12.18% for sorghum. Contamination rates range from 37.89% to 52.71% and from 41.89% to 55.11% respectively for millet samples from attics and markets. For sorghum, contamination rates vary from 42.39% to 61.36% for attics samples and from 43.46% to 74.47% for markets samples. The highest total flora was obtained in zone III (40×10^3 CFU/g) for millet and in zone V (113.2510^3 CFU/g) for sorghum. With regard to fungal load, the highest values were obtained in zones II and IV (2.5×10^3 CFU/g) for millet and in zone IV (14.64×10^3 CFU/g) for sorghum. The 535 molds strains identified mainly belong to the genera *Aspergillus* (52%) and *Penicillium* (27%) followed by *Fusarium* (10%) and *Mucor* (9%).

Keywords

Millet, Sorghum, Cereals, Contamination, Molds, *Aspergillus*, *Penicillium*, *Fusarium*.

Article Info

Accepted:
26 July 2018
Available Online:
10 September 2018

Introduction

Cereals are the staple foods of animal and human nutrition. They contribute to about half of the world's food protein, mainly in

developing countries (Schonfeldt *et al.*, 2012). In the semi-arid regions of Africa and Asia, millet and sorghum are the most cultivated cereal crops. Naturally drought-resistant, they provide food and nutrition security for

millions of people in these regions (Kayodé, 2006). Millet and sorghum occupy an important place in the Beninese food scene, due to the wide range of food products derived from these two speculations. The production of millet and sorghum is very strongly localized in the northern Benin. In the Atacora region, cereals help meet the energy needs of school-aged children in times of plenty (43%) and wedding (55%) (Mitchikpè, 2007).

Unfortunately, millet and sorghum do not escape from problem of cereals fungal contamination, which, beyond the economic impact, poses a real public health problem. Indeed, Beninese ambient humidity and temperatures promote fungal growth in the field and during storage. Indeed, in the cropping cycles of these two cereals in Benin, humid weather prevails after flowering until the maturity of the grain and before harvest.

Although fungal contamination continue to cause economic losses for producers, processors and consumers, the primary danger concerns the mycotoxins produced by them and its effects on consumer health (AFSSA, 2009). In Africa, the sanitary impact of mycotoxins is preponderant because the cereal production is generally intended for the self-consumption (Dieme *et al.*, 2016). It is estimated that more than 5 billion people in developing countries are at risk of exposure to chronic mycotoxicosis (Shephard, 2008). Large parts of the exposed people are in Africa. Mycotoxins are among the most potent mutagenic and carcinogenic substances known. The prolonged exposure through diet has been associated with cancer and diseases of the kidneys, liver and immune system.

Faced with this thorny problem of fungal contamination and to fill the lack of knowledge on the level of millet and sorghum fungal contamination at Benin, the present study assesses the occurrence and identifies

the mycotoxinogenic fungi in millet and sorghum stored and consumed in Benin.

Materials and Methods

Study area and sample collection

Sampling was carried out in the five Agro-Ecological Zones of Benin (AEZ I, II, III, IV and V) (Figure 1) where millet and sorghum are produced according to data from the Beninese Department of Agriculture, Livestock and Fisheries (MAEP, 2016). Benin is localized in West Africa (south of Sahara), in the tropical zone between Equator and Tropic of Cancer, precisely between the parallel 6° 30' and 12° 30' of North latitude and meridians 1° and 30° 40' of East longitude. It lasted three months, from October 2015 to January 2016. Six villages or city districts were prospected in each agro-ecological zone. In each village or city districts eight samples of each crop are collected from producers (4) and sellers (4). In AEZ III and AEZ IV with more than six millet and sorghum-producing communes, we chose according to the production level and accessibility of the fields and markets, six municipalities with a village or district of town by municipality. In the other three zones (AEZ I, II and V) with less than six communes where these two cereals are produced, multiple samplings were carried out per municipality so as to have 6 villages or town districts in each of these agro-ecological zones. Table 1 presents the characteristics of the different agro-ecological zones concerned as well as the villages and city districts investigated.

The sampling plan used is the random one. Thus, 500g of millet and sorghum grains were either directly taken from producers' attics or purchased from grain retailers in the markets. The collection was made in sterile plastic bags that are immediately delivered under a cold

regime to the laboratory and stored at 4°C (72 h at most) for analysis. A total of 240 millet and 240 sorghum samples were collected and analyzed.

