Raw cashew nut quality as function of contamination by mycotoxins and other secondary metabolites of *Aspergillus* spp. and farmer practices

Leo Yendouban Lamboni

Thesis committee

Promotors

Prof. Dr M.A.J.S. van Boekel
Special Professor Dairy Science & Technology
Wageningen University & Research

Prof. Dr E.J. Smid
Personal chair at the Laboratory of Food Microbiology
Wageningen University & Research

Co-promotor

Dr A.R. Linnemann
Assistant professor, Food Quality and Design
Wageningen University & Research

Other members

Prof. Dr P.C. Struik, Wageningen University & Research

Dr H.J. van der Fels-Klerx, Wageningen University & Research

Dr S.E. Schoustra, Wageningen University & Research

Prof. Dr B. de Meulenaer, Ghent University, Belgium

This research was conducted under the auspices of the Graduate School VLAG
(Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Raw cashew nut quality as function of contamination by mycotoxins and other secondary metabolites of *Aspergillus* spp. and farmer practices

Leo Yendouban Lamboni

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 28 May 2018
at 4 p.m. in the Aula.

Leo Yendouban Lamboni

Raw cashew nut quality as function of contamination by mycotoxins and other secondary metabolites of *Aspergillus* spp. and farmer practices
191 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2018)
With references, with summaries in English and French

ISBN: 978-94-6343-752-3
DOI: 10.18174/442674

CONTENTS

List of terms

Fruit: in botany, a fruit is the seed-bearing structure in flowering plants (also known as angiosperms) formed from the ovary after flowering.

Nut: in botany, a nut is a dry, hard fruit that does not split open at maturity to release its single seed (indehiscent).

Kernel: in botany, a kernel is the edible central part of a seed, nut or fruit within the shell or the fruit stone.

Shell: a hard, usually fibrous outer layer of some fruits, and especially nuts.

Achene: (also referred to as **akene, achenium** or **achenocarp**) is a type of simple dry fruit, monocarpellate (formed from one carpel) and indehiscent (they do not open at maturity). Achenes contain a single seed that nearly fills the pericarp, but does not adhere to it.

Chapter 1

General introduction and thesis outline

1.1. Background of the research

Africa is considered as one of the least industrialized parts of the world. The Food and Agriculture Organization of the United Nations (FAO) estimated that 85% of the African population relies on agriculture for subsistence (FAO, 2015). Crop production, with no or little mechanization, does not ensure sufficient income for small-scale farmers in the rural areas. To overcome the gap in their income, many farmers adopt the production of cash crops with high revenues in addition to the cultivation of subsistence crops (Achterbosch *et al.*, 2014). The income from cash crops is estimated at 50% of the farmers' revenues and thus contributes significantly to the national gross domestic product (Bonaglia and Fukasaku, 2003; Degla, 2012; Samen, 2010). In West Africa, important cash crops include coffee, cocoa, cotton and cashew nut. The most important cashew nut producers in West Africa are Ivory Coast, Nigeria, Guinea-Bissau and Benin (Cashew Handbook, 2014).

The Beninese cashew represents the second export product after cotton (Dah-Dovonon and Idrissou-Yaya, 2009). However, this export commodity is facing a challenge since international trade regulations are becoming restrictive on food safety and quality aspects in relation to mycotoxins (ACi, 2012a; European Commission, 2010; Van Egmond *et al.*, 2007). For raw cashew nuts, the international trade is regulated based on several quality parameters, including nut count, water content, insect infestation and fungal contamination which can lead to the accumulation of mycotoxins in the kernels (Cashew Handbook, 2014). Nut count and water content are mostly controlled by sorting and grading of cashew nuts (UNIDO, 2011). For mycotoxin contamination, the European Union implemented the most extensive and detailed regulations for food associated mycotoxins worldwide, including 13 different mycotoxins or groups, and 40 different toxin-food commodity combinations (Commission of the European Communities, 2006; Van Egmond *et al.*,

2007). Therefore, within the Beninese context, improving the quality parameters of cashew nuts is necessary to help small-scale farmers to realise a better quality.

1.2. The cashew tree and its products

Cashew (*Anacardium occidentale* L.) (**Fig. 1.1**) originates from north-eastern Brazil (Mitchell and Mori, 1987). The tree was discovered by the Portuguese in the 16th century as a potential commodity. The first record of cashew cultivation in Africa is from Mozambique at the end of the 18th century, although Johnson (1973) mentioned it might have been introduced fifty years before. Later, cashew cultivation was extended to India and Asia. Cashew trees were first introduced in Benin in 1950 (Chabi Sika *et al.*, 2015a). Nowadays cashew is cultivated in many tropical countries in large holdings and plantations as well as in the smallholder environment for its highly appreciated nuts (Orwa *et al.*, 2009).

Fig. 1.1. The cashew tree in a tropical region

Source: https://www.feedipedia.org/sites/default/files/images/cashew_tree_gaba.jpg.

Belonging to the family of *Anacardiaceae*, cashew is a fast growing, evergreen tree that can grow to a height of 8 - 20 m depending on soil characteristics and climate (Ibouraiman *et al.*, 2016). The tree is genuinely tropical and does not tolerate frost. Although the cashew tree can tolerate high temperatures and conditions of drought, an optimal temperature of 27°C and an average yearly rainfall of 1,500 mm provide ideal conditions for growth. As a perennial tree crop, it does not require intensive care. Nevertheless, cultural practices like fertilization, irrigation, spraying against pests and pruning are advised for optimal yields (GTZ, 2006). The tree starts flowering by the 3rd year depending on the variety. The full production capacity is attained after 8 years and can last for about 20 to 30 years (Behrens, 1996). **Fig. 1.2** shows the main production countries of cashew nuts from West to East Africa.

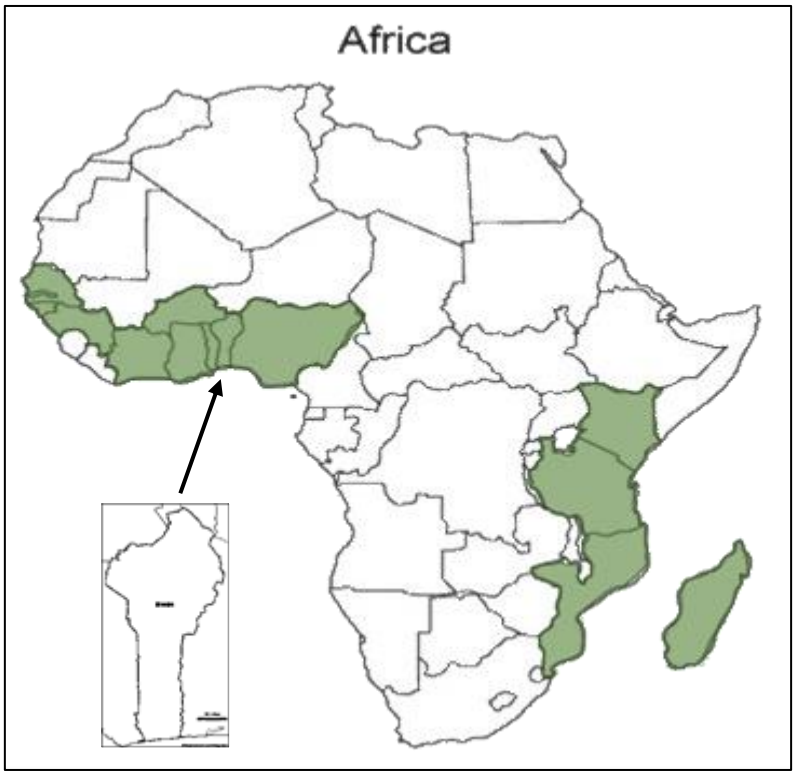


Fig. 1.2. Map of Africa showing the main cashew nut producing countries

Source: Free map of Africa, (from www.mapsfordesign.com) coulored and modified by Leo Yendouban Lamboni.

(From left to right and from top to bottom: Senegal, Gambia, Guinea-Bissau, Guinea, Cote d'Ivoire, Burkina-Faso, Ghana, Togo, Benin, Nigeria, Kenya, Tanzania, Mozambique, and Madagascar).

The cashew fruit (**Fig. 1.3**) consists of a false fruit (apple) and an attached nut. The apple represents 90% of a good and mature fruit. The nut is commonly used as a commercial product while the apple is used to produce wine, juice and vinegar (Lowor *et al.*, 2016). Several cultivated varieties of cashew are reported for various countries: Brazil (Assuncao and Marcadante, 2003; Souza *et al.*, 2016); Costa Rica (Schweiggert *et al.*, 2016); India (Das and Arora, 2017; Prabhakaran Nair, 2010); Benin (Michodjehoun-Mestres *et al.*, 2009; Chabi Sika *et al.*, 2015a,b), but in most countries including Benin, biotypes are identified on the basis of the shape, size and colour of the false fruit as well as the nut size (Chabi Sika *et al.*, 2015b). A list of these cultural varieties is given by Papademetriou and Herath (1998).

Fig. 1.3. Mature cashew fruits of a yellow and a red variety

The cashew nut (**Fig. 1.4**), called the true fruit, is a kidney-shaped achene that does not split open after drying. The inner part of the nut, the cashew kernel, is a large curved 2-3 cm seed covered by a membrane called testa, inside the cashew nut shell (**Fig. 1.4**). The kernels, which have a high nutritional and commercial value, are used for human consumption after shelling (Catarino *et al.*, 2015). **Table 1.1** shows the nutritional value of cashew kernels as recorded by the United States Department of Agriculture (USDA). According to the USDA, the cashew kernel is high in calories, providing 553 kcal (2,314 kJ) per 100 g of nuts. It contains vitamin B_1 (thiamine), vitamin B_6 (pyridoxine) and vitamin E, representing more than 30% of the recommended dietary allowance, and minerals such as copper, iron, magnesium, manganese, phosphorus and zinc.

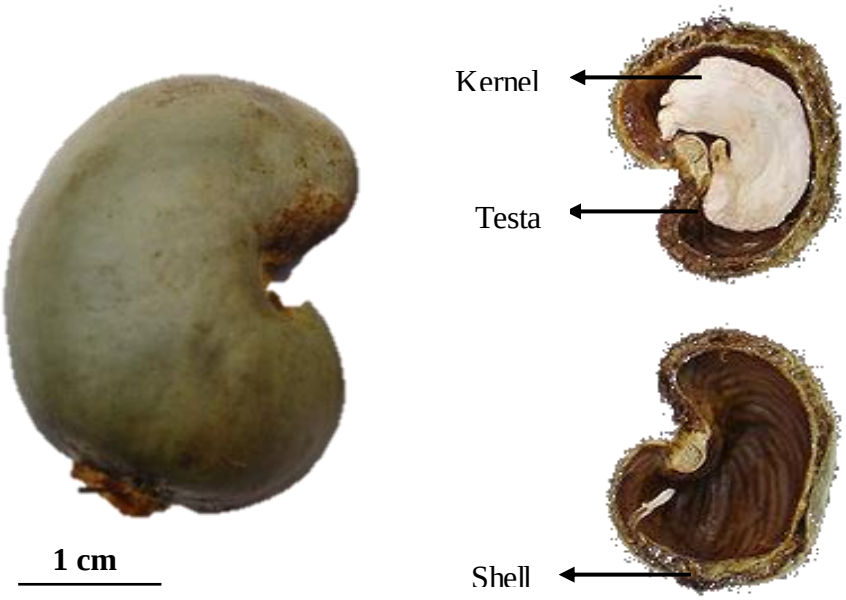


Fig. 1.4. A cashew nut with a cross-section showing the cashew kernel

Table 1.1. Nutritional value of the cashew kernel[α]

Composition per 100 g edible portion[β]						
Compound	**Value**	**% of RDA**		**Compound**	**Value**	**% of RDA**
Energy	553 kcal (2,314 kJ)	28		Total fat	43.9 g	146
Carbohydrates	30.2 g	23		Dietary fibre	3.3 g	8.5
Protein	18.2 g	32.5				
Vitamins				**Minerals**		
Folates	25 µg	6		Sodium	12 mg	1
Niacin	1.1 mg	6.5		Potassium	660 mg	14
Pantothenic acid	0.9 mg	17		Calcium	37 mg	4
Pyridoxine (Vit B_6)	0.4 mg	32		Copper	2.2 mg	244
Riboflavin	0.1 mg	4.5		Iron	6.7 mg	83.5
Thiamine (Vit B_1)	0.4 mg	35		Magnesium	292 mg	73
Vitamin C	0.5 mg	1		Manganese	1.7 mg	72
Vitamin E	5.3 mg	35		Phosphorus	593 mg	85
Vitamin K	34.1 µg	3		Selenium	19.9 µg	36
Phyto-nutrients				Zinc	5.8 mg	52.5
Lutein-zeaxanthin	22 µg	--				

[α] Raw cashews have been heat-treated to safely remove kernel from shell, but not further roasted;

[β] maximum number of data points = 2;

RDA = Recommended Dietary Allowance;

Source: USDA National Nutrient Database for Standard Reference Release 28 slightly revised May, 2016 Full Report (all Nutrients) 12087, Nuts, cashew nuts, raw).

1.3. Economic importance of cashew nuts

The global cashew nut production during 2013-14 was estimated to be 2.7 million tonnes from an area of 5.3 million hectares (Cashew Handbook, 2014). Half of this production came from Asian countries. West Africa contributed nearly one-third (36%) while Latin America and East Africa contributed about 11 and 8%, respectively (ITC, 2013). The major cashew nut producing countries are Indonesia (0.134 million tonnes), Brazil (0.132 million tonnes), India (0.774 million tonnes), Côte d'Ivoire (0.480 million tonnes) and Vietnam (0.267 million tonnes) (FAOSTAT, 2016).

Benin is ranked 8th on the world market and 3rd in West Africa in terms of cashew nut exports (ACi, 2010a). The production in 2015 was estimated at 128.000 tonnes from a total of 468.000 hectares, which generated a turnover of 150 million US dollars (FAOSTAT, 2016). Cashew production in Benin accounts for 8% of national export revenues and 25% of the agricultural export revenues (ACi, 2010a). Benin exports 95% of its production as unshelled nuts to several countries including India (Cashew Handbook, 2014). The cashew trading period is from February to June, corresponding to the harvesting period that lasts for several weeks, depending mainly on the weather conditions (ITC, 2013). Cashew nut trading represents the main employment and is a major source of income for many collectors, traders and exporters in Benin (Degla, 2012).

1.4. Quality parameters of raw cashew nut

Four main physical criteria are used for grading cashew nuts: water content, nut count (*i.e.* the number of nuts per kilogram); outturn and proportion of damaged nuts. These criteria are influenced by a wide range of factors including cashew variety, growing conditions, harvesting and post-harvest handling, and the storage conditions (ITC, 2013).

1.4.1. Water content

The water content should be measured and monitored from harvesting to trading. The water content of cashew nuts at harvest depends on weather conditions, the water content of the soil on which the nuts have fallen, the weed growth density under the tree and the time passed between the moment of nuts falling on the ground and their harvest. Before storage, it is recommended that water content is between 7 and 10% (ACi, 2012a; UNIDO; 2011). If the water content exceeds 10%, the nuts are sensitive to mould growth; if it is lower than 7% the nuts wither and lose weight. Sun drying of cashew nuts may last for maximum 3 days (Asogwa *et al.*, 2008; Dendena and Corsi, 2014) to reduce the water content, to mature the seed in the infra-red and ultra-violet rays and to

retain flavour and quality of the kernels (ITC, 2013; Papademetriou and Herath, 1998). In rural areas, sun drying of cashew nuts can be done on specially prepared drying floors or mats made of palm leaves. The drying areas should be smooth and slightly sloping, so as to allow rainwater to run off. The nuts are rolled over on a regular basis to ensure the drying of both sides (Azam-Ali and Judge, 2001).

1.4.2. Nut count

The nut count represents the number of in-shell nuts per kilogram. A nut count of 170 nuts/kg is considered excellent, 180-190 nuts/kg is very good, 190-200 nuts/ kg is good, while nut counts of 200-210 are middle, 210-220 low middle, 220-230 limit acceptable and higher counts are considered of poor quality (http://www.export-forum.com/africa/raw_cashew_nuts_info.htm; ACi, 2012a). In West Africa, depending on the year, most of the in-shell cashew nuts have a nut count of 190 to 210. Adeniyi and Adedeji (2015) reported a nut count ranging from 112 to 258 nuts per kg in several states of Nigeria. Associated with the outturn, the nut count provides information on the size of kernels.

1.4.3. Nut outturn

The outturn, also called KOR (Kernel Output Ratio), represents the quantity of good kernels found in an 80 kg bag after shelling. The unit of this measurement is the British pound (lb). For example, an outturn of 54 lb/bag means that 54 lb of cashew kernels can be obtained from an 80 kg bag of in-shell nuts, i.e. a yield of about 30% as the conversion factor from lb to kg is 0.45. This unusual unit of measure in lb/80 kg bag reflects the Africa – India nature of in-shell cashew trade. African raw cashew is sold in 80 kg jute bags, while weights in India are measured in pounds. Alternatively, the outturn can be expressed in grams of kernels per kg of in-shell nut where 1 pound equals to 0.45 kg or 1 kg equals 2.2 lb. Cashew kernels are priced according to the value of the outturn (ITC, 2013).