Moisture of grains

Moisture expresses the amount of water in the grain. It was determined by desiccation method. Indeed, the flours (millet or sorghum) obtained after grains milling were dried at 130 °C for 4h. Moisture is expressed as a percentage (AFSCA, 2013).

Identification of fungi isolated from millet and sorghum

Fungi were isolated using the "Direct Plating" method according to Tahani *et al.*, (2008) with some modifications. This method does not provide a direct indication of the extent of fungal invasion in individual particles but provides insight into the level of grains infestation (Pitt and Hocking, 2009).

Each sample (50g) are disinfected on the surface with active chlorine solution (1%). One hundred (100) grains of previously disinfected sample are selected and distributed in seven (7) Petri dishes containing sterile Whatmanfilter paper No 1. The filter paper is pre-soaked with 3.5 ml of sterile sodium chloride solution (7.5%). The dishes are incubated for 10 to 15 days at 25°C and a percentage of the contaminated grains were calculated. The tests were realized in triplicate.

The fungi grown on the grains were transplanted on MEA (Malt Extract Agar: Malt extract, Peptone, Glucose and Agar), CYA (Czapek Yeast Agar: K₂HPO₄, Czapek concentrate, metal solution, yeast extract, sugar and Agar) and PDA (Potato Dextrose Agar: Potatoes infusion, dextrose and agar) for 5 days. The transplanted strains are

successively purified until pure strains are obtained. These fungal strains are identified using Pitt and Hocking (2009) method based on the macroscopic and microscopic characteristics.

Microbial load

The enumeration of grains microbial density was done using the suspension-dilution method. The dilution method is appropriate for evaluation of the microbial density of liquid products, flour products and grains (Lana *et al.*, 2003). So, the samples were aseptically ground. Ten (10) grams of flour was mixed with 90 ml of Buffered Peptone Water. The mixture was stirred with an orbital shaker for 30 minutes. One (01) milliliter of this mixture was inoculated in Petri dishes depth with 20 ml of PCA (Plat Count Agar) and incubated at 30°C for 72 h (Total Mesophilic Flora) and 0.1ml in surface on PDA (Potato Dextrose Agar) incubated at 25°C for 5 days (Fungi).

Statistical analysis

Grain contamination data were subjected to Analysis of Variance (ANOVA) at probability level of 5% and the Student Newman-Keuls test using Statistical Analysis System (SAS) software version 8.1. The Pearson correlation test was done on Minitab V.17 with all studied variables. The geographical map of sampling areas was realized using the ArcMap software version 9.2.

Results and Discussion

Moisture content of millet and sorghum grains

Grain moisture levels ranged from 9.12% (AEZ I) to 10.427% (AEZ III) for millet and from 10.71% (AEZ I) to 12.18% (AEZ V) for sorghum (Table 2). The ANOVA test reveals a

very high significance difference of grains (millet and sorghum) moisture content according to the agro-ecological zones ($P < 0.001$). According to SNK test, the moisture content of millet grains of the AEZs II, III, IV and V are not different, but it's are different to moisture content of millet grains of AEZ I. According sorghum moisture content, SNK test grouped the AEZs in three clusters; first cluster contained only AEZ V, second contained only AEZ III and the cluster third grouped the AEZs I, II and IV. The grains (millet and sorghum) sampled in AEZs III and V are the most moisture content.

Fungal contamination rate of millet and sorghum grains

Fungal contamination rates of millet and sorghum grains vary by sampled localities. Highly significant difference have obtained between contamination rates of millet grains from producers (attics) from one AEZ to another while this difference is significant for samples collected from markets (Figure 2A). The contamination rates range from 37.89% (AEZ II) to 52.71% (AEZ V) and 41.89% (AEZ I) to 55.11% (AEZ IV) respectively for samples from producers' attics and those from markets. For sorghum grains (Figure 2B), very highly significant difference have obtained between contamination rates both from producers (attics) and markets. The contamination rates vary from 42.39% (AEZ III) to 61.36% (AEZ I) for attic samples and from 43.46% (AEZ II) to 74.47% (AEZ V) for grain collected in markets. By combining both sample types (attic and market), the contamination rates vary from 42.79% to 50.94% and 46.92% to 67.42% respectively for millet and sorghum. Globally the samples collected from AEZs IV and V has highest fungal contamination rates and separately grouped by SNK test. Globally, the fungal contamination rate of millet and sorghum grains was not different from attic samples to

market samples ($p > 0.05$) (Table 3), but it different from millet samples to sorghum samples ($p < 0.05$). Sorghum grains therefore were significantly more contaminated than millet grains.

Microbial density of millet and sorghum grains

The fungal and total mesophilic aerobic density of millet and sorghum samples is shown in figures 3. The mesophilic aerobic (Figure 3A) and fungal (Figure 3B) densities were very highly different from one AEZ to another ($p < 0.001$). The total mesophilic aerobic densities varies from 2.90×10^3 CFU/g (AEZ II) to 40×10^3 CFU/g (AEZ III) for millet grains and from 30×10^3 CFU/g (AEZ I) to 113×10^3 CFU/g (AEZ V) for sorghum grains. The density of mesophilic aerobic flora was not statistically different between AEZs I, IV and V (according to SNK test), but it different to densities of AEZs II and III. The fungal flora ranges from 0.651×10^3 CFU/g (AEZ III) to 3×10^3 CFU/g (AEZ V) for millet and from 3.95×10^3 CFU/g (AEZ II) to 14.64×10^3 CFU/g (AEZ IV) for sorghum.

Correlation test

There is a moderate positive relationship between moisture and contamination rate ($P = 0.187$), and total flora ($P = 0.331$) of millet. So when the moisture content of millet increases, the rate of contamination and total flora also increase. Similarly, there is a negative correlation between moisture and the fungal load of millet ($P = 0.875$). But for all these relations $P > 0.05$. It is therefore not possible to say that these different relationships are significant. We also note a positive relationship between the contamination rate and the fungal load of millet ($P = 0.274$) and conversely a moderate negative relationship between the contamination rate and the total flora of millet ($P = 0.995$). But there is still no

significant relationship between these variables. For sorghum, we note moderate positive relationships between moisture content and contamination rate ($P = 0.446$), and fungal load ($P = 0.363$). There is also a high and significant positive relationship between sorghum moisture content and its mesophilic aerobic flora ($P = 0.014$). Increasing the moisture content would therefore increase the rate of contamination, the fungal load and the mesophilic aerobic flora of sorghum. The same relationships are observed between the contamination rate and the fungal load ($P = 0.192$) as well as the total flora ($P = 0.295$) of sorghum.

Mycology

Six (06) and four (04) fungal genus were identified respectively from sorghum and millet samples (Table 3). The main genus identified for sorghum grains are: *Aspergillus* (52%) and *Penicillium* (27%) followed by *Fusarium* (10%) and *Mucor* (9%). The genus *Alternaria* and *Cladosporium* are at an equal rate of contamination (1%). Millet samples were found to be contaminated with the same genus as sorghum with the exception of *Alternaria* and *Cladosporium*. Thus, *Aspergillus* (61%), *Penicillium* (20%), *Mucor* (13%) and *Fusarium* (6%) were isolated from millet (Table 3). Twenty fungal species were isolated and identified from both millet and sorghum samples. The species belong to *Aspergillus* genus are mostly contaminated millet and sorghum grains in Benin.

Table 4 presents the mold species isolated and identified in the millet and sorghum samples produced and consumed in Benin. One hundred and forty nine (149) fungal strains were isolated and identified from millet samples and 386 in sorghum samples. In all, nine species of *Aspergillus*, two species of *Alternaria*, three species of *Fusarium* and three species of *Penicillium* have been

identified. Unidentified species of *Cladosporium*, *Penicillium* and *Mucor* have also been found. *Aspergillus flavus* and *Aspergillus niger* are the two species mostly isolated with respectively 25 and 11% in millet seeds and 15 and 13% in sorghum grains. The other species most found in millet samples are *Aspergillus parasiticus* (8%), *Aspergillus versicolor* (5%) followed by *Aspergillus fumigatus* and *Penicillium griseofulvum* with an occurrence rate of 4% each. In sorghum samples, *Penicillium digitatum* (9%), *Aspergillus parasiticus* (8%), *Aspergillus versicolor* (7%) are the other most recovered species. In both cereals, some species are represented with only one isolate. These are *Aspergillus candidus*, *Aspergillus ochraceus* and *Alternaria infectoria* (Table 5).