1.4.4. The proportion of damaged nuts

The rate of damaged nuts indicates the percentage of damaged in-shell nuts, such as immature, empty, moth-eaten, oil stained, mouldy and rotten nuts. Damaged nuts should be less than 10%; up to 24%, a lot is considered as acceptable. A lot with more than 24% of damaged nuts is rejected (ITC, 2013).

Other quality criteria for in-shell cashew are the foreign matter content (which should not exceed 5%) and the float rate (*i.e*. the percentage of cashew nuts that will float when put into water: immature and unfilled nuts will float while mature nuts will sink; a float rate < 18% is the requirement) (Papademetriou and Herath, 1998). The quality of in-shell cashew directly determines the quality of cashew kernels produced and therefore the profit after processing. An example is the low quality of a very large part of the West African in-shell cashew exported, in reference to the above-mentioned parameters, which forms the basis for the very high percentages of low grade, non-exportable cashew kernels obtained after processing in India and Vietnam. Ivory Coast produces nearly half a million tonnes of in-shell cashew, but less than a third of the nuts are of superior quality (ITC, 2013). This is also the case in Nigeria (Asogwa *et al*., 2008), Benin (Chabi Sika *et al*., 2015a) and Ghana (Gyedu-Akoto *et al.*, 2014). Therefore, proper harvesting and post-harvesting handling such as drying are essential to contribute to the improvement of the quality of the crop (ITC, 2013).

The measurement of the quality parameters is often performed by buyers or traders as a requirement before shipping. In African countries like Benin, traders often require a third party inspector like SGS® (Société Générale de Surveillance) with standardized procedures for verification, testing and certification of the product intended for shipment.

1.5. Mycotoxins associated with cashew nuts

As a result of the recommended harvesting practice for cashew nuts, namely to collect the nuts after the fruits have fallen from the tree, fungal contamination occurs via the soil (Cashew Handbook, 2014). Indeed, it is well known that fungal contamination of foodstuffs is mainly via contact during harvesting, with spores present in the soil, but also via insect infestation of damaged or cracked crops, and by cross-contamination during processing (De Saeger, 2011; Lamboni and Hell, 2009). Cashew nuts are susceptible to fungal growth because of their intrinsic characteristics of a high water activity, water content, and nutrient composition that favour the growth of fungi (Tolosa *et al.*, 2013). The predominant fungi reported on cashew nuts are *Aspergillus niger*, *A. flavus*, *Mucor* spp. and *Rhizopus* spp. (Adebajo and Diyaolu, 2003; Freire *et al.*, 1999; Gyedu-Akoto *et al.*, 2014; Milhome *et al.*, 2014; Suleiman, 2010). This fungal contamination can occur pre- and post-harvest, enabling the production of mycotoxins (Milhome *et al.*, 2014).

Mycotoxins are toxic compounds produced by fungi, mostly by saprophytic moulds growing on a variety of foodstuffs (Logrieco, 2010, Yang *et al.*, 2014). They form a group of structurally diverse secondary metabolites (Turner *et al.*, 2009). Some moulds are capable of producing more than one mycotoxin and some mycotoxins are produced by more than one fungal species (Sakuda and Kimura, 2010). *Aspergillus* is one of the most important mycotoxins producing genera with more than twenty species able to produce mycotoxins (Varga *et al.*, 2011).

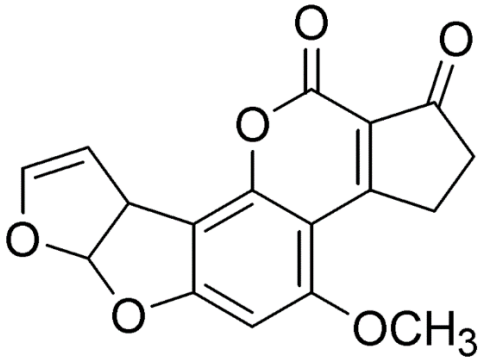
Mycotoxins have adverse effects on humans, animals, and crops and result in illnesses and economic losses (Zain, 2011). The economic impact of mycotoxin contamination of food and feed crops includes loss of human and animal life, increased cost for health and veterinary care, reduced livestock production, disposal of contaminated foods and feeds, and investment in research and applications to reduce the severity of the mycotoxin problem (Chassy, 2010, Zain, 2011). The ingestion of mycotoxins may be pathogenic in humans as they may lead to serious health problems,

such as liver, kidney, or nervous system damage, immunosuppression, biphasic cellular response, and carcinogenesis (Bennett and Klich, 2011; Diesing *et al.*, 2011; Maresca and Fantini, 2010). The most studied mycotoxins (**Fig. 1.5**) in fruits and nuts and their processed products are aflatoxins (Abdulla, 2013; Acevedo *et al.*, 2011; Gyedu-Akoto *et al.*, 2014) and ochratoxin A (Frisvad *et al.*, 2011; Gerez *et al.*, 2014; Nielsen *et al.*, 2009).

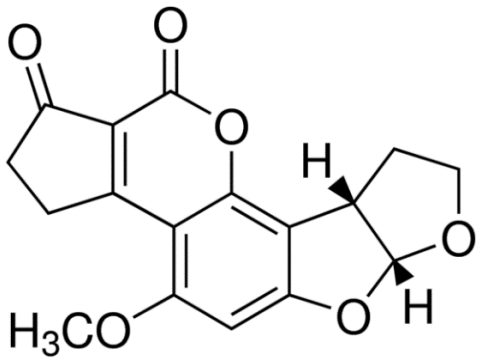
Aflatoxins (Af) are a group of closely related metabolites produced mainly by *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. They are difuranocoumarin derivatives and the main components of this group are AfB_1, AfB_2, AfG_1 and AfG_2 (Turner *et al.*, 2009).

Aflatoxins were first detected and characterized in the 1960s (Asao *et al.*, 1965) and have been found in a variety of agricultural and food products and are classified as the most prevalent in West Africa (Bankole *et al.*, 2006; Darwish *et al.*, 2014; Hell *et al.*, 2003, 2008). Around 20 chemically related compounds are called by the term aflatoxins. AfB1 is the most toxic variant and has been classified by the International Agency for Research on Cancer as a group 1 carcinogen, primarily affecting the liver (Fung and Clark, 2004; IARC, 1993).

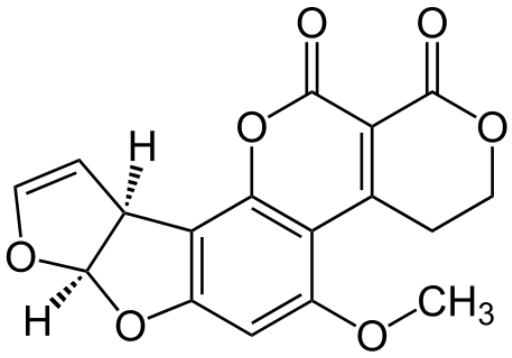
Detectable levels of aflatoxins in cashew nuts were reported in several countries including Venezuela (Acevedo *et al.*, 2011), Iraq (Abdulla, 2013), Brazil (Milhome *et al.*, 2014) and Ghana (Gyedu-Akoto *et al.*, 2014). The European commission's regulations set limits for AfB_1 and total aflatoxins of 5 and 10 µg/kg, respectively, for cashew nuts to be subject to sorting or other physical treatment, and of 2 and 4 µg/kg, respectively, for processed nuts intended for direct human consumption (European Commission, 2010).



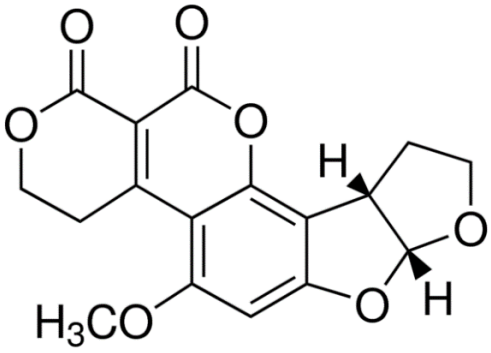
Aflatoxin B_1



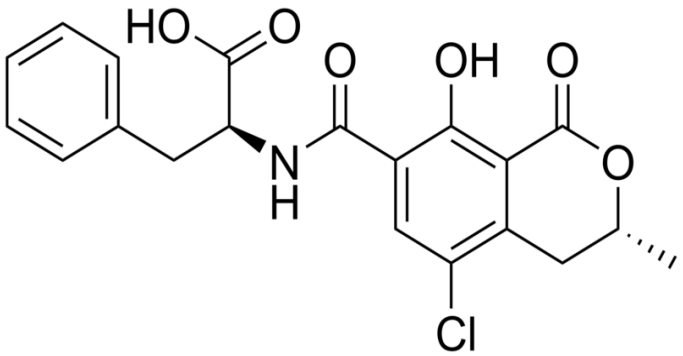
Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2



Ochratoxin A

Fig. 1.5. Chemical structures of aflatoxin (Af) B_1, AfB_2, AfG_1, AfG_2 and ochratoxin A

Ochratoxin A (OTA) was originally described as a metabolite of *Aspergillus ochraceus* and was found soon after from several related *Aspergillus* species (Pardo *et al.*, 2005). In warm temperate and tropical zones, OTA is commonly associated with *A. ochraceus* and species belonging to *A.* section *Nigri*, called also black aspergilli. The significance of black aspergilli as toxin-producing fungi has changed since the evidence that they can produce mycotoxins that are considered a newly emerged risk for human and animal health (Accensi *et al.*, 2004; Ostry *et al.*, 2013).

1.6. Rationale of the study

The Republic of Benin has a population of 10.3 million people with more than 80% earning their living from agriculture (FAO, 2015). Most are subsistence farmers, growing crops on small family plots. Poor infrastructure and poor agricultural management practices are some of the challenges these farmers face.

Since the 1970s, cotton is the most important cash crop in Benin, contributing approximately 35% to the country's export revenues and providing an income to roughly 3 million people. However, cotton productivity and profitability have declined in recent years due to, in part, poor management practices (Mensah *et al.*, 2012). To counter this, the government has launched a program in 2007 to promote along with the cotton and the traditional subsistence crop, three agricultural products with high export revenues: cashew nuts, shea butter and pineapple. The main objective of the program is to help farmers sustainably boost and diversify their crop production for household consumption and sale. Subsequently, cashew nut has increased interest and cashew plantations estimated to be 10.000 ha in 1990 grew to more than 190.000 ha in 2008 according to FAOSTAT data. Since cashew is an export product, it should conform to regulations on quality, trade and export to enter international markets (ACi, 2010a).

The contamination of the raw product is a key point for the quality of the end product. Several authors have reported the presence of mycotoxigenic fungi on cashew nuts around the world including Brazil (Milhome *et al.*, 2014), Venezuela (Acevedo *et al.*, 2011), Iraq (Abdulla, 2013) and Nigeria (Adebajo and Diyaolu, 2003), but these publications focused on processed and retail cashew nuts. Whereas raw cashew nuts from Benin have an easy access to the international market, limited information is available about their fungal infection and the subsequent contamination with mycotoxins. Aflatoxins remain the referenced mycotoxin on raw and processed cashew nuts and are considered as a real health issue; their presence in foodstuffs is carefully regulated (European Commission, 2010; van Egmond and Jonker, 2008; van Egmond *et al.*, 2007). Therefore, there is a need to fill the knowledge gap about fungal and subsequent aflatoxin contamination of cashew nut.

Other mycotoxins may contaminate raw cashew nuts as a consequence of the wide range of fungal contamination that occurs on cashew nuts. Ochratoxin A contamination, for instance, has been reported on pistachios, another member of the cashew family (Brandt, 2007; Palumbo *et al.*, 2015; Zaied *et al.*, 2010). Therefore, there is a need to broaden the knowledge to other mycotoxins and secondary metabolites that can be present on raw cashew nuts.

While the beneficial effects of sorting have been demonstrated for Brazil nuts (De-Mello and Scussel, 2009; Pacheco and Martins, 2013) and for pistachios (Shakerardekani *et al.*, 2012), only few reports were available on the impact of this post-harvest practice on raw cashew nuts in the Beninese context. In Tanzania, cashew nuts are sorted and sold in 2 grades at different prices, which stimulates farmers to improve the quality of their harvest (UNIDO, 2011) while in Benin, raw cashew nuts are shipped without sorting. It was therefore necessary to investigate the effect of sorting of raw cashew nuts from Benin, as a low cost technology applicable at small-scale farmers level on their final quality. Moreover, it is reported that the socio-economic aspects of the farmers and their practices may influence the quality of the cashew nuts. But most available studies

focused on the cashew value chain (Dendena and Corsi; 2014; Krepl *et al.*, 2016). Reports on farmers' practices and the constraints to produce good raw quality nuts are lacking. Therefore, the need to investigate the influence of the socio-economic aspects of the farmer on the quality of raw cashew nuts arises. Filling both knowledge gaps may disclose sources of information for extension services to improve the quality of raw cashew nuts and stimulate Beninese policy makers to investigate the way of grading the cashew nuts.

1.7. Objectives of the study

This thesis reports on the occurrence of mycotoxins and other secondary metabolites of *Aspergillus* sp. on raw cashew nuts and ways to improve nut quality. The specific objectives were:

(1) - to evaluate the occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels;

(2) - to investigate the diversity in secondary metabolites, including mycotoxins from strains of *Aspergillus* section *Nigri* isolated from raw cashew nuts;

(3) - to assess the influence of sorting and grading on quality parameters of raw cashew nuts;

(4) - to study the impact of small-scale farmers' practices on the quality of raw cashew nuts.

1.8. Outline of thesis

This **chapter 1** formulates the justification and the relevance of the research, its objectives and outline. It provides general information about the cashew tree, the cashew nut, the economic importance of cashew and the parameters used to assess the quality of cashew nuts intended for the international market. Furthermore, a summary on the mycotoxin issue in cashew nuts and the

regulations on quality and safety are provided. **Chapter 2** describes the investigation of the occurrence of fungal contamination on raw cashew kernels focusing on two main sections of the genus *Aspergillus*: *Flavi* and *Nigri*. Also the cencentrations of aflatoxin in raw kernels and some quality parameters applicable at the small-scale farmer's gate such as the nut count and the water content, were evaluated. This was meant to assess the quality of the cashew nut from Benin. **Chapter 3** focuses on the secondary metabolites including mycotoxins from strains of *Aspergillus* section *Nigri*. The objective here was to investigate the occurrence of other mycotoxins from black aspergilli that are less studied on cashew kernels. **Chapter 4** assesses the influence of sorting and grading on quality parameters of raw cashew nuts. The rationale of this investigation is to improve the cashew nut quality by sorting them into two grades. This can give an incentive to policy makers to implement sorting and grading as a low cost technology for better revenues for small-scale farmers. **Chapter 5** evaluates the impact of small-scale farmers' practices on the quality of raw cashew nuts to enable the implementation of good agricultural and good harvesting practices that may improve the quality of the raw cashew nut. The final chapter, **Chapter 6,** presents a general discussion on the findings, together with concluding remarks on the extend to which this study has achieved its objectives. Finally, recommendations are given for further research.

Chapter 2

Occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels (*Anacardium occidentale* L.) from Benin

This chapter has been published as:

Yendouban Lamboni, Jens C. Frisvad, Kerstin Hell, Anita R. Linnemann, Rob M.J. Nout, Manuele Tamo, Kristian F. Nielsen, Martinus A.J.S. van Boekel, Eddy J. Smid (2016). Occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels (*Anacardium occidentale* L.) from Benin. *LWT-Food Science and Technology*, 70, 71-77.

Doi:10.1016/j.lwt.2016.02.017

Abstract

The objective of this study was to evaluate the presence of *Aspergillus* section *Flavi* and *A.* section *Nigri* in cashew nuts harvested in the Northern Guinea (NG) and Southern Sudanian (SS) agro-ecological zones of Benin. Also, the presence of aflatoxins was investigated. For detection of fungal contamination, a total of 100 kernels/sample (with disinfection) and 40 kernels/sample (without disinfection) were plated. Seventy samples from fourteen villages were used. Aflatoxins occurrence was analysed on 84 samples by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The average water content and the cashew nuts count were respectively 8.6% and 172 nuts/kg in NG and 8.7% and 174 nuts/kg in SS. Significant differences between villages in both zones were found for both water content and nuts count. In disinfected samples, strains of *Aspergillus* section *Nigri* were predominant, in NG and SS zones (90.2% and 87.2%) respectively. When non disinfected kernels were plated, *A.* section *Nigri* was predominant in both NG and SS zones, with percentages of 89.7% and 93.4%, respectively. None of the 84 nuts samples were positive for natural occurrence of aflatoxins with a detection limit of 0.05 - 0.2 µg/kg.

Keywords: *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri*, aflatoxins, cashew nuts, Benin

2.1. Introduction

The cashew (*Anacardium occidentale* L., Anacardiaceae) originates from north-eastern Brazil and was introduced, in the sixteenth century, in other tropical regions of the world. Its edible part is one of the major agricultural export crops in Benin. In Benin, in 2012 cashew nut trees were cultivated on about 468.000 hectares. The average production over the last 5 years was approximately 128 tons per year. In 2011, the annual turn-over for cashew exports was estimated at 150 million US dollars (FAOSTAT, 2015). Beninese cashew nuts account for 8% of national export revenues and 25% of agricultural export revenues (ACi, 2010a).