Moisture is an important criterion that informs about quality of cereals. It is one of the parameters that promotes the development of molds and the production of mycotoxins. According to AFSSA (2009), toxigenic molds can develop in all climates, on all solid or liquid carriers containing nutrients and having a water activity value greater than 0.6. The moisture levels we obtained ranged from 9.12 to 10.424% for millet and from 10.71 to 12.187% for sorghum, which predisposes the samples to fungal contamination. The extreme northern agro-ecological zone of the country has the lowest values of grain moisture content. Its climate is of the Sudano-Sahelian type, the temperatures are excessive and reach 40 ° C in the shade in the dry season (SICC, 2018). This could explain why grains grown in this area are drier than those grown in other parts of the country. The results obtained, however, are in line with European Commission regulations (2006/576/EC) and those of the Codex Alimentarius which limit the maximum moisture content allowed for millet and sorghum in the range of 13-15% (Kouable, 2010). But the values obtained in our study are higher than those obtained by

Toffa (2015) which amounted to 10% in sorghum grown in Nigeria, a Sahelian country. According to Gacem *et al.*, (2011), storage molds are able to grow on substrates containing 10 to 18% moisture, with an optimum growth of between 11 and 13%.

Despite the healthy appearance of the millet and sorghum grains in stock analyzed, their infestation rates have been very high and vary depending on the collection site. In fact, the samples from grain sales markets appear to be more contaminated than those from storage attics. In sales markets, cereals are often exposed in the air in basins and are therefore exposed to contamination by spores and bacteria. According to Toffa (2015), one of the particularities of the grain sales markets is that they are sometimes very exposed to the pollutants of urban life: sand dust, exhaust fumes from vehicles, debris lifted by the wind etc. This condition could explain the high contamination rates obtained in samples from markets. Also, the work of Tovide *et al.* (2017) showed that cereal producers do not hesitate to use cotton inputs to process their grain in storage, thus reducing the risk of contamination but creating a hazard to consumers' health. In all cases, the fungal loads obtained in the millet and sorghum samples are above the recommended standards. In fact, the fungal flora counts obtained in most millet and sorghum samples are above the standard of 10^3 CFU/g for cereals. Also, the sorghum microbial density (mesophilic aerobic flora and fungal flora) was very highest than that millet globally and in each AEZ. Sorghum grain (4 mm) is known to be larger than millet grain (2.5 mm) (Cruz *et al.*, 1988). So our results show that the moisture of sorghum samples is higher than that of millet. All of this may explain why sorghum grains are more contaminated than millet grains. Of the two batches of samples

studied, the genus *Aspergillus* and *Penicillium* were the most dominant genus of isolates with the same fungal strains but in different proportions. Gacem *et al.*, (2011) in Algeria and Tahani *et al.*, (2008) in Morocco noted the predominance of these two fungal genus in cereals reserved for human consumption. According to Moreno *et al.*, (2009) and Makun *et al.*, (2009) the genus *Aspergillus* and *Penicillium* proliferate mainly during storage on substrates with a moisture content of between 10 and 18%. However, the high humidity favors rather the growth of *Penicillium* in favor of that of the *Aspergillus* which dominates them. The lack of high temperature ventilation would favor the growth of *Aspergillus* and *Penicillium* fungi (Riba *et al.*, 2005). Millet and sorghum produced and consumed in Benin are stored under similar conditions, which could explain why these two fungal genus predominate during our work.

The results of the mycological analysis of the two cereals also revealed the presence of the genus *Fusarium*, which is in agreement with the results obtained by Toffa (2015) who also isolated *Fusarium* species from millet and sorghum produced in Niger. *Fusarium* species are mainly considered as field molds, which require relatively high moisture and moisture content, so they are not competitive under storage conditions (Makun *et al.*, 2007).

However, some species may persist in large numbers in stored grain (Pitt and Hocking, 2009). Other strains that have been isolated from sorghum samples belong to the genus *Mucor*, *Alternaria* and *Cladosporium*. These genera are naturally present on crops in open fields and in the soil. The persistence of these genera in cereals seems to be due to the high humidity during storage (Gacem *et al.*, 2011).

Table.1 Sampling zones

Agro-Ecological Zone (AEZ)	Agro-ecological characteristics	Municipalities	Villages or city districts
AEZ I: Benin Northern Extreme Zone	Climate: Sudano-Sahelian with one rainy season Pluviometry: 700-900 mm Temperature: 40° on average (April-June) and from 12°C to 25°C (November to March)	Malanville	Goro-Bani Bodjécali Bangou
		Karimama	Toura Fadama Fandou
AEZII: Cotton Zone of North Benin	Climate: Sudanese with one rainy season and one dry season Pluviometry: 800-1200 mm Temperature: 28°C on average	Banikoara	Sompérékou Founougo
		Kandi	Tissérou Koussè
		Gogounou	Sori Sonkorou
AEZIII: Southern Borgou Food Zone	Climate: Sudanese with one rainy season Pluviometry: 900-1300 mm Temperature: 29°C on average	N'Dali	Gounin
		Nikki	Koussoukou
		Bembèrèkè	Gamia
		Kouandé	Sakabou
		Pèrèrè	Gorobani
		Sinendé	Gakpérou
AEZIV: West-Atacora Zone	Climate: Mountain with slight variations from one locality to another Pluviometry: 800-1500 mm Temperature: 26°C on average	Cobly	Tapoga
		Ouaké	Komdé
		Djougou	Kolokondé
		Copargo	Pabegou
		Natitingou	Koussantikou
		Tanguiéta	GoroBani
AEZV: Cotton Zone of Central Benin	Climate : Sudano-Guinean with two rainy seasons in southern and one rainy season in northern Pluviometry : 700-1400 mm Temperature : 27°C on average	Bassila	Manigri
			Adjimon
		Parakou	Dokparou
		Tchaourou	Bétérou Tchalla
		Savalou	Gobada

Table.2 Moisture content of millet and sorghum grains

Agro-Ecological Zone (AEZ)	Moisture (%)	
	Millet	Sorghum
I	9,12 ±0,53 ^b	10,71 ±0,73 ^c
II	9,97 ±0,73 ^a	10,96 ±0,85 ^c
III	10,427 ±0,69 ^a	11,67 ±0,77 ^b
IV	10,21 ±0,77 ^a	11,06 ±0,80 ^c
V	10,424 ±0,95 ^a	12,18 ±0,76 ^a
Signification	***	***

The means in the same column with the same letters are not significantly different at probability level of 5% according to Student Newman-Keuls test. nd = p > 0.05 (no difference); * = p < 0.05(significant); ** = p < 0.01 (highly significant); *** = p < 0.001 (very highly significant).

Table.3 Global contamination rate of millet and sorghum

Sample type	Contamination rate (%)	
	Millet	Sorghum
Attics	43.98±14.22 ^a	54.51±14.45 ^a
Markets	48.61±13.87 ^a	59.33±16.16 ^a
Signification	nd	nd

The means in the same column with the same letters are not significantly different at probability level of 5% according to Student Newman-Keuls test. nd = p > 0.05 (no difference); * = p < 0.05(significant); ** = p < 0.01 (highly significant); *** = p < 0.001 (very highly significant).

Table.4 Fungal species contamination rate of millet and sorghum samples

Fungal genus	Percentage of contamination (%)	
	Sorghum	Millet
<i>Alternaria</i>	1	--
<i>Cladosporium</i> spp	1	--
<i>Aspergillus</i>	52	61
<i>Fusarium</i>	10	6
<i>Penicillium</i>	27	20
<i>Mucor</i> spp	9	13

Table.5 Fungal species isolated from Millet and Sorghum grains

Molds	Millet		Sorghum	
	Number of isolates	Contamination rate / species	Number of isolates	Contamination rate / species
<i>Aspergillus flavus</i>	37	25 %	60	15%
<i>Aspergillus candidus</i>	1	1%	3	1%
<i>Aspergillus fumigatus</i>	6	4%	10	3%
<i>Aspergillus niger</i>	17	11%	50	13%
<i>Aspergillus niveus</i>	5	3%	10	3%
<i>Aspergillus ochraceus</i>	1	1%	4	1%
<i>Aspergillus parasiticus</i>	12	8%	30	8%
<i>Aspergillus terreus</i>	4	3%	4	1%
<i>Aspergillus versicolor</i>	7	5%	28	7%
<i>Cladosporium spp</i>	0	0	4	1%
<i>Alternaria alternata</i>	0	0	2	1%
<i>Alternaria infectoria</i>	0	0	1	0
<i>Fusarium graminearum</i>	5	3%	10	3%
<i>Fusarium poae</i>	0	0	8	2%
<i>Fusarium proliferatum</i>	5	3%	20	5%
<i>Penicillium spp</i>	20	13%	50	13%
<i>Penicillium citrinum</i>	4	3%	8	2%
<i>Penicillium digitatum</i>	0	0	36	9%
<i>Penicillium griseofulvum</i>	6	4%	12	3%
<i>Mucor spp</i>	19	13%	36	9%
Total of isolates	149	100%	386	100%