Nuts are susceptible to fungal growth according to their intrinsic characteristics of water activity, water content, nutrient composition and pH which favour the growth of fungi (Tolosa *et al*., 2013). According to Milhome *et al*. (2014), cashew nuts are subject to pre and post-harvest fungal contamination facilitating the production of mycotoxins.

Mycotoxins are secondary metabolites produced by certain type of filamentous fungi. *Aspergillus* is one of the most important genera including section *Flavi* with more than twenty species (Varga *et al*., 2011). The predominant fungi reported on cashew nuts are *Aspergillus niger*, *A. flavus*, *Mucor* spp. and *Rhizopus* spp. (Adebajo and Diyaolu, 2003; Freire *et al*., 1999) with *A. flavus* and *A. parasiticus* being the main producers of aflatoxins.

Around 20 chemically related compounds are called by the term aflatoxins in which the most important are AfB_1, AfB_2, AfG_1 and AfG_2. AfB_1 is the most toxic variant and has been classified by the International Agency for Research on Cancer as a group 1 carcinogen primarily affecting the liver (IARC, 1993). The European commission's regulations set limits for AfB_1 and total aflatoxins of 5 and 10 µg/kg, respectively, for cashew nuts to be subject to sorting or other physical treatment, and of 2 and 4 µg/kg respectively, for processed nuts intended for direct human consumption (European Commission, 2010).

Several authors have reported the presence of mycotoxigenic fungi on cashew nuts and cashew products (Abdulla, 2013; Freire *et al.*, 1999; Lawal and Fagbohun, 2014). Detectable levels of aflatoxins on processed cashew nuts were reported in several countries including Venezuela (Acevedo *et al.*, 2011), Iraq (Abdulla, 2013) and Brazil (Milhome *et al.*, 2014) but on raw cashew nuts, there is few data about natural occurrence of aflatoxins.

The aim of this study is to evaluate the occurrence of *Aspergillus* section *Flavi* and *A.* section *Nigri* on raw cashew nuts from northern Benin, and to investigate the presence of aflatoxins in these kernels. This is also the first report to describe the incidence of fungal contamination and presence of aflatoxins in raw cashew kernels from Benin.

2.2. Materials and methods

2.2.1. Sampling area - geographic and climatic characterization

Cashew nuts were sampled in production areas covering two agro-ecological zones of Benin in 2013: Northern Guinea (NG) and Southern Sudanian (SS). NG lies within latitudes 8º1' and 10º6' N whereas SS lies within latitudes 9º4' and 12º3' N. NG and SS are covered by a unimodal rainfall distribution averaging 1000 mm annually, and maximum temperatures varying from 28ºC to 40ºC.

Fourteen villages were selected for sampling: Alafiarou, Bante, Ina, Kilibo, Tchaourou, Tchatchou and Toui (in NG) and Birni, Chabikouma, Kolokonde, Nagayile, Patargo, Penessoulou and Pira (in SS). These villages were selected based on their accessibility (**Fig. 2.1**).

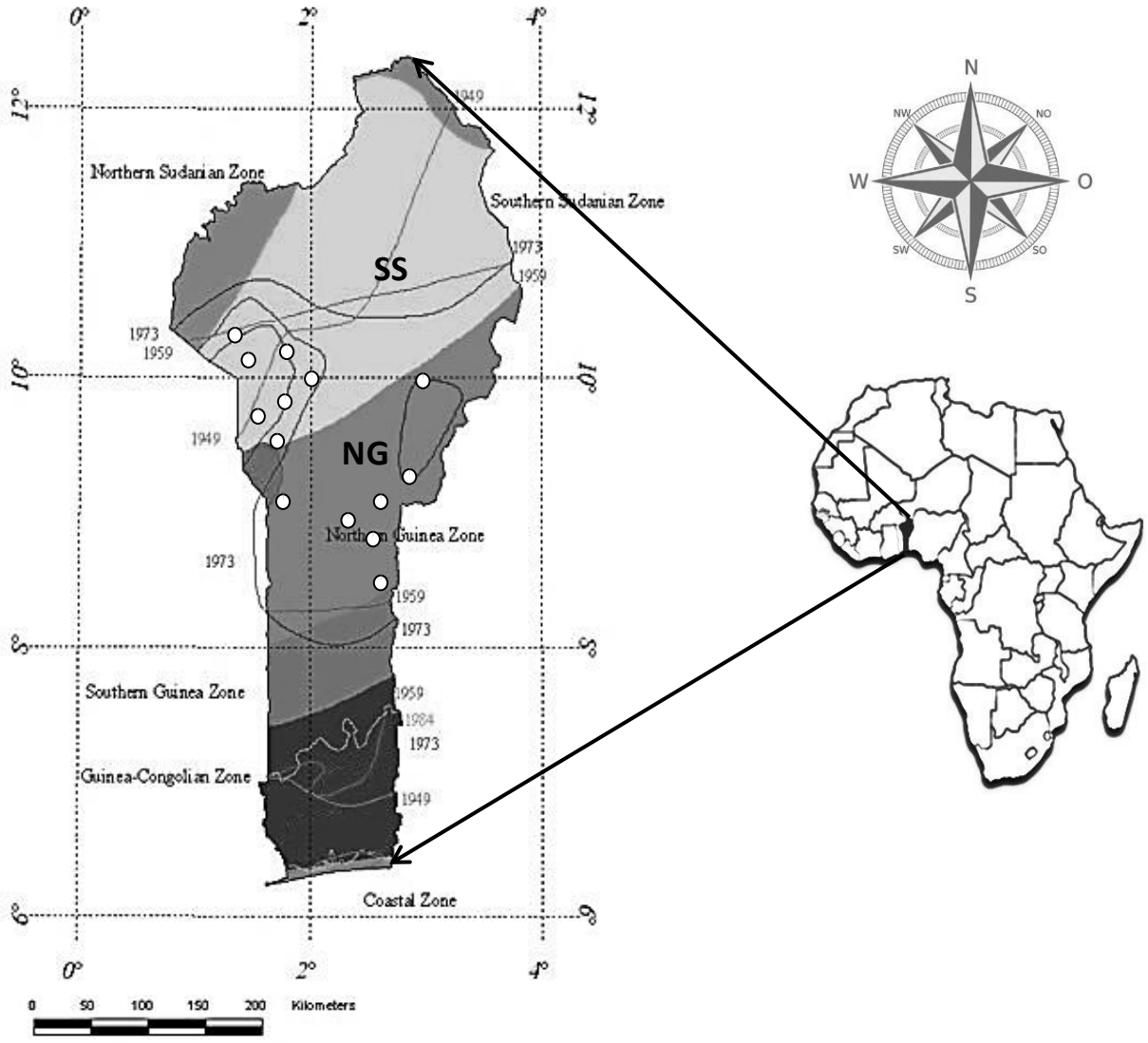


Fig. 2.1. Location of the sampling areas in two agro-ecological (NG, SS) zones of Benin

NG = Northern Guinea

SS = Southern Sudanian

Source: Map modified from Wezel *et al*., 1999.

2.2.2. Sampling procedure

Sampling was done according to Whitaker *et al*. (2010) and the European Regulation (EC No. 401/2006, 2006) describing the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs for small lots. In each village, ten farmers, with cashew farm's area of 5 hectares or more, were identified, and harvested cashew nuts were sampled. Polyethylene bags, jute bags, plastic or metal basins and in piles on the ground, are the most common storage systems used in the study area. The available nuts, were thoroughly mixed and 10 different incremental samples were taken to approximately 3 kg from each farmer. The samples were then labelled, placed in paper bags and transported within the next 3 days to the laboratory. A total of 140 samples were collected. To prevent postharvest changes, samples were stored at 4ºC for further analysis except sub-samples for water content analysis.

2.2.3. Cashew kernels extraction

The water content, microbial contamination and the aflatoxins content were evaluated on cashew kernels. From each nut, the shell was cut in two pieces, using a sharp scalpel and the two cotyledons were extracted. The extraction was done under aseptic conditions. Cross contamination was prevented by disinfecting the scalpels with 90% ethanol. The extracted kernels were put in plastic bags and kept in a refrigerator at 4ºC for further analysis.

2.2.4. Evaluation of nuts count and water content

The count is defined as the number of nuts per kilogram (ACi, 2012a). Evaluation was done by mixing each sample and counting 3 replicates of one kilogram.

The water content was determined using the oven-drying method prescribed by ISO 665-2000 (UNECE, 2002). In duplicate, 10 g ± 1 mg of crushed kernels were placed in a metal box, dried at 103 ± 2°C for 6 hours at atmospheric pressure, with further drying for 3 hours until constant mass

was reached. The mean water content was calculated and expressed as percentage on wet weight basis.

2.2.5. Mycobiota isolation and identification

α. Culture media

For mycobiota isolation, several media were used: Dichloran 18% glycerol agar (DG18, Oxoid) (Pitt and Hocking, 1997) for growth of spores present on cashew kernels; Czapek yeast autolysate (CYA) agar (Frisvad and Samson, 2004), Malt extract agar (MEA, Oxoid), (Samson *et al*., 2004) and Yeast extract sucrose agar (YES) (Pitt and Hocking, 1997) for isolation, morphological observation and identification.

β. Surface disinfection plating

Seventy samples were used for surface disinfection. In order to evaluate the presence of fungi on 100 cotyledons per village, 5 replicates of 4 cotyledons from 5 subsamples per village were randomly picked and surface sterilized for 2 min in 0.4% aqueous solution of sodium hypochlorite, followed by three subsequent rinsings of 2 min with sterile ultrapure water (*Millipore Synergy® UV*, Molsheim, France). With sterile forceps, the cotyledons were plated together equi-spaced from each on DG18; two of the cotyledons having their inner surface turned up and the remaining two having their outer surface turned up (**Fig. 2.2**) according to Adebajo and Diyaolu (2003). Plates were incubated in perforated plastic bags at 25ºC in the dark for 7 days. After the incubation period, the number of cotyledons on which fungal growth was noticed and which showed morphologies consistent with *Aspergillus* section *Flavi* and *A.* section *Nigri*, were counted separately. These strains were isolated and inoculated at three points equidistant from the centre on CYA, MEA and YES and incubated at 25ºC in the dark for 7 days for morphological observation and identification. The strains identified in *Aspergillus* section *Flavi* were *A. flavus, A. tamari, A. costaricaensis, A.*

minisclerotigenes and *A. nomius*. Other species belonging to *Eurotium* and *Rhizopus* and *Mucor* were also recorded.

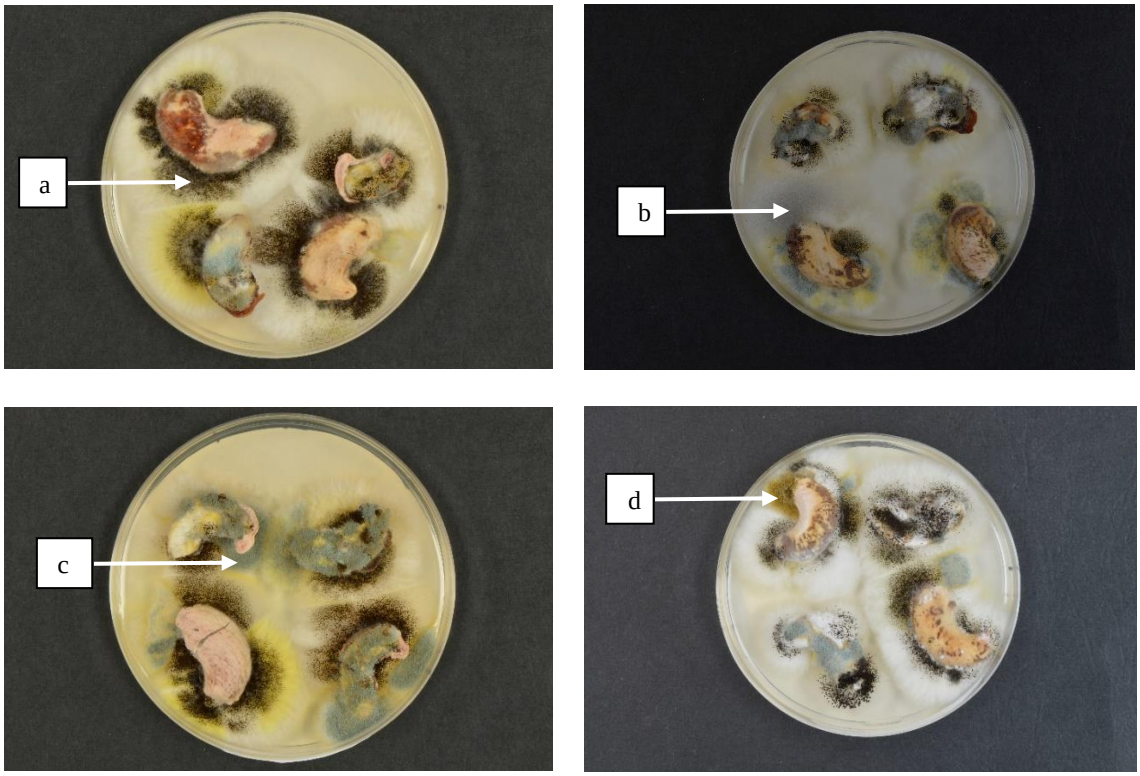


Fig. 2.2. Fungi growth on cashew kernels cultured on Dichloran Glycerol 18% agar (DG18) showing in each petri dish four cotyledons with two having their inner surface turned up and the remaining two having their outer surface turned up (Pictures by Leo Yendouban Lamboni**)**

a = *Aspergillus* section *Nigri*;

b = *Rhizopus* sp.;

c = *Eurotium* sp.;

d = *A.* section *Flavi.*

γ. Non-disinfection plating

The above mentioned plating method was used for direct plating of cashew cotyledons on DG18 without any surface disinfection. A total of 40 cotyledons (2 replicates of 4 from 5 subsamples per village) were plated and all the strains belonging to *Aspergillus* section *Flavi* and *A.* section *Nigri* were transferred to CYA, MEA and YES for identification.

Colony morphology, spore characteristics, mycelium growth, reverse plate observation and microscopic mounts were used for the identification of the strains according to taxonomic schemes and illustrations in Pitt and Hocking (2009), Samson *et al.* (2002; 2007; 2014) and Varga *et al.* (2011).

2.2.6. Statistical analysis

Simple descriptive analysis was used to evaluate the occurrence and the percentage of fungi. The frequencies of fungi occurrence were calculated as a percentage of cotyledons contaminated. One-way ANOVA followed by Tukey HSD test ($p \leq 005$) was used to separate average means of water content and nuts counts. T-test was used to separate means across the two agro-ecological zones. In the case there was significant difference between infection percentage of fungi within villages, Bonferroni and Holm multiple comparison test was used to separate means.

2.2.7. Aflatoxin extraction and quantification

Certified mixed aflatoxins standards with a concentration of 2.0 ng/µl for AfB_1, 2.01 ng/µl for AfB_2 and 0.5 ng/µl for AfG_1 and AfG_2, purchased from Biopure, (Tulln, Austria), were used for quantification. Stock solutions were prepared in methanol and kept at -20ºC. All solvents used for chemical analysis were LC-MS grade. Methanol, formic acid and acetonitrile were purchased from Sigma Aldrich (Denmark).

Raw cashew kernels were analysed for determination of the concentration of AfB_1, AfB_2, AfG_1 and AfG_2. In duplicate, 3 subsamples from the 14 villages were analysed, giving in total 84 samples. From each sample, 25 g of kernels were crushed until the size of particles obtained was no greater than 3 mm. Next, 5 ml of an acetonitrile:water (85:15, v/v) mixture was added to 1 g of ground cashew kernels and extracted for 90 min using a GLF rotary shaker (Microlab, Aarhus). Samples were then centrifuged for 5 min at a speed of 4900 rpm. The extracts (80 µl + 20 µl of internal standard) were then transferred into glass vials and analysed by LC-MS/MS without any further treatment. The internal standard used was ^{13}C labelled AfB_1.

The analyses were performed using an UHPLC-MS/MS on an Agilent 1290 Infinity UHPLC with auto-sampler system coupled to an Agilent 6490 iFunnel Triple quadrupole MS (Agilent Technologies, Santa Clara, CA, Denmark) equipped with electrospray ionization (ESI) source (Nielsen et al., 2015) operating in both negative and positive ionization modes. The injection volume was 2 µl. Separation was performed by using an Agilent Poroshell 120 Phenyl Hexyl column (2.7 µm, 100 x 2.1 mm) operating at 40°C and a flow of 0.4.

Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined to test the validity of the HPLC method used. Linearity was estimated by construction of a five-point calibration curve using Aflatoxins standards of 1–50 µg/kg of each aflatoxin. The recovery was ascertained by spiking 2 µg/kg of AfB_1 and AfG_1, and 1 µg/kg of AfB_2 and AfG_2 to the clean cashew nut powder. The recoveries ranged from 84.2% to 102.9%. LOD and LOQ were determined in spiked sample as 3 and 10 times the standard deviation of the response over the slope of the calibration curve, respectively (Nielsen *et al.*, 2015).