Fig.3 Microbial density of millet and sorghum according to AEZs. (A): mesophilic aerobic flora; (B): fungal flora. On each graph and for same histogram type, the means with the same letters are not significantly different at probability level of 5% according to Student Newman-Keuls test; nd = $p > 0.05$ (no difference); * = $p < 0.05$ (significant); ** = $p < 0.01$ (highly significant); *** = $p < 0.001$ (very highly significant).

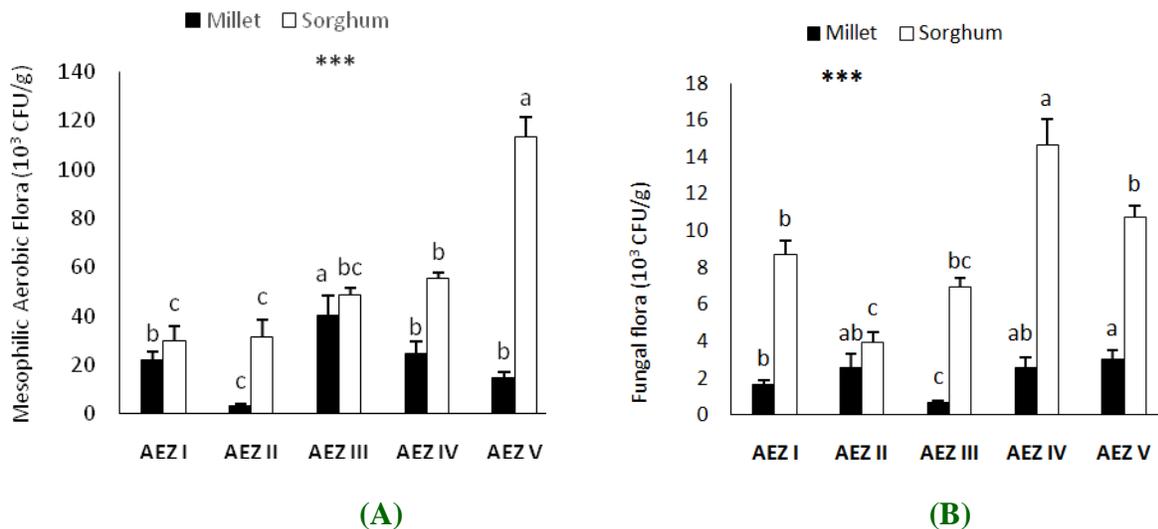


Fig.1 Geographical location of sampling areas

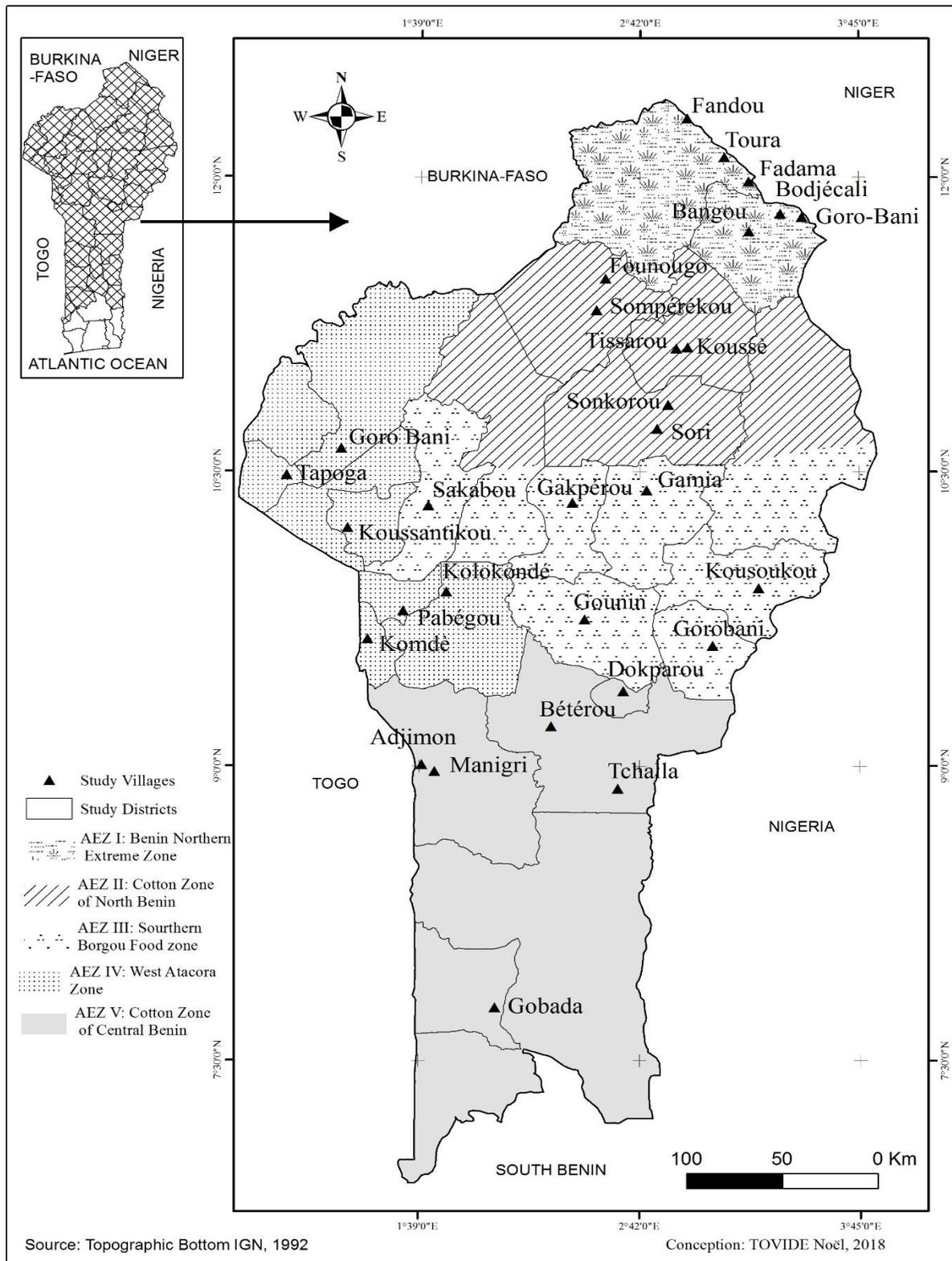
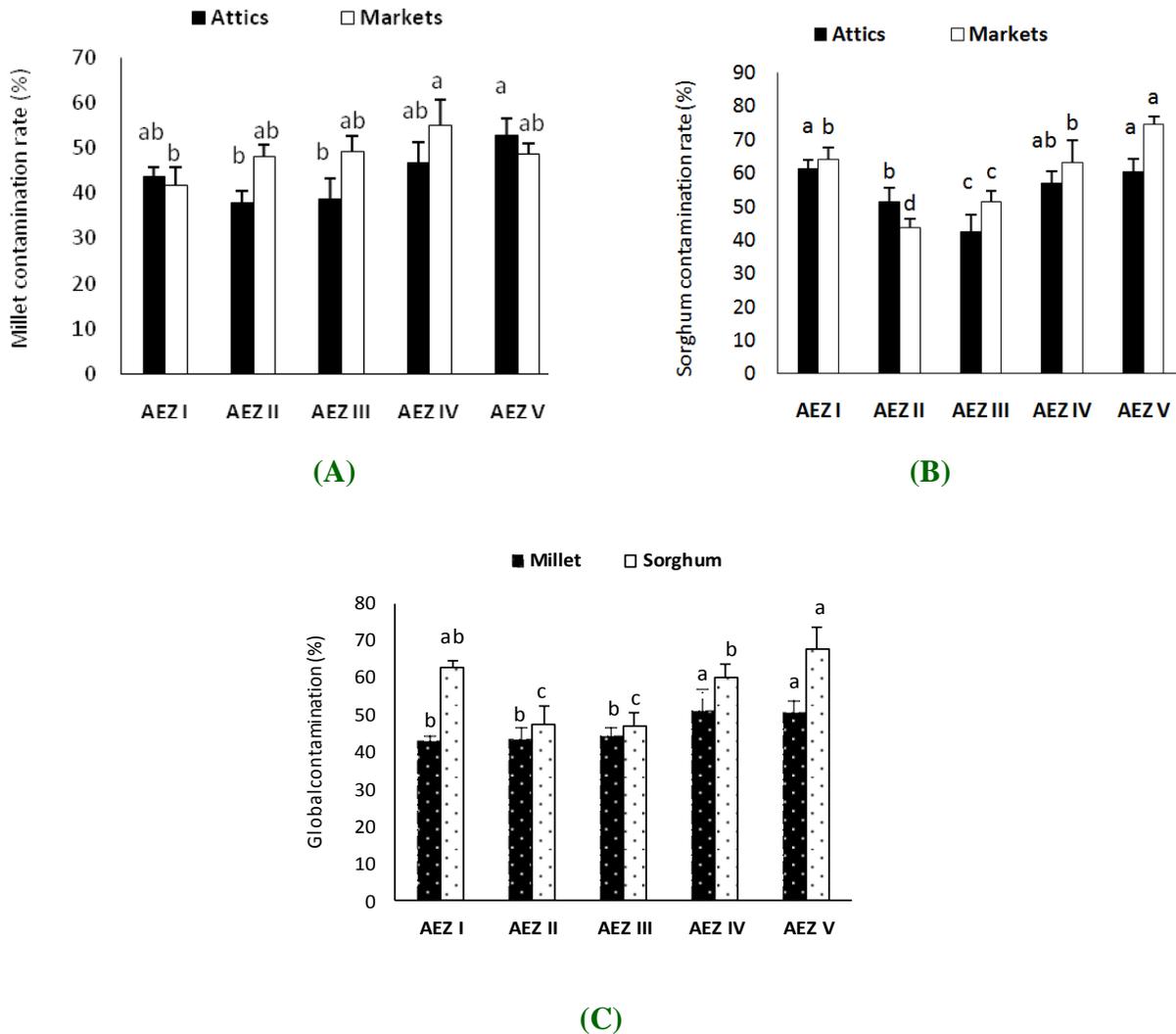