2.3. Results

2.3.1. Cashew nuts count and water content of cashew kernels

In NG, the average cashew nuts count ranged from 160 nuts/kg (Kilibo) to 185 nuts/kg (Bante) with a mean value of 172 nuts/kg whereas in SS it ranged from 152 nuts/kg (Pira) to 189 nuts/kg (Chabikouma) with mean value of 174 nuts/kg (**Table 2.1**). Significant differences were noticed between the average nuts counts in both NG and SS with p-values of 0.0008 and 0.0002, respectively. The average nuts count within the country was 171 nuts/kg.

The average water content ranged from 5.7% (Ina) to 10.8 % (Kilibo) in NG, with a mean value of 8.6%. In SS, it ranged from 6.3% (Penessoulou) to 10.3 % (Patargo), with mean value of 8.7% (**Table 2.1**). There were significant differences between the average water contents both in NG and SS (p = 0.0002; 0.0176). The average water content within the country was 8.6%.

2.3.2. Occurrence and distribution of fungi

a. Surface disinfected samples

Using disinfected kernels, 14 and 17 of the 35 samples from respectively NG and SS contained strains of *A.* section *Flavi* (**Table 2.2**). The range of incidence of contamination varied in NG from 2.0 to 20.5% with mean value of 6.7% whereas it varied in SS from 0.5 to 9.5% with mean of 4.6%. Strains of *A.* section *Nigri* were noticed in all 35 samples in both zones. In NG the incidence of *A.* section *Nigri* varied from 82.5 to 96.0% with the mean of 90.2%. The range and the mean in SS were 70.5 to 98.5% and 87.2%, respectively (**Table 2.2**).

Table 2.1. Averages nut count and water content of cashew nuts from sampling sites in Northern Guinea (NG) and Southern Sudanian (SS) agro-ecological zones of Benin in 2013

Agro ecological zone	Village	Cashew nut count (Nuts/kg)[b]	Water content (%)c
NG [a]			
	Alafiarou[b]	178.3 **ab**	9.2 **ab**
	Bante	184.7 **b**	10.5 **b**
	Ina	179.9 **b**	5.7 **a**
	Kilibo	160.1 **a**	10.8 **b**
	Tchaourou	166.4 **ab**	8.1 **ab**
	Tchatchou	169.8 **ab**	8.5 **ab**
	Toui	163.0 **ab**	7.4 **ab**
mean		171.7 ± 9.3	8.61 ± 1.8
SS			
	Birni	177.8 **ab**	8.5 **ab**
	Chabikouma	188.6 **b**	9.9 **ab**
	Kolokonde	179.3 **b**	8.5 **ab**
	Nagayile	180.5 **ab**	7.3 **ab**
	Patargo	172.8 **ab**	10.3 **b**
	Penessoulou	165.4 **ab**	6.3 **a**
	Pira	152.5 **a**	9.9 **ab**
mean		174.8 ± 11.8	8.68 ± 1.5

a = NG = Northern Guinea; SS = Southern Sudanian;

b = Number of replication was 10 per village.

NB: Cashew nut count and water content followed in the column by the same letter are not significantly different from each other (One way ANOVA, Tukey HSD Test at $p \leq 0.05$).

Table 2.2. Relative frequency (%) of *Aspergillus* section *Flavi* and *A.* section *Nigri* and other species isolated from surface disinfected kernels in different locations in the two agro-ecological zones of Benin

Agro-ecological zone[a]	Location	Contaminated nuts (%)	Number of strains[b]	*Flavi* (%)	*Nigri* (%)	*Eurotium* (%)	*Rhizopus* (%)	*Mucor (%)*
NG	Alafiarou	100	43	2.5	92.5	40.5	2.5	0
	Bante	99.5	62	20.5	96.0	56.7	5.5	1.5
	Ina	99.5	53	9.0	91.5	76.0	2.0	1.5
	Kilibo	100	55	5.0	91.5	90.0	4.0	0
	Tchaourou	100	66	5.0	88.0	86.0	14.5	0.5
	Tchatchou	100	56	2.0	89.5	75.0	7.0	1.5
	Toui	100	53	3.0	82.5	84.0	1.5	1.0
			Mean	6.7	90.2			
			Median	5.0	91.5			
		Number of positive/total samples		14/35	35/35			
		Number of positive/total kernels		48/700	633/700			
SS	Birni	99.5	47	0.5 **a**	88.7 **ab**	47.3	4.17	0.5
	Chabikouma	100	58	4.5 **b**	95.5 **b**	33.5	5.5	0.5
	Kolokonde	92.5	61	6.5 **b**	77.0 **a**	75.5	5.5	1.0
	Nagayile	99.5	53	3.5 **b**	87.5 **ab**	69.3	2.5	0
	Patargo	99.5	56	7.0 **b**	98.5 **b**	62.5	1.5	0.5
	Penessoulou	100	70	9.5 **b**	70.5 **a**	94.0	11.0	2.0
	Pira	100	51	0.5 **a**	93.0 **ab**	96.5	0.5	0
			Mean	4.6	87.2			
			Median	4.5	88.9			
		Number of positive/total samples		17/35	35/35			
		Number of positive/ total kernels		34/700	637/700			

a = NG = Northern Guinea; SS = Southern Sudanian; **b** = on a total number of 100 kernels plated per village.

NB: Means of contamination of cashew nuts by strains of *A* section *Flavi* and *A.* section *Nigri* followed by the same letter are not significantly different from each other (One way ANOVA, Tukey HSD Test at $p \leq 0.05$).

In both NG and SS, strains of *A*. section *Nigri* were the most commonly isolated followed by *Eurotium, A*. section *Flavi, Rhizopus* and *Mucor* (**Table 2.2**). The total number of strains isolated varied from 43 to 66 and from 47 to 70 in NG and SS, respectively. In NG, the highest contamination level of *A*. section *Flavi* was noticed in Bante (20.5%), whereas strains of *A*. section *Nigri* were homogeneously distributed across all locations. In SS, the highest contamination level of *A*. section *Flavi* was noticed in Penessoulou (9.5%) together with the lowest contamination level of *A*. section *Nigri* (70.5%). In SS, there was a significant difference between the contamination levels by strains of both *A*. section *Flavi* and *A*. section *Nigri* ($p = 0.0281$; $p = 0.0011$) (**Table 2.2**).

ß. Non - disinfected samples

Two replications of 5 subsamples from the 14 locations were directly plated on culture media. Eleven and 19 of the 35 samples from respectively NG and SS contained strains of *A*. section *Flavi* (**Table 2.3**). The range of incidence of contamination varied from 1.3 to 32.5% in NG, with mean value of 10.0%, whereas it varied in SS from 0 to 35% with mean of 15.7%. All the samples from both zones were contaminated with *A*. section *Nigri*. The incidence of contamination varied from 75 to 98.8% (mean of 89.7%) and 83.8 to 100% (mean of 93.7%) in NG in SS, respectively (**Table 2.3**).

Aspergillus species belonging to section *Nigri* were isolated in all locations in both the NG and the SS zones and they were the most commonly occurring species (**Table 2.3**). They were followed by *Eurotium*, *Rhizopus, A*. section *Flavi* and *Mucor*. The number of strains isolated varied from 18 to 31 and 21 to 33 in both NG and SS, respectively. The same trend as on surface disinfected samples was noticed for the highest contaminated location in NG, being Bante (32.5%). In SS, the highest contamination level of *A*. section *Flavi* was recorded in Penessoulou (35.0%) with the lowest contamination of *A*. section *Nigri* (83.75%). There was a significant difference ($p = 0.0255$) between levels of contamination of *A*. section *Flavi* in NG. In SS, significant differences were noticed

between of contamination levels by both *A*. section *Flavi* and *A*. section *Nigri* (p = 0.0238; p = 0.0090) (**Table 2.3**).

2.3.3. Aflatoxins in cashew nuts

The aflatoxin content was investigated using UHPLC-MS/MS. For AfB_1, AfB_2, AfG_1 and AfG_2, LOD were 0.2, 0.2, 0.05 and 0.3 µg/kg whereas LOQ were 0.9, 0.7, 0.2 and 1.1 µg/kg, respectively. Linearity of the measurements was checked for a standard solution of aflatoxins on spiked matrix. Calibration curves were y_{AfB1} = 2E+06x + 53.437, y_{AfB2} = 335489x – 31.754, y_{AfG1} = 4E+06 + 26.496 and y_{AfG2} = 745588x + 24.423 with correlation coefficients (R^2) of 0.9993, 0.9908, 0.9968 and 0.9994, respectively.

Among the 84 cashew nut samples analysed, no sample revealed an aflatoxin content above the LOD.

Table 2.3. Relative frequency (%) of *Aspergillus* section *Flavi* and *A.* section *Nigri* and other species isolated from non- disinfected kernels in different locations in the two agro-ecological zones of Benin

Agro-ecological zone[a]	Location	Contaminated nuts (%)	Number of strains[b]	*Flavi* (%)	*Nigri* (%)	*Eurotium* (%)	*Rhizopus* (%)	*Mucor (%)*
NG	Alafiarou	100	21	6.5 **ab**	95	52.5	7.5	1.3
	Bante	100	31	32.5 b	96.30	60.0	28.8	2.5
	Ina	100	22	10.0 **ab**	98.8	62.5	10.0	0
	Kilibo	100	18	10.0 **ab**	75	92.5	0	0
	Tchaourou	100	29	6.3 **ab**	91.3	88.8	15.0	3.8
	Tchatchou	100	26	1.3 **a**	93.8	73.8	17.5	2.5
	Toui	100	20	3.8 **a**	78.8	91.3	1.3	0
			Mean	10.0	89.7			
			Median	6.5	93.8			
		Number of positive/total samples		11/35	35/35			
		Number of positive/total kernels		30/280	253/280			
SS	Birni	100	28	25.0 **ab**	93.8 **ab**	42.5	15.0	0
	Chabikouma	100	28	13.8 a**b**	93.8 a**b**	45.0	22.5	2.5
	Kolokonde	100	31	17.5 a**b**	92.5 **ab**	41.3	3.0	1.5
	Nagayile	100	26	7.5 a**b**	97.5 **b**	58.8	12.5	1.3
	Patargo	100	22	11.3 a**b**	100 **b**	67.5	1.3	0
	Penessoulou	100	33	35.0 **b**	83.8 **a**	93.8	27.50	2.5
	Pira	100	21	0 **a**	92.5 **ab**	97.5	2.5	0
			Mean	15.7	93.4			
			Median	13.8	93.8			
		Number of positive/total samples		19/35	35/35			
		Number of positive/ total kernels		43/280	263/280			

a = NG = Northern Guinea; SS = Southern Sudanian; **b** = on a total number of 40 kernels plated per village

NB: Means of contamination of cashew nuts by strains of *A* section *Flavi* and *A.* section *Nigri*, followed in column by the same letter are not significantly different from each other (One way ANOVA, Tukey HSD Test at $p \leq 0.05$).

2.4. Discussion

The international trade of cashew nuts is regulated based on several quality parameters including water content, nuts count and aflatoxin content (Cashew Handbook, 2014). These parameters give information about the quality of the product. Namdeo *et al.* (2007) concluded that the water in Indian cashew nuts inversely affected the price and the grade, and the higher the number of nuts per kilogram, the poorer their quality. According to ACi, (2012a) the nuts counts are usually in the range of 150 to 210 nuts/kg with 170 nuts/kg to 190 nuts/kg being nuts of excellent or very good quality, and the recommended water content for storage is 12% or less. The cashew counts in our study were on average 172 nuts/kg and 174 nuts/kg in NG and SS, respectively. These values agreed with those recently mentioned by Adeniyi and Adedeji (2015) in a similar study in Nigeria.

The average water contents of cashew nuts measured in our study were 8.6% and 8.7. These values were below the maximum water level allowed for export, being 12% (ACi, 2012a). Similar studies carried out in Nigeria by Adeniyi and Adedeji (2015) recorded water contents of 7.6% and 7.4% in North-Central region. This study area, regarding latitude, is similar to the Northern Guinea and Southern Sudanian regions of Benin, with similar temperature and relative humidity. The water content noticed across both agro-ecological zones can also be explained by the fact that our samples were collected directly from the farmers' storage structure, where harvested cashew nuts might have had time to lose water since harvest.

The recommended harvesting practice for cashew nuts is to collect the nuts when the fruits fall, ensuring that only ripe nuts are collected; but it makes microbial contamination possible via the soil (Cashew Handbook, 2014). Indeed, it is well known that microbial contamination of foodstuffs is mainly via contact during harvesting, with spores present in the soil, but also via insect infestation to damaged or cracked crops, and by cross-contamination during processing (De Saeger, 2011; Lamboni and Hell, 2009).

In this study, the mycobiota encountered on raw cashew kernels predominantly belonged to the *Aspergillus* genera, namely *Flavi* and *Nigri* groups. The species of these genera are mainly post-harvest fungi reported to be predominant in NG and SS of Benin (Cardwell and Cotty, 2002). Our results showed that *A.* section *Nigri*, *Eurotium*, *A.* section *Flavi*, *Rhizopus* and *Mucor* were present on cashew nuts in both zones. Several studies on raw cashew kernels showed similar results. Either at farmer's and retailer's gates (Freire and Kozakiewicz, 2005) or in storehouses (Adeniyi and Adedeji, 2015; Gyedu-Akoto *et al.*, 2014), *Aspergillus* strains mainly *A. niger* followed by *A. flavus*, and *Penicillium* were predominantly recorded on raw cashew nuts.

Although several strains belonging to *A.* section *Flavi* were recorded in our samples, the UHPLC-MS/MS analysis for aflatoxins content revealed that all tested samples were below the detection limit. Indeed, surface contamination with potentially toxigenic fungi does not always mean the presence of mycotoxins. It is well known that fungal growth and toxin production depend on several factors including water activity, temperature, food substrate and strain of the mould (Milhome *et al.*, 2014).

Several studies reported the presence of mycotoxins on nuts and nutty products including almonds and hazelnuts (El tawila *et al.*, 2013), pistachios (Dini *et al.*, 2013) and Brazil nuts (Reis *et al.*, 2012) but there are limited studies that investigated the natural occurrence of aflatoxins on raw cashew nuts. Freire *et al.* (1999) reported that cashew nuts from Brazil were aflatoxin free. In Ghana, Gyedu-Akoto *et al.* (2014) reported a maximum of 0.09 ppb as total aflatoxins on raw kernels, which is far lower than the 20 ppb limit of Ghana standards authority and the World Health Organisation (WHO), and also the EU limits of 4 µg/kg (European Commission, 2010). Moreover, there were many studies on the occurrence of aflatoxins on processed cashew nuts, but most of them reported also no presence of aflatoxin, or aflatoxins levels lower than the EU limits. Leong *et al.* (2010) concluded that cashew nuts purchased from retail outlets in Malaysia were aflatoxins free. Abdulla

(2013) and Gyedu-Akoto *et al*. (2014) reported total aflatoxins concentration to be only 0.185 ppb and 0.3 ppb on roasted kernels from Iraq and Ghana, respectively.

In contrast, there were some reports on the presence of aflatoxins on processed cashew nuts that exceeded the EU limits (Milhome *et al*., 2014; Acevedo *et al*., 2011). The presence of aflatoxin on processed nuts could be explained by possible fungi growth during storage or shipment prior to processing.

Thus far, the overall findings presented in this paper show that raw cashew nuts from Benin are within the limits of water content and nuts count for export, and have aflatoxin levels below the limit of detection. The absence of aflatoxin in cashew nut could be explained by its thick shell that is the first barrier for microbial contamination (Lund *et al*., 2000). Also, cashew nuts are known to contain tannins, which potentially supress aflatoxins formation (Molyneux *et al*., 2007).

Despite the fact that freshly harvested cashew nuts from Benin seem to be safe with regard to aflatoxins, it is highly and mainly contaminated by strains of *A*. section *Nigri* from which *A. niger* is known to produce ochratoxin A and fumonisin (Frisvad *et al*., 2011; Gerez *et al*., 2014; Nielsen *et al*., 2009). Due to the high level of contamination by strains of *A*. section *Nigri*, further investigation will focus on the screening of ochratoxin A and fumonisin in raw nuts. Unfortunately, there is no EU and WHO regulations regarding these mycotoxins, for raw or processed cashew nuts, that could help ranging cashew nuts as good or bad quality. Nevertheless, good agricultural practices need to be strengthened to ensure that the quality is kept constant or improved based on known regulations, namely water content, nuts count and aflatoxin level.

2.5. Conclusion

The occurrence of *Aspergillus* section *Flavi* and *A.* section *Nigri* was investigated together with the presence of aflatoxins in raw cashew kernels. We conclude that Beninese cashew nuts are contaminated by strains of *A.* section *Nigri*, while few with strains of *A.* section *Flavi* where isolated. The presence of strains from the *Flavi* group did not result in aflatoxin contamination since samples were below the detection limit. Therefore, based on available regulations on water content, nuts count and aflatoxin level, Beninese cashew nuts were in the range of good quality for export.

2.6. Acknowledgments

The first author (Y. L.) gratefully thanks Wageningen University for financial support, the International Institute of Tropical Agriculture in Benin for support in sampling, and the Department of System Biology of Technical University of Denmark for providing the infrastructure of laboratories for mycological and aflatoxin analyses. We thank Agilent Technologies for the Thought Leader Donation of the UHPLC-MS/MS system.

Chapter 3

Diversity in secondary metabolites including mycotoxins from strains of *Aspergillus* section *Nigri* isolated from raw cashew nuts from Benin, West Africa

This chapter has been published as:

Lamboni, Y., Nielsen, K. F., Linnemann, A. R., Gezgin, Y., Hell, K., Nout, M. J. R., Smid, E. J., Tamo, M., van Boekel, M. A. J. S., Hoof, J. B., & Frisvad, J. C. (2016). Diversity in Secondary Metabolites Including Mycotoxins from Strains of *Aspergillus* Section *Nigri* Isolated from Raw Cashew Nuts from Benin, West Africa. *PloS one*, *11*(10), e0164310.

Doi:10.1371/journal. pone.0164310

Abstract

In a previous study, raw cashew kernels were assayed for the fungal contamination focusing on strains belonging to the genus *Aspergillus* and on aflatoxins producers. These samples showed high contamination with *Aspergillus* section *Nigri* species and absence of aflatoxins. To investigate the diversity of secondary metabolites, including mycotoxins, the species of *A.* section *Nigri* may produce and thus threaten to contaminate the raw cashew kernels, 150 strains were isolated from cashew samples and assayed for their production of secondary metabolites using liquid chromatography high resolution mass spectrometry (LC-HRMS). Seven species of black aspergilli were isolated based on morphological and chemical identification: *A. tubingensis* (44%), *A. niger* (32%), *A. brasiliensis* (10%), *A. carbonarius* (8.7%), *A. luchuensis* (2.7%), *A. aculeatus* (2%) and *A. aculeatinus* (0.7%). From these, 45 metabolites and their isomers were identified. Aurasperone and pyranonigrin A, produced by all species excluding *A. aculeatus* and *A. aculeatinus*, were most prevalent and were encountered in 146 (97.3%) and 145 (95.7%) isolates, respectively. Three mycotoxins groups were detected: fumonisins (B_2 and B_4) (2.7%) ochratoxin A (13.3%), and secalonic acids (2%), indicating that these mycotoxins could occur in raw cashew nuts. Thirty strains of black aspergilli were randomly sampled for verification of species identity based on sequences of β-tubulin and calmodulin genes. Among them, 27 isolates were positive to the primers used and 11 were identified as *A. niger*, 7 as *A. tubingensis*, 6 as *A. carbonarius*, 2 as *A. luchuensis* and 1 as *A. welwitschiae* confirming the species names as based on morphology and chemical features. These strains clustered in 5 clades in *A.* section *Nigri*. Chemical profile clustering also showed also 5 groups confirming the species specific metabolites production.

Keywords: Diversity; secondary metabolites; mycotoxins; DNA sequencing; *Aspergillus* section *Nigri*; cashew nuts; Benin

3.1. Introduction

Aspergillus section *Nigri* also known as black aspergilli are among the most common fungi responsible for food spoilage and bio-deterioration of other materials (Schuster *et al.*, 2002), also causing substantial impact on food safety due to their mycotoxins production. They are known to produce on the one hand, the mycotoxins ochratoxin A (Chiotta *et al.*, 2013), fumonisins B_2, B_4 and B_6 (Månsson *et al.*, 2009, Nielsen *et al.*, 2009) as well as numerous other compounds with poorly investigated activities (Blumenthal e*t al.*, 2004; Nielsen *et al.*, 2009). On the other hand, black aspergilli are also reported to be of biotechnological importance due to their use in the fermentation industry, for example in their ability to produce hydrolytic enzymes and organic acids (Varga *et al.*, 2000). *Aspergillus luchuensis* is reported to be extensively used in Asia for koji production (Hong *et al.*, 2013). Moreover, many *A. niger* processes have been classified as GRAS (Generally Recognized As Safe) by the Food and Drug Administration of the US government (Schuster *et al.*, 2002) despite the ability of *A. niger* to produce ochratoxin A and fumonisins. However these mycotoxins seem not to be produced under submerged conditions (Frisvad *et al.*, 2011).

Black aspergilli are one of the most complicated species complexes to classify and identify, and the taxonomy of strains in the *A.* section *Nigri* has been studied and debated for decades. In 1934, Mosseray described 35 species of black aspergilli (Mosseray, 1934). Later, that number was reduced to 12 species by Raper and Fennell (Raper and Fennell, 1965). In 1984, based on morphological features, Al-Musallam (1980) revised the taxonomy of *niger* group to 7 species: *A. japonicus*, *A. carbonarius*, *A. ellipticus*, *A. helicothrix*, *A. heteromorphus*, *A. foetidus* and *A. niger*. While, in 2009, Nielsen *et al.* (2009) reported 18 species in the black aspergilli group with *A. niger*, *A. tubingensis*, *A. brasiliensis*, *A. acidus*, *A. carbonarius* and *A. ibericus* as the most common ones. In 2012, Jurjević *et al.* (2012) added *A. floridensis* and *A. trinidadensis* as new species to the *A.* section *Nigri*. Recently, Varga *et al.* (2011) revisiting the species in *A.* section *Nigri*, added 4 other new species and concluded that the black aspergilli group includes 26 taxa. Therefore, a polyphasic taxonomic

approach (Oliveri *et al.*, 2008), has been used to accurately identify black aspergilli at species level. These include morphological, physiological and biochemical characteristics of the isolates, e.g. using high performance liquid chromatography mass spectrometry (HPLC-MS) as well as DNA sequence analysis. The latter is presently based on the use of β-tubulin (Glass and Donaldson, 1995) and calmodulin (Carbone and Kohn, 1999) genes (Hong *et al.*, 2013; Varga *et al.*, 2011), as the ITS regions does not provide sufficient resolution (Balajee *et al.*, 2009).

Nuts are nutritious human foodstuffs (USDA, 2015) because of their high content of protein, carbohydrates, vitamins, essential minerals and especially unsaturated fatty acids. Nuts are consumed in both developing and developed countries by all age groups and across all social strata (Škrbić *et al.*, 2014). Among tree nuts, cashew nuts are known for their high minerals content (e.g. copper, iron and phosphate) and vitamins (e.g. thiamine, vitamin E and pyridoxine) (USDA, 2015). In tropical regions of Africa, 48% of the world's cashew nuts are produced, making them crops of high economic importance (Gyedu-Akoto *et al.*, 2014), and in 2011, cashew nut export contributed about 150 million US dollars to the gross domestic product of Benin (FAOSTAT, 2015) accounting for 8% of the national export revenues.

Since stored nuts generally have a low water activity, their spoilage association consists mainly of fungi and members of *A.* section *Nigri* have been reported to contaminate cashew nuts (Adeniyi and adedeji, 2015).

Although strains of *A.* section *Nigri* have been found on cashew nuts, very little is known about the risk of mycotoxins contamination from black aspergilli on cashew kernels. Therefore, the objective of this study was to screen the mycotoxins and other metabolites that can be produced by *A.* section *Nigri* strains isolated from raw cashew kernels and, based on their metabolite production, to determine which species are prevalent on kernels from Benin. To accomplish these goals, isolated black aspergilli were assayed for their mycotoxins and other secondary metabolites diversity by LC-

HRMS on a LC-time-of-flight mass spectrometry instrument (LC-TOFMS) and representative strains were identified using molecular methods.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

All solvents used for chemical analysis were LC-MS grade. Methanol, acetonitrile, 2-propanol, formic acid were LC-MS grade, while ethyl-acetate and dichloromethane were HPLC grade. All were purchased from Sigma Aldrich (Fluka Analytical, Denmark). Purified water was obtained by using a Milli-Q water purification system (Millipore Synergy® UV, Molsheim, France).

3.2.2. Fungal isolates and growth conditions

One hundred and fifty strains belonging to *Aspergillus* section *Nigri* isolated from cashew nut samples from northern Benin were used in this study. These strains were obtained from Lamboni *et al.* (2016). Cashew nuts were sampled in the main cashew production area covering two agro-ecological zones lying within latitudes 8º1' and 12º3' N and longitudes 0º8' and 3º8' E, with an unimodal rainfall distribution averaging 900 mm to 1000 mm annually and maximum temperatures varying from 28ºC to 40ºC. Based on the agreement between the International Institute of Tropical Agriculture located in Benin and the Beninese Government, any other specific permissions were not required for sampling cashew nut within the study area. In total, 70 nuts samples were randomly selected in fourteen different locations.

After collection, the cashew shell was cut and the kernel (2 cotyledons) aseptically extracted, plated on dichloran 18% glycerol agar (DG18, Oxoid, Basingstoke, Hampshire, UK) (Pitt and Hocking, 1997) and incubated at 25ºC in the dark for 7 days. Four cotyledons were plated per Petri dish, either in five replicates for surface sterilization (SS) (0.4% aqueous solution of sodium hypochlorite) or in

two replicates for direct plating (DP), giving a total of 1960 cultured cotyledons. Both culturing methods were used to enable the growth of conidia present in the inner and the outer part of the cotyledons. According to taxonomic schemes and illustrations in Samson *et al.* (2002), colonies belonging to *A.* section *Nigri* were first isolated on Czapek yeast autolysate agar (CYA) (Pitt and Hocking, 1997) and later 3 point inoculated on Yeast extract sucrose agar (YES) (Pitt and Hocking, 1997). The plates were incubated at 25ºC in the dark for 5 days. From the centre of fugal colonies, three 5-mm agar plugs were taken with an aseptic steel drill and pooled together into the same vial and stored at 4ºC for further extraction.

3.2.3. Plug extraction

A one step extraction method was used by adding 0.5 ml of a mixture of ethyl acetate-dichloromethane-methanol (3:2:1, v/v/v) with 1% (v/v) formic acid to the vials containing the agar plugs. The plugs were then extracted in an ultrasonic bath for 60 min. The supernatant was transferred to a new vial, evaporated to complete dryness using N_2 flow, and re-dissolved in 500 µl of methanol assisted by ultrasonication for 20 min, and the aliquots filtered into an HPLC vial using a 0.45 µm polytetrafluoroethene (PTFE) filter.

3.2.4. UHPLC-QTOF-MS analysis

Analyses were performed using ultra-high-performance liquid chromatography (UHPLC) with diode array detector and maXis 3G QTOF mass spectrometer (MS) (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source (ESI) and connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, USA) equipped with a Kinetex 2.6-µm C_{18}, 100 mm × 2.1 mm column (Phenomenex, Torrance, CA) (Klitgaard *et al.*, 2014). A linear water-acetonitrile gradient was used (buffered with 20 mM formic acid) starting from 10% (v/v) acetonitrile and increased to 100% in 10 min, maintaining for 3 min before returning to the starting conditions. MS was performed in ESI^+, the scan range m/z 100–1000, with a mass accuracy < 1.5 ppm (Klitgaard *et al.*, 2014).

UV/VIS spectra were collected at wavelengths from 200 to 700 nm. Data processing was performed using DataAnalysis 4.0 and Target Analysis 1.2 (Bruker Daltonics) by the aggressive dereplication approach (Klitgaard *et al.*, 2014), using a database of 495 known and putative compounds from black aspergilli, tentatively identifying them based on accurate mass (deviation < 1.5 ppm) and isotopic pattern (isotope fit < 50) (Klitgaard *et al.*, 2014). For saturated peaks (>10^6 counts/sec) a manual verification of the accurate mass was made in the front and the tail of the peak. A further database of 1500 reference standards, tentatively identified compounds were also used along with a small 50 compounds database of peaks observed in sample blanks. All major peaks (observed in the BPC chromatograms) not tentatively identified by the approach were added to the search list as unknown compounds for mapping.

3.2.5. DNA sequencing of *Aspergillus* section *Nigri* strains

α. Aspergillus isolates and growth conditions

Thirty strains of *Aspergillus* section *Nigri* isolated from raw cashew nuts were randomly selected for diagnostic PCR and sequencing. The strains were 3 point inoculated on separate Petri dishes containing Czapek yeast autolysate (CYA) agar and incubated in micro perforated plastic bags at 25ºC for 7 days in the dark, to ensure extensive conidiation of the colonies. From these cultivations on solid media, we prepared stock suspensions for further inoculations and harvested conidia to make suspensions in 5 ml glass tubes containing autoclaved milli-Q water supplemented with 0.05% Tween 80. Conidia were inoculated at 3 points equidistant from the centre, on CYA and incubated in micro perforated plastic bags in the dark at 25ºC for 3 days to favour mycelial growth and reduce the total conidiation as this would inhibit tissue-PCR. The Petri dishes were kept at 4ºC for sampling.

β. Tissue-PCR for molecular identification of fungal isolates

Tissue PCR alleviates the need for genomic DNA extraction, as fungal mycelial tissue was the direct source for template DNA in PCR reactions amplifying partial genes encoding calmodulin and β-

tubulin. PCR tubes containing a total volume of 40 µl had the following components mixed in milli-Q H_2O; 1X Phire PCR buffer (ThermoFisher Scientific, USA), 200 µM dNTP mix (Invitrogen, Merelbeke, Belgium), 0.25 µM forward and reverse primers and 0.7 U Pfu X7 polymerase (Nørholm, 2010). We based molecular identification of the thirty strains on the amplification of two partial genes encoding β-tubulin and calmodulin. The selected β-tubulin primers were T10-F-ACGATAGGTTCACCTCCAGAC (O'Donnell and Cigelnik, 1997), and Bt2b-R-ACCCTCAGTGTAGTGACCCTTGGC (Glass and Donaldson, 1995), and for calmodulin Cmd5-F CCGAGTACAAGGARGCCTTC and Cmd6-R CCGATRGAGGTCATRACGTGG (Hong *et al.*, 2006). A sterile pipette tip was used to streak 1 to 3 mm of peripheral mycelium in two replicates. Distribution of the fungal tissue on the pipette tip to PCR tubes resulted in two tubes with different amounts of biomass and thereby template DNA for PCR.

The amplification was performed in Agilent SureCycler 8800 Thermal Cycler (Agilent Technologies Inc., Santa Clara CA, USA). The amplification process consisted of an initial denaturation step of 30 min at 98°C to release template DNA from fungal debris, followed by 35 cycles of touch-down PCR with 10 s at 98°C (denaturation), 30 s at 61-52°C (primer annealing) and 1 min at 72°C (extension), and a final extension step of 5 min at 72°C. We verified purity of the amplification products by agarose gel electrophoresis in 1% TAE buffer (tris-acetate-EDTA (Ethylenediaminetetraacetic acid)) stained by SYBR Safe DNA Gel Stain (ThermoFisher Scientific, USA) and visualized by UV-light. If more than one product was observed after electrophoresis, PCR products were purified prior to DNA sequencing using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK limited, Buckinghamshire, UK). PCR products were sequenced by GATC Biotech (Constance, Germany).

3.2.6. Phylogenetic analysis of sequence data

The identity of the β-tubulin and calmodulin gene sequences was determined using Basic Local Alignment Search Tool for nucleotide (BLASTN) algorithm in the National Centre for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). They were then transformed into multi FASTA format using DNA Baser software. Phylogenetic analyses and molecular evolutionary were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 6.0 (Tamura *et al.*, 2013). Sequences were pairwise aligned by Clustal W method (Thompson *et al.*, 1994) and trimmed both sides up to the same nucleotide position. Phylogenetic trees were prepared using the maximum likelihood method. Evolutionary distances were calculated by using the Jukes-Cantor model (Jukes and Cantor, 1969) embedded in the MEGA package. Bootstrap values were calculated from 1000 replications after complete deletion of all positions containing gaps or missing data. To compare with cluster output of DNA Baser, secondary metabolites of strains were grouped using MultiExperiment Viewer (MeV v4.2).

3.3. Results

3.3.1. Mycotoxins and other metabolites diversity from *Nigri* group on cashew nuts

The mycotoxins and other metabolites produced by strains of *Aspergillus* section *Nigri* on YES agar are presented in **Table 3.1**. From the 150 isolates used for metabolites profiling, 66 strains (44%) belonged to *A. tubingensis*, 48 strains (32%) to *A. niger* (with a chemical profile similar to *A. welwitschiae*), 15 strains (10%) to *A. brasiliensis*, 13 strains (8.7%) to *A. carbonarius*, 4 strains (2.7%) to *A. luchuensis* (synonyms to *A. kawachi* or *A. acidus*), 3 strains (2%) to *A. aculeatus* and 1 strain (0.7%) to *A. aculeatinus*.

In total, 45 metabolites including their isomers were identified during UHPLC-QTOF-MS analysis within retention times (RT) ranging from 1.56 min (nigragillin) to 10.1 min (aflavinine). Aurasperone C (positive in 97.3%), aurasperone F (96.7%), pyranonigrin A (96.7%), and fonsecin (96%) were the metabolites identified in most of the strains of *A.* section *Nigri*. The metabolites that were rarely produced by strains of *A.* section *Nigri* were secalonic acids (2%), tubingensins (2%), antafumicins (2.7%), fumonisins (2.7%), kotanin (4%) and ochratoxin A (5.3%) (**Table 3.1)**.

The detection of orlandin, kotanin and fumonisin B_2, B_4, B_6 was specific for *A. niger* whereas the presence of antafumicin A and B was specific for *A. luchuensis*. Secalonic acids were specific for *A. aculeatus*.