Fig.2 Contamination rate of millet and sorghum. (A and B): according to sample types (attic and market) and AEZs; (C): according to crop types and AEZs. On each graph and for same histogram type, the means with the same letters are not significantly different at probability level of 5% according to Student Newman-Keuls test; nd = $p > 0.05$ (no difference); * = $p < 0.05$ (significant); ** = $p < 0.01$ (highly significant); *** = $p < 0.001$ (very highly significant)



Mucor are fungi that quickly invade isolation environments and hinder the growth of other fungal species and colonize cereals, fruits and vegetables. These results corroborate those of Tabuc (2007) who claimed that the main species of molds that contaminate cereals still belong to the genus *Aspergillus*, *Penicillium* and *Fusarium*.

In conclusion, the results of our work showed that the contamination rate of millet and

sorghum differs from one agro-ecological zone to another. This difference is related to the climatic conditions which are favorable to the growth of the molds but also to the storage conditions. Mycological analyzes have shown the presence of the genus *Aspergillus*, *Penicillium* and *Fusarium*, which are considered as the most common and most destructive fungi for foods. These genus are described in several research works as being molds that mainly produce mycotoxins. Due

to the contamination rate of the samples analyzed, the consumption of products derived from these grains may constitute a danger to the consumer because these molds are likely to secrete mycotoxins which represent serious threats to the health of humans and animals. The development of these molds on these substrates can have several consequences on the food, the quality of the finished product and the health of humans and animals. It is therefore necessary to conduct further studies on the fungal contamination of millet and sorghum to see if these isolated molds are likely to produce mycotoxins.

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How to cite this article:

Noël Tovide, Kifouli Adéoti, Pacôme A. Noumavo, Kamal Garba, Brice Ohin, Akim Soninhekpon, Fidèle Tchobo, Christophe Gandonou, Fatiou Toukourou and Farid Baba-Moussa. 2018. Occurrence of Molds and Identification of Mycoflora Contaminating Millet and Sorghum Produced and Consumed in Benin. *Int.J.Curr.Microbiol.App.Sci.* 7(09): 3750-3763. doi: <https://doi.org/10.20546/ijcmas.2018.709.464>