Table 3.1. Mycotoxins and other secondary metabolites of *Aspergillus* section *Nigri* isolated from cashew nuts

		Mycotoxins/metabolites[a]	R.T. (min)	n (pos)	*A. tubingensis*	*A. niger / welwitschiae*	*A. brasiliensis*	*A. carbonarius*	*A. luchuensis*	*A. aculeatus*	*A. aculeatinus*
		Total strains = 150			*66(7)*[b]	*48(11)*	*15(0)*	*13(7)*	*4(2)*	*3(2)*	*1(1)*
1	**A**	Fumonisin B_2*	5.37	04	_	+ (8.3)[c]	_	_	_	_	_
2		Fumonisin B_4*	5.78	04	_	+ (8.3)	_	_	_	_	_
3		Ochratoxin B	5.90	08	_	+ (10.4)	_	++ (23.1)	_	_	_
4		Ochratoxin A	6.63	20	_	+ (14.6)	_	++ (100)	_	_	_
5		Secalonic acids A, D, F	7.30	03	_	_	_	_	_	+ (100)	_
6		Nigragillin	1.56	129	+++ (95.5)[c]	++ (93.8)	+ (100)	_	+ (100)	_	_
7		Pyranonigrin A	2.27	145	+++ (97.0)	++ (100)	++ (100)	+ (100)	++ (100)	_	_
8	**B**	Nigerazine A, B	2.98	03	_	+ (4.2)	_	_	_	_	++ (100)
9		Nigerapyrone E	3.18	99	++ (42.4)	++ (87.5)	++ (80.0)	+ (100)	+ (50.0)	_	_
10		Tensyuic acid A / F	3.18	116	+ (51.5)	+++ (83.3)	+ (66.7)	+ (100)	+++ (100)	_	_
11		Pyranonigrin B / C	3.60	65	++ (37.9)	++ (33.3)	++ (53.3)	+ (100)	++ (75.0)	_	_
12		Pyranonigrin D	3.77	17	+ (1.5)	+ (2.1)	++ (6.7)	+++ (100)	+++ (25.0)	_	_
13		Fonsecin	4.30	144	+++ (97.0)	++ (100)	++ (100)	+++ (100)	+ (100)	_	_
14		Tensidol A	4.42	72	+ (31.8)	++ (75.0)	+ (93.3)	_	_	_	_
15		Pyrophen	4.45	79	+ (28.8)	+++ (91.7)	++ (93.3)	_	+ (50.0)	_	_
16		Atromentin	4.52	145	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	_	_
17		Tensyuic acid B	4.52	67	+ (31.8)	+ (41.7)	+ (66.7)	++ (100)	+ (25.0)	_	_
18		Funalenone	5.08	137	++ (100)	++ (97.9)	++ (93.3)	+ (46.2)	++ (100)	_	_
19		Rubrofusarin	5.27	142	++ (95.5)	+++ (97.9)	++ (100)	+++ (100)	++ (100)	_	+ (100)

20	Orlandin	5.32	07	_	+ (14.6)	_	_	_	_	_
21	Asperazine	5.35	70	+++ (100)	_	_	_	+ (100)	_	_
22	Tensyuic acid C or D	5.41	39	+ (7.6)	+ (25.0)	+ (20.0)	++ (100)	+ (25.0)	_	_
23	Nigerasperone A	5.42	57	+ (28.8)	++ (58.3)	++ (60.0)	_	_	_	_
24	Tensidol B (Pestalamide A)	5.42	70	++ (31.8)	++ (68.8)	+ (86.7)	_	_	_	_
25	Fonsecin B	5.58	140	++ (97.0)	++ (93.8)	+ (100)	+++ (100)	+ (100)	_	_
26	Malformin A_2	5.69	102	+++ (83.3)	+ (72.9)	+ (93.3)	_	_	_	_
27	Tubingensin A or B	5.90	03	+ (3.0)	_	_	_	+ (25.0)	_	_
28	Malformin C	6.12	116	+++ (93.9)	++ (81.3)	+ (100)	_	+ (50.0)	_	_
29	Kotanin	6.57	06	_	+ (12.5)	_	_	_	_	_
30	Nominine	10.1	08	++ (7.6)	_	_	_	+ (25.0)	++ (66.7)	_
31	Antafumicin A or B	3.56	04	_	_	_	_	+ (100)[c]	_	_
32	Aurasperone C	5.95	146	+++ (100)	++ (100)	+ (100)	++ (100)	++ (100)	_	_
33	Aurasperone F	6.29	145	+++(100)	+++ (100)	++ (100)	++ (100)	+ (75.0)	_	_
34	Aurasperone E	6.58	133	+ (87.9)	++ (95.8)	++ (100)	+ (100)	++ (75.0)	_	_
35	Aurasperone B	6.61	141	+ (97.0)	++ (95.8)	++ (100)	++ (100)	++ (75.0)	_	_
36	Flavasperone	7.03	137	+++ (90.9)	+++ (95.8)	+++ (100)	++ (100)	+ (50.0)	_	_
37	Nafuredin	9.22	55	++ (33.3)	++ (56.3)	+ (20)	_	+ (75.0)	_	_

* Small peak of fumonisin B_6 were also detected when B_4 and B_2 was detected;

[a] Divided in 2 groups: A = mycotoxins known as toxic compounds for humans, B = other secondary metabolites;

[b] Number of strains of each species, with in brackets, number of strains selected for DNA sequencing;

(+) Detected with relative abundance within a line (+++ high production, ++ average production and + low production); (-) not detected; n (pos) = total number of positive strains for a given mycotoxin/metabolite; R. T. = retention time in min using ultra-high-performance liquid chromatography quadrupole time of flight mass spectrometry. Positive electrospray ionization (ESI^+, *m/z* 100-1000);

[c] In bracket is the percentage of strains that produced a given mycotoxin/metabolite.

Secalonic acids, atromentin, asperazine and aurasperone C were produced consistently by all the strains in a species. The mycotoxin fumonisin B_2 (2.7%) was detected in strains belonging to *A. niger* whereas ochratoxin A (13%) and ochratoxin B (5.3%) were produced by strains of both *A. niger* and *A. carbonarius*.

For example, in **Table 3.1** with UHPLC-QTOF MS, nigragillin was produced by 129 (86%) of the 150 strains studied. Nigragilin was produced by 95% of the strains of *A. tubingensis* (64/66), 94% of *A. niger* (46/48) and 100% of both the strains of *A. brasiliensis* (15) and *A. luchuensis* (4).

An example of Base Peak Chromatogram (BPC) of *A. niger* extract is shown in **Fig. 3.1** where several compounds were identified including fumonisin B_2 and fumonisin B_4.

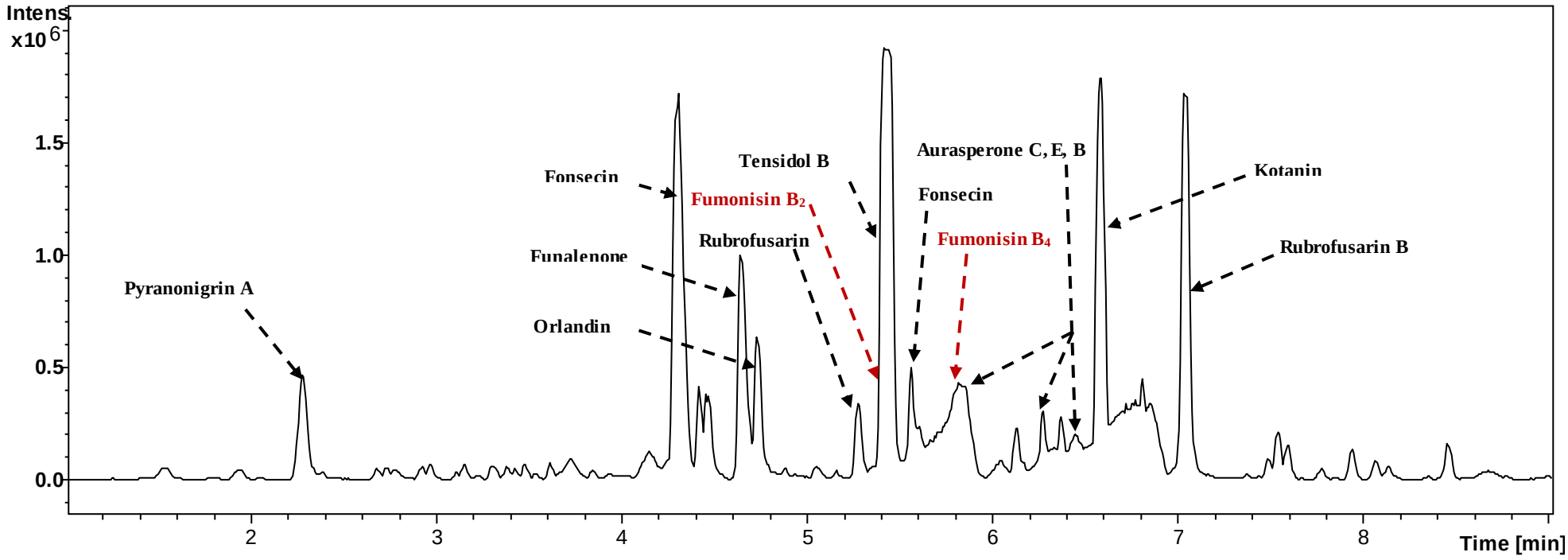


Fig. 3.1. Base Peak Chromatogram (BPC) of *Aspergillus niger* extract

The Analysis was done by reversed phase ultra-high-performance liquid chromatography quadrupole time of flight mass spectrometry. Positive electrospray ionization (ESI^+, *m/z* 100-1000). *A. niger* was cultured on yeast extract sucrose agar for 5 days in dark. The BPC showed the production of fumonisin B_2 and fumonisin B_4 and other secondary metabolites.

3.3.2. Phylogenetic analysis

We examined the genetic relatedness of 30 randomly taken strains of *Aspergillus* section *Nigri* using nucleotide sequences of β-tubulin and calmodulin genes. **Table 3.2** summarizes the species names based on their metabolite production which was confirmed by sequencing data. Eleven isolates were identified as *A. niger*, 7 as *A. tubingensis*, 6 as *A. carbonarius*, 2 as *A. luchuensis*, 2 as *A. aculeatus*, 1 as *A. aculeatinus* and 1 as *A. welwitschiae*. Three of the isolates (2 of *A. aculeatus* and 1 of *A. aculeatinus*) did not match with the β-tubulin and calmodulin genes used.

The phylogenetic relationship between the isolates of *A.* section *Nigri* species was illustrated in the maximum likelihood method analysis. Twenty seven isolates matched either with β-tubulin and/or calmodulin genes during PCR amplification. The 27 sequences were aligned and resulted in the formation of 5 clades (**Fig. 3.2**). All the *A. niger* strains (11) clustered together, as did the *A. tubingensis* strains (7) and *A. carbonarius* strains (6). *A. welwitschiae* strain was separated from *A. niger* sequences with 98% bootstrap value. Two strains of *A. luchuensis* clustered to form a clade.

The hierarchical clustering based on chemical compounds of the 27 strains, showed 5 major clusters similar to the genetic clades (**Fig. 3.2**). Some of the strains in *A. tubingensis* group clustered (A) whereas the others clustered together with strains of the *A. luchuensis* clade (E). Strains of *A. niger* clustered in 2 groups (B and C) whereas *A. welwitschiae* joined strains of *A. carbonarius* to form cluster D (**Fig. 3.2**). The hierarchical clustering revealed aflavinin, tubingensin, orlandin, secalonic acids, fumonisins and ochratoxins as key compounds for chemical clustering (**Fig. 3.3**).

Table 3.2. *Aspergillus* section *Nigri* strains isolated from cashew nuts characterized by sequence analysis

	Samples Id	Sequencing Id	method [a]	Location [b]	Species [c]	GenBank accession number [d]
1	8714	Lyl1	SS	Penessoulou	*A. tubingensis*	KX769852
2	8715	-	SS	Penessoulou	***A. aculeatus***	-
3	8716	-	SS	Penessoulou	***A. aculeatus***	-
4	8726	-	SS	Kolokonde	***A. aculeatinus***	-
5	8726	Lyl2	SS	Kolokonde	*A. niger*	KX769853
6	8741	Lyl3	SS	Patargo	*A. niger*	KX769854
7	8743	Lyl42	SS	Patargo	*A. carbonarius*	KX769875
8	8749	Lyl33	SS	Pira	*A. niger*	KX769866
9	8755	Lyl4	SS	Nagayile	*A. niger*	KX769855
10	8764	Lyl5	SS	Birni	*A. niger*	KX769856
11	8765	Lyl6	SS	Birni	*A. luchuensis*	KX769857
12	8775	Lyl43	SS	Alafiarou	*A. carbonarius*	KX769876
13	8710	Lyl7	DP	Penessoulou	*A. welwitschiae*	KX769858
14	8718	Lyl8	DP	Kolokonde	*A. niger*	KX769859
15	8735	Lyl9	DP	Chabikouma	*A. niger*	KX769860
16	8739	Lyl45	DP	Patargo	*A. carbonarius*	KX769878
17	8741	Lyl11	DP	Patargo	*A. luchuensis*	KX769861
18	8749	Lyl12	DP	Pira	*A. niger*	KX769862
19	8761	Lyl13	DP	Nagayile	*A. tubingensis*	KX769863
20	8768	Lyl14	DP	Birni	*A. niger*	KX769864
21	8809	Lyl44	DP	Tchaourou	*A. carbonarius*	KX769877
22	8714	Lyl15	DP	Penessoulou	*A. carbonarius*	KX769865
23	8799	Lyl34	DP	Kilibo	*A. tubingensis*	KX769867
24	8792	Lyl35	SS	Toui	*A. tubingensis*	KX769868
25	8800	Lyl36	SS	Kilibo	*A. tubingensis*	KX769869
26	8714	Lyl37	SS	Penessoulou	*A. tubingensis*	KX769870
27	8718	Lyl38	SS	Kolokonde	*A. tubingensis*	KX769871
28	8739	Lyl39	SS	Patargo	*A. niger*	KX769872
29	8758	Lyl40	SS	Nagayile	*A. niger*	KX769873
30	8801	Lyl41	SS	Kilibo	*A. carbonarius*	KX769874

[a] SS = strains isolated after surface sterilization of cashew kernels; DP = strains isolated after direct plating of cashew kernels. In both methods, cashew kernels were first plated on Dichloran 18% glycerol agar (DG18, Oxoid). Strains belong to *A.* section *Nigri* were isolated on Czapek yeast autolysate agar (CYA) and later inoculated on Yeast extract sucrose agar (YES). The plates were incubated at 25 ºC in the dark for 5 days;

[b] All locations are in northern Benin within latitudes 8º1' and 12º3' N and longitudes 0º8' and 3º8' E, with an unimodal rainfall distribution of 900 mm to 1000 mm annually and maximum temperatures varying from 28 ºC to 40 ºC;

[c] No PCR products was obtained for the partial β-tubulin and calmodulin gene sequences for the species in bold. Their identification relied only on morphology and their metabolite profile;

[d] GenBank accession numbers based on partial calmodulin genes used during PCR amplification.

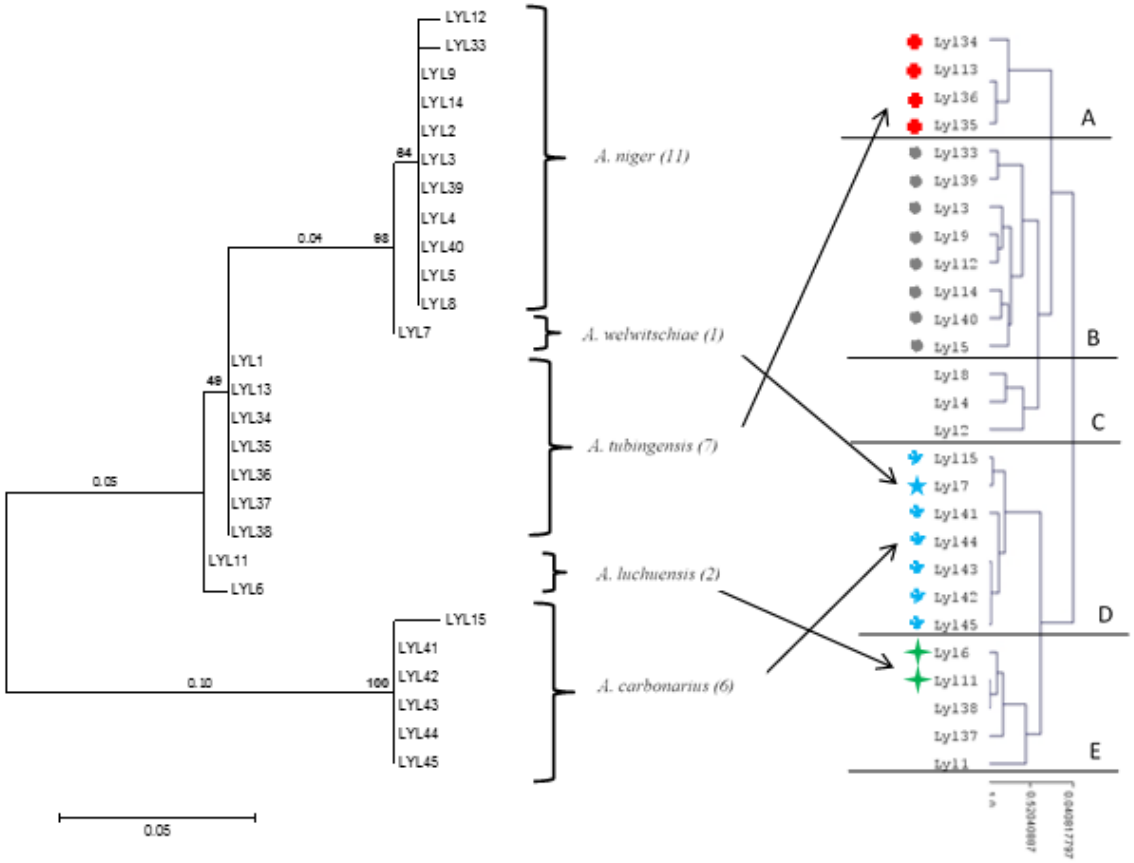


Fig. 3.2. Phylogenetic trees based on combined sequences data of β-tubulin and calmodulin of 27 strains of *Aspergillus* section *Nigri*, with clustering based on metabolites profile

Phylogenetic analyses and molecular evolutionary were conducted using MEGA version 6.0. The identity of the gene sequences was determined using BLASTN algorithm. The alignment was performed using the Clustal W program. Nucleotide divergences were estimated according to the Jukes-Cantor model. Numbers above branches are bootstrap values. The evolutionary history was inferred using the maximum likelihood method. The chemical clustering was performed using MultiExperiment Viewer (MeV v4.2).

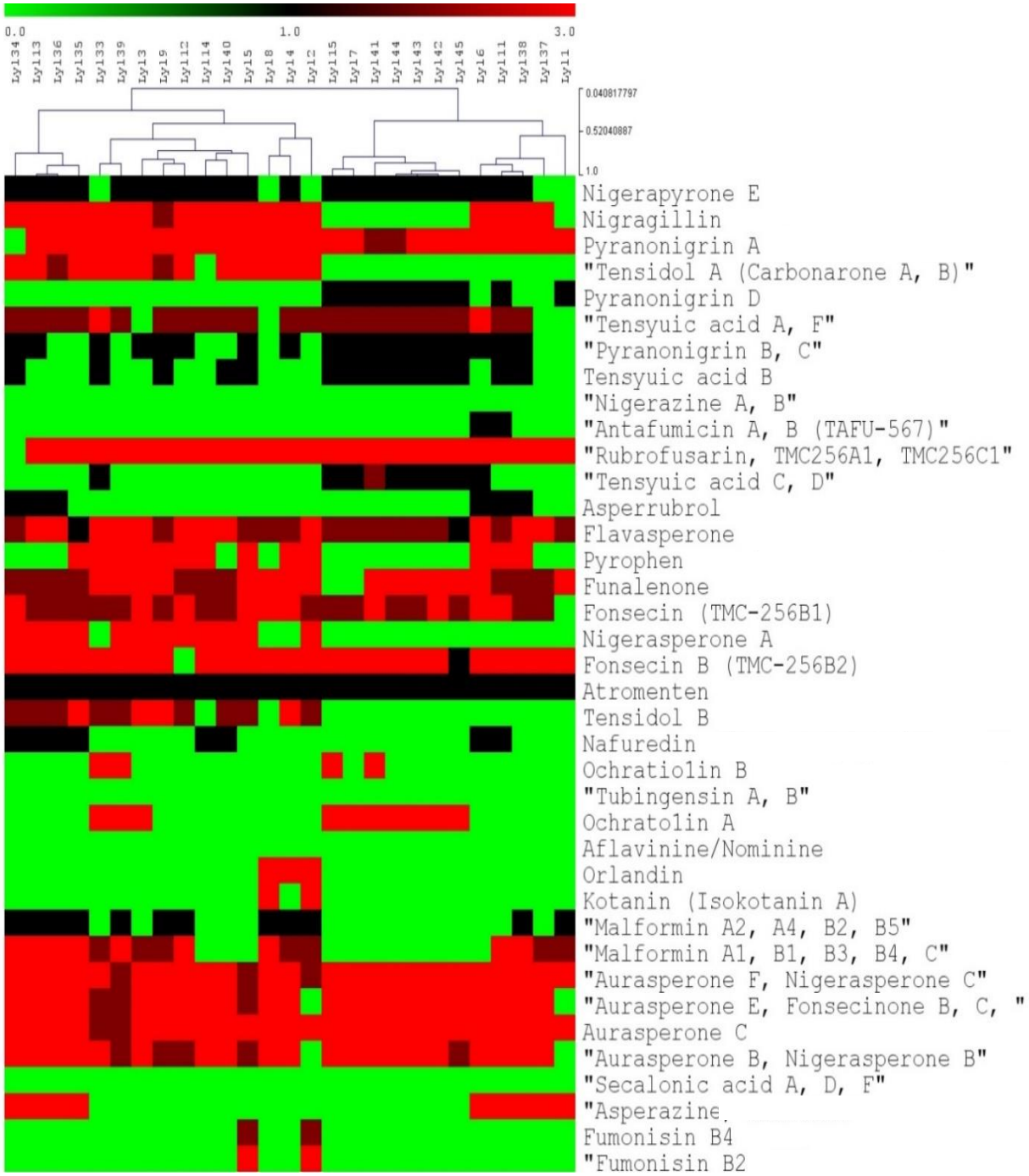


Fig. 3.3. Hierarchical clustering based on metabolites profile of strains of *A.* section *Nigri*

NB: The chemical clustering was performed using MultiExperiment Viewer (MeV v4.2);
In brackets (“ ”) are the chemicals with high probability of being species specific metabolites.

A. tubingensis (Lyl34, Lyl13, Lyl36, Lyl35, Lyl38, Lyl37, Lyl1)
A. niger (Lyl33, Lyl39, Lyl3, Lyl9, Lyl12, Lyl14, Lyl40, Lyl5, Lyl8, Lyl4, Lyl2)
A. carbonarius (Lyl15, Lyl41, Lyl44, Lyl43, Lyl42, Lyl45)
A. welwitschiae (Lyl7)
A. luchensis (Lyl6, Lyl11).

3.4. Discussion

One hundred and fifty strains of *Aspergillus* section *Nigri* were used for mycotoxin and other secondary metabolite profiling using ultra-high-performance liquid chromatography. All the 45 chemical compounds identified pertained to black aspergilli as previously described by Nielsen *et al.* (2009). Some of these natural products are known to be toxic to human and animals. These where classified in **Table 3.1** as group A and included fumonisins and ochratoxin A, which were reported for *Aspergillus niger* previously (Varga *et al.*, 2010). According to Mogensen *et al.* (2010) and Noonim *et al.* (2009), up to 75% of *A. niger* isolates produce fumonisins and 41% produce ochratoxins. Also, Massi *et al.* (2016) reported 74% of *A. niger* to be fumonisin B_2 producers while 32% were ochratoxin A producers. In our results, among the 48 strains of *A. niger* isolated, only 9% (4 strains) produced fumonisins and 15% (7 strains) produced ochratoxins. The difference could be due to the type of commodities from which the *A. niger* strains were isolated. The effect of the food matrix was demonstrated by Vaamonde *et al.* (2003) who studied the variability of aflatoxin production by strains of *A.* section *Flavi* in peanut, wheat and soybean. The isolates of Mogensen *et al.* (2010) were from raisins and those from Noonim *et al.* (2009) from dried coffee bean samples. The food matrix of raisins (16% of moisture, 25 to 30% of sugars and <1% of lipids) is different from that of dried green coffee (12 – 13% of moisture, <1% of sugars and 4 to15% of lipids) and much more different from that of cashew nuts (8 to 9% of moisture, 1 to 8% of sugars and 60 to 64% of lipids). Isolates from Massi *et al.* (2016) were from different food commodities: dried fruits, Brazil nuts, coffee beans, grapes cocoa and onions. In addition, cashew nuts also have a thick shell that constitutes a first barrier to microbial contamination (Lund *et al.*, 2000). It is known that cashew shells contain tannins that are able to suppress mycotoxin formation (Molyneux *et al.*, 2007) and probably alter the gene expression by the fungi. More so, on Pistachio nuts, Marin *et al.* (2008) noticed only 5% of *A. niger* to be ochratoxin A positive.

The geographic origin of a strain can reportedly influence its mycotoxin production. Isolates of *A. flavus* from various geographic regions have revealed differences in the proportions of isolates that produce low, medium and high amount of aflatoxins (Donner *et al.*, 2009). This could also apply to fumonisin and ochratoxin production by black aspergilli. Samson *et al.* (2004) reported ochratoxin production from species of *A.* section *Nigri* isolated from different food matrices collected from various regions. Moreover, Perrone *et al.* (2006) reported that 33% of *A. niger* isolated from grapes in Italy produced ochratoxin A. In our study we did not notice simultaneous production of both fumonisins and ochratoxins from the same strains of *A. niger*, where Frisvad *et al.* (2011) reported that up to 10% of *A. niger* strains may produce both mycotoxins. Ochratoxin A production rate can be overestimated in some studies as HPLC with fluorescence detection (even using immunoaffinity purification), can provide false positives (Storari *et al.*, 2012) which unfortunately has been extensively reported for *A. tubingensis* (Medina *et al.*, 2005; Perrone *et al.*, 2006). Ochratoxin A production from *A. tubingensis* was not detected during our screening process, confirming the report of Nielsen *et al.* (2009) and Storari *et al.* (2012) concerning this. Also, in accordance with Frisvad *et al.* (2011), strains of *A. tubingensis*, *A. brasiliensis* and *A. luchuensis* did not produce fumonisins or ochratoxins.

A. carbonarius isolates always produced ochratoxin A as reported by several authors (Abarca *et al.*, 2003; Chiotta *et al.*, 2011; Nielsen *et al.*, 2009). Our result was in accordance with this consistent production since all the 13 isolates of *A. carbonarius* showed ochratoxin A production.

Secalonic acids, reported as toxic metabolites of *A. aculeatus* (Andersen *et al.*, 1977) were noticed during our analysis, confirming their production by *A. aculeatus* as mentioned by Parenicová *et al.* (2001). These toxic compounds were not produced by *A. aculeatinus* from cashew nuts which is in contrast to the report by Noonim *et al.* (2008).

Some secondary metabolites detected during our analysis, such as Aurasperones, Nigragillin, Malformins and Nigerazine and grouped as B in Table 1 are reported to be toxic compounds to plants, bacteria, and mice (Blumenthal, 2004; Ghosal *et al.*, 1979). Asperazine was reported to have significant *in vitro* cytotoxicity against human leukemia (Varoglu *et al.*, 1997) but no *in vivo* including bioavailability studies confirmed this. Malformins are currently being investigated for anti-cancer drug potential (Wang *et al.*, 2015). Altogether there are very few studies on other effects than *in-vitro* or in older studies intraperitoneal injection of compounds. However these do not include degradation in the body nor bioavailability of the compounds, and with a definition of mycotoxin being toxic through a natural route of exposure, such studies can only be considered indicative, but also highlights the need for testing these compounds in relevant animal models under relevant exposure conditions. Similar problems are reported in the *Aspergillus glaucus* group (and formerly *Eurotium*) (Blaser *et al.*, 1980; Greco *et al.*, 2015).

DNA sequencing using β-tubulin and calmodulin genes was performed to validate morphology and extrolite profile based on identification of our isolates and their association to *A.* section *Nigri*. The use of a polyphasic approach, to identify and validate to species level isolates of fungi, was described by Frisvad (2011), Oliveri *et al.* (2008). Our DNA sequences confirmed the species name identified by morphological and chemical characteristics and the phylogenetic tree shows that the main clades belong to the black aspergilli. Perrone *et al.* (2006) in a cluster analysis of 94 isolates of *A.* section *Nigri* identified the same clades confirming the fact that *A. tubingensis* and *A. niger* are the main clades of *A.* section *Nigri* as reported by Nielsen *et al.* (2009). Moreover, Samson *et al.* (2014) confirmed the presence of these 4 different clades in *A.* section *Nigri* and grouped them as biseriate group of *Aspergillus* section *Nigri* in contrast to uniseriate group of *Aspergillus* including *A. aculeatus*.

The cluster analysis of the 27 strains using their metabolite profiles was similar to the clustering based on sequencing data. The secondary metabolites have been previously used most often in species recognition due to their high species specificity (Larsen *et al*., 2005). Samson *et al.* (2014) mentioned that isolates of *Aspergillus* species usually produce a diverse range of secondary metabolites that are characteristic of the different groups of section of *Aspergillus*. They also reported that the production of a particular secondary metabolite is an efficient identification aid for allocating a species to section while profiles of secondary metabolites can be very effective in identifying an *Aspergillus* isolates to species. With few exceptions, this was effective during our analysis where the combined production of orlandin, fumonisins and kotanin was specific to *A. niger*, and the production of antafumicin A and B was specific to *A. luchuensis*.

3.5. Conclusion

The diversity in secondary metabolites including mycotoxins from isolates of *Aspergillus* section *Nigri*, analysed using UHPLC-QTOF-MS, revealed several metabolites produced by 7 different species that contaminated cashew nuts samples from Benin. In pure cultures on a laboratory medium, ochratoxin A and fumonisins, the 2 main toxic compounds from black aspergilli, were produced by strains of 2 predominant species in *A.* section *Nigri*, namely *A. niger* / *A. welwitchiae* and *A. carbonarius*, although *A. carbonarius* is unable to produce fumonisins. Ochratoxins and fumonisins were produced by a relatively little proportion of the isolates of *A. niger* and *A. carbonarius*, but it is well know that species of *A.* section *Nigri* are the most isolated on cashew kernels, given a substantial number of species that may produce mycotoxins in cashew nuts. Even though the presence of fungi has not always meant the presence of mycotoxins, the production of ochratoxin A and fumonisins by isolates on *A.* section *Nigri* on cashew nuts could constitute an additional and hidden problem in term of mycotoxins content, and can negatively affect cashew nut safety and the nutritional quality of the nuts.

There are no regulations on ochratoxin A and fumonisins for raw and processed cashew nuts like those of EU and WHO on aflatoxins. Nevertheless, these findings suggest more investigations in order to detect the presence and the levels of ochratoxin A and fumonisins and to evaluate their exact contribution to the total level of mycotoxins in cashew kernels. But immediate actions should emphasize on the prevention by strengthening post-harvest practices that can lower fungal contamination along the cashew nut value chain, mainly during nut storage, where high contamination of species belonging to black aspergilli are noticed.

3.6. Acknowledgments

The first author (YL) gratefully thanks Wageningen University, the International Institute of Tropical Agriculture in Benin, and the EEC project MycoRed (KBBE-2007-222690-2). The DTU Metabolomics Platform, Department of Systems Biology, Technical University of Denmark is acknowledged for access to UHPLC- QTOF instrument.

3.7. Author contributions

Conceptualization: YL KFN KH; Data curation: YL KFN YG; Formal analysis: YL KFN YG; Funding acquisition: KFN MT MAJSB JBH JCF; Investigation: YL KFN YG JBH JCF; Methodology: YL KFN YG JBH JCF; Project administration: KFN MAJSB JCF; Resources: YL KFN YG KH EJS MT JBH JCF; Software: YL KFN EJS; Supervision: KFN MT JBH JCF; Validation: YL KFN JBH JCF; Visualization: YL KFN EJS JBH JCF; Writing – original draft: YL; Writing – review & editing: YL KFN ARL KH MJRN EJS MAJSB JBH JCF.

Chapter 4

Influence of sorting and grading on water content, nut count and fungal contamination of raw cashew nuts (*Anacardium occidentale* L.)

This chapter has been submitted for publication:

Yendouban Lamboni, Martinus J. R. Nout, Eddy J. Smid, Kerstin Hell, Manuele Tamo, Martinus A. J. S. van Boekel, Anita R. Linnemann*. Influence of sorting and grading on water content, nut count and fungal contamination of raw cashew nuts (*Anacardium occidentale* L.).

Abstract

Cashew nut is of economic importance in West Africa and one of the major cash crops in Benin. Its access to the international market is restricted by regulations on quality parameters of raw nuts. In order to improve the quality, we investigated the potential on low cost technology applicable at small scale famers' level to augment the score of customary quality parameters. One hundred and forty samples of raw nuts were collected in the Northern Guinea (NG) and Southern Sudanian (SS) agro-ecological zones in Benin. The samples were first sorted in two grades. The results showed that nut counts in Grade 1 were from 151 to 174 nuts/kg and from 142 to 182 nuts/kg in NG and SS, respectively. In Grade 2, nut counts were from 168 to 202 nuts/kg in NG and from 171 to 197 nuts/kg in SS. There was significant difference between Grade 1 and Grade 2 in both agro-ecological zones. We recorded a water content of 8.6% for both Grade 1 and Grade 2 in NG. Significant difference was noticed in water content in SS where it was 9.7% and 6.4% for Grade 1 and Grade 2, respectively. After disinfection of nuts, 42.9% and 50.0% of samples showed significant differences between Grade 1 and Grade 2 for contamination by *A.* section *Flavi* and *A.* section *Nigri*, respectively. Non-disinfected samples showed the same significant difference for 35.7% of *Aspergillus* section *Flavi* contaminated nuts and 28.6% of *A.* section *Nigri* contaminated nuts. These findings indicate that sorting and grading impacted the nut count within villages and across agro ecological zones, and the fungal contamination within villages. These quality parameters are improved and therefore can have a positive impact on the marketability of raw cashew nuts, increasing the potential profit of small-scale farmers.

Keywords: Sorting and grading; Fungal contamination; nut count; *Anacardium occidentale*; Agro-ecological zone; Benin

4.1. Introduction

Cashew (*Anacardium occidentale* L.) is the most important tree nut in West Africa. This region represents 38% of the global cashew nut production areas. Nigeria, Ivory Coast, Benin and Guinea-Bissau had the highest cashew nut production in 2013, with in total 1,718,195 tonnes, giving a gross production value of 637 million US dollars (FAOSTAT, 2016). Benin was ranked as the 3rd production country in West Africa, with 180,000 tonnes and an annual export turn-over estimated at 150 million US dollars (FAOSTAT, 2016). Beninese cashew nuts account for 8% of national export revenues and 25% of agricultural export revenues of the country (ACi, 2010a). More than 95% of raw cashew nuts from Benin are exported, amounting to about 15 to 19% of the total raw cashew nut import of India (Cashew Handbook, 2014).

The variability in the market supply of cashew nuts from many tropical regions of the world has induced an increase in quality requirements for export. Kashani-Nejad *et al.* (2003) reported that proper harvesting and postharvest handling are key operations in obtaining maximum yield of good quality nuts. To secure the quality of raw cashew nuts, they should be harvested as soon as possible after they have fallen on the ground (Azam-Ali and Judge, 2001). This avoids quality loss and minimises fungal contamination (Kader, 2013). A survey on harvesting practices in Ghana showed that only 45.3% of the farmers picked their nuts daily from the fields (Gyedu-Atoko *et al.*, 2014).

Quality of raw cashew nuts is determined by size of the nut expressed as nut count (i.e. number of raw nuts per kg), water content, incidence of insects and or fungal damage and out-turn, also known as the kernel output ratio after removing the shell (ACi, 2012a, b). The first three quality attributes can be evaluated and influenced at the level of the small-scale farmer while the last parameter is mostly evaluated at trader or processor level. The Ghana export promotion council recommends a water content of max 9% for export, while the recommendation in West and East Africa is a 8 – 10% water content after 3 days of drying, with a nut count ranging from 170 to 190

nuts/kg (ACi, 2012a, UNIDO, 2011). A recent study showed the average water content and nut count of cashew nuts harvested in Benin to be 8.7% and 171 nuts/kg, respectively (Lamboni *et al.*, 2016). Generally, tropical conditions such as high temperatures and relative humidity, and unexpected rains during harvest, in combination with poor harvesting practices and improper storage, lead to proliferation of fungi on cashew nuts with the risk of mycotoxin formation (Milhome *et al.*, 2014). The European commission' regulations set limits for aflatoxin B_1 and total aflatoxins of 5 and 10 µg/kg, respectively, for cashew nuts to be subject to sorting or other physical treatment, and of 2 and 4 µg/kg respectively, for processed nuts intended for direct human consumption (European Commission, 2010). Contamination is usually associated with shelled nuts. Most fungi encountered on cashew kernels belong to *Aspergillus* section *Flavi*, *A* section *Nigri*, *Rhizopus* spp. and *Penicillium* spp. (Adeniyi and Adedeji, 2015; Freire and Kozakiewicz, 2005; Lamboni *et al.*, 2016).

Several authors have reported a consequential effect of sorting on the quality of Brazil nuts (*Bertholletia excelsa*) (De-Mello and Scussel, 2009; Pacheco and Martins, 2013) and pistachios (*Pistacia vera*) (Shakerardekani *et al.*, 2012) but the impact of this practice on the quality of raw cashew nuts has not been determined. In Tanzania, cashew nuts are sorted and sold in 2 grades at different prices, which stimulates farmers to improve the quality of their harvest (UNIDO, 2011). In Benin, raw cashew nuts are shipped without sorting. The aim of the present study is to evaluate the influence of sorting and grading on quality parameters that are easily applicable by small-scale farmers, such as water content, nut count and fungal contamination of raw cashew nuts.

4.2. Materials and Methods

4.2.1. Sampling

Cashew nuts were sampled in March 2013, in the main production areas of Benin, which cover two agro-ecological zones: the Northern Guinea (NG) and the Southern Sudanian (SS) zones. NG lies within latitudes 8º1' and 10º6' N whereas SS lies within latitudes 9º4' and 12º3' N. NG and SS are covered by a unimodal rainfall distribution averaging 1000 mm annually, and maximum temperatures varying from 28ºC to 40ºC. Based on their accessibility, 14 villages were selected for sampling: Alafiarou, Bante, Ina, Kilibo, Tchaourou, Tchatchou and Toui in NG and Birni, Chabi-kouma, Kolokonde, Nagayile, Patargo, Penessoulou and Pira in SS (**Fig. 2.1**). A total of 140 cashew nut samples were collected according to Whitaker *et al*. (2010). Sub-samples were taken for immediate analysis of water content. The remainder of the samples was stored at 4ºC, no more than 3 days, to prevent postharvest changes before further determination of nut count and fungal occurrence.

4.2.2. Sorting and grading

Based on visual examination of the shell of the raw nuts, the nuts were sorted into 2 grades. First (good) grade (Grade 1) consisted of nuts with less than 50% of their shell surface affected by fungi and/or insect injuries, whereas second (bad) grade (Grade 2) were nuts that had 50% or more of their shell surface covered by fungi and/or insect symptoms. Sorting and grading were performed on a clean desk, wearing gloves to avoid additional fungal contamination. To minimise cross contamination, nuts were flattened as one layer and carefully separated.

4.2.3. Cashew nut count

The cashew count is defined as the number of nuts per kilogram (ACi, 2012a). The count was performed on samples by mixing each sorted sample and counting 3 replicates of one kilogram.

4.2.4. Extraction of cashew kernels

Each raw cashew nut was cut into 2 pieces using a sharp scalpel and next the two cotyledons were extracted. This extraction was under aseptic conditions. Cross contamination was prevented by disinfecting the scalpel with 90% ethanol. The extracted kernels were put in plastics bags and kept at 4°C for further analysis of water content and fungal contamination.

4.2.5. Evaluation of water content

The water content of cashew kernels was determined using the oven-drying method prescribed by ISO 665-2000 (UNECE, 2002). In duplicate, 10 g ± 1 mg of crushed kernels were placed in a metal box, dried at 103 ± 2°C for 6 hours at atmospheric pressure, with further drying for 3 hours until constant weight was reached. The mean water content was calculated and expressed as percentage on wet weight basis.

4.2.6. Isolation and identification of mycobiota

α. Culture media

For mycobiota isolation, several media were used: Dichloran 18% glycerol agar (DG18, Oxoid) (Pitt and Hocking, 1997) for growth of conidia present on cashew kernels; Czapek yeast autolysate (CYA) agar (Frisvad and Samson, 2004), Malt extract agar (MEA, Oxoid), (Samson *et al*., 2004) and Yeast extract sucrose agar (YES) (Pitt and Hocking, 1997) for isolation, morphological observation and identification.

β. Plating after surface disinfection

Seventy samples were used for surface disinfection. In order to evaluate the presence of fungi on 100 cotyledons per village, 5 replicates of 4 cotyledons from 5 sub-samples per village were randomly picked and platted according to the method described in Lamboni *et al*. (2016). A plating

technique of Adebajo and Diyaolu (2003) was used with two of the cotyledons having their inner surface turned up and the remaining two having their outer surface turned up (see supplementary material **Suppl. Figure 1**). After the incubation period, the number of cotyledons on which fungal growth was observed and which showed morphologies consistent with *Aspergillus* section *Flavi* and *A.* section *Nigri*, were counted separately. These strains were isolated and inoculated according to Lamboni *et al.* (2016) for morphological observation and identification.

γ. Plating without prior disinfection

The above mentioned plating method was also used for direct plating of cashew cotyledons on DG18 without prior surface disinfection. A total of 40 cotyledons (2 replicates of 4 from 5 subsamples per village) were plated and all the strains belonging to *Aspergillus* section *Flavi* and *A.* section *Nigri* were again transferred to CYA, MEA and YES for identification.

Colony morphology, conidiation characteristics, mycelium growth, reverse plate observation and microscopic mounts were used for the identification of the strains according to taxonomic schemes and illustrations in Pitt and Hocking (2009), Samson *et al.* (2002) and Samson *et al.* (2014). Colonies of fungi with morphology not similar to either *A.* section *Flavi* or *A.* section *Nigri* were classified as 'other fungi' and included *Eurotium*, *Rhizopus* and *Mucor*. Based on strain morphology, *A. flavus, A. tamari, A. costaricaensis, A. minisclerotigenes, A. nomius* were identified in the section *Flavi*.

4.2.7. Statistical analysis

Data on cashew nut count, water content and frequency of fungi contamination were subjected to ANOVA using the mixed model procedure in SAS version 9.3 (SAS institute Inc., 2011). Variances were stabilized with the following transformations: $x' = \log (x + 1)$ for nut count; and $x' = \arcsin\sqrt{p}$, $p = x/100$ for water content and frequencies of fungi contamination. Within villages, the *t*-test was used to separate mean values of grades. Correlations were computed to establish the

interactions between nut count, water content and frequencies of fungi contamination. Means are presented untransformed in graphs and tables.

4.3. Results

4.3.1. Cashew nut count

The average cashew nut counts in Grade 1 and Grade 2 are shown in **Fig. 4.1** for both agro-ecological zones. In Northern Guinea (NG), nut count in Grade 1 ranged from 151 nuts/kg (Kilibo) to 174 nuts/kg (Bante) whereas it ranged from 168 nuts/kg (Toui) to 202 nuts/kg (Alafiarou) for Grade 2. The means were 160 nuts/kg and 187 nuts/kg for Grade 1 and Grade 2, respectively. Statistical analysis revealed that Grade 1 showed significant differences for nut count within villages (p = 0.0008) (**Fig. 4.1**). Also, significant differences were found between the nut counts of Grade 1 and Grade 2 in 5 villages in NG (Bante, Ina, Kilibo, Tchaourou and Tchatchou). In the Southern Sudanian (SS) zone, nut count ranged from 143 nuts/kg (Pira) to 182 nuts/kg (Chabikouma) with an average of 165 nuts/kg for Grade 1, whereas for Grade 2 it ranged from 171 nuts/kg (Pira) to 197 nuts/kg (Chabikouma) with an average of 187 nuts/kg (**Fig. 4.1**). Significant differences were found within villages in SS for Grade 1 (p = 0.0002). Nut count of Grade 1 was significantly different from that of Grade 2 in 2 villages in the SS zone (Patargo and Penessoulou). Overall, the nut counts in the survey were 163 nuts/kg and 187 nuts/kg for Grade1 and Grade 2, respectively, with a significant difference between these 2 grades.

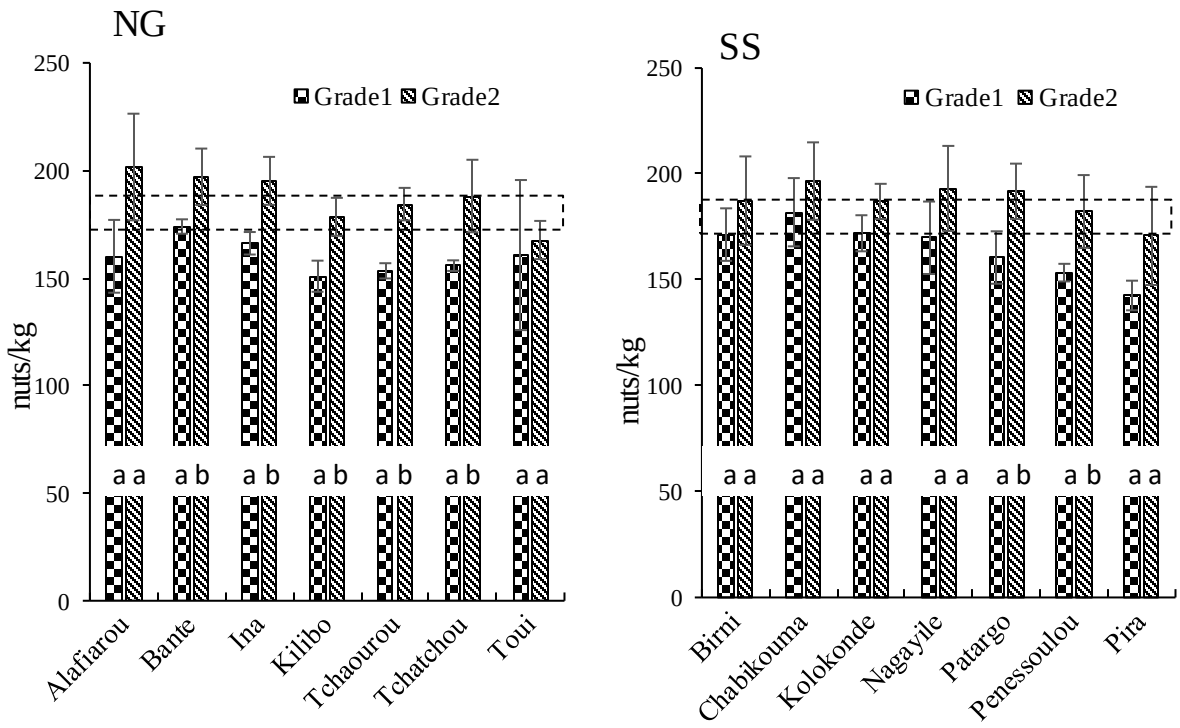


Fig. 4.1. Average nut count (± SD) of Grade 1 and Grade 2 cashew nuts from the Northern Guinea (NG) and the Southern Sudanian (SS) agro-ecological zones of Benin

NB: - The dashed lines indicate the cashew nut count as required for export from, i.e. 170 to 190 nuts/kg (ACi, 2012a);

- Cashew nut count (bars) from the same village with the same letter are not significantly different from each other (T-test at $p \leq 0.05$).

4.3.2. Water content of cashew kernels

Fig. 4.2 presents the average water contents of Grade 1 and Grade 2 cashew kernels from the villages within the NG and SS zones. In NG, the water content ranged from 6.3% (Ina) to 11.1% (Bante) with an average of 8.6% for Grade 1. For Grade 2, it ranged from 5.2% (Ina) to 9.7% (Bante) with an average of 8.6%.A significant difference was noticed for Grade 1 between the average water content of villages within NG (p = 0.0002). In SS, the water content ranged from 6.4% (Penessoulou) to 12.4% (Patargo) with an average of 9.7% for Grade 1, whereas as it ranged from 6.3% (Penessoulou) to 8.4% (Birni) with average of 7.6% for Grade 2 (**Fig. 4.2**). The average water contents of cashew nuts in the survey were 9.2% and 8.1% for Grade 1 and Grade 2, respectively.

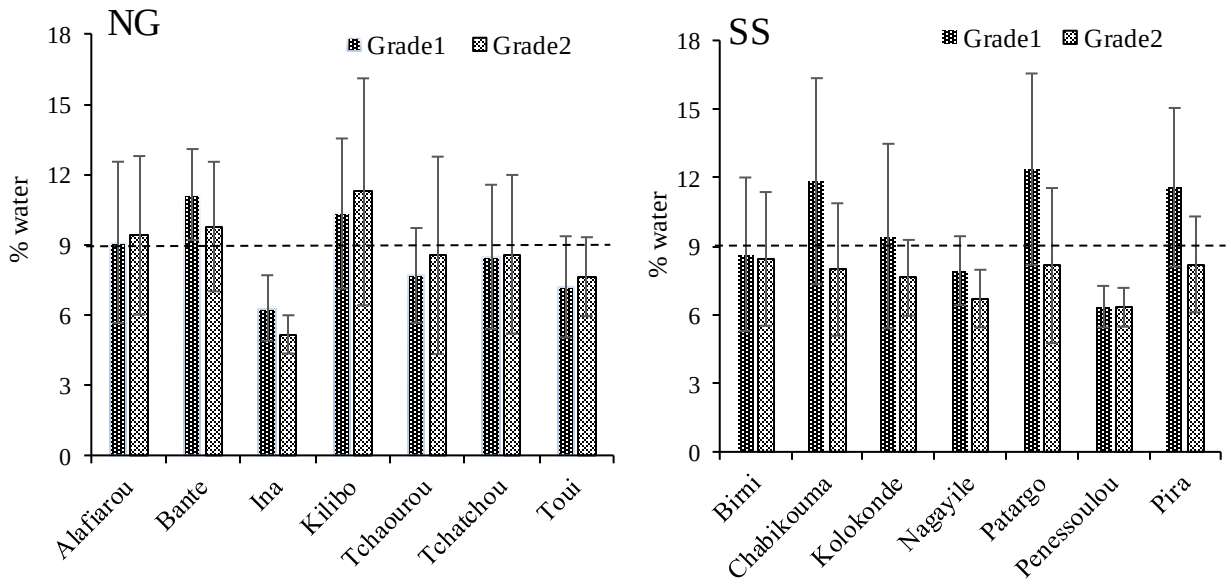


Fig. 4.2. Average water content (± SD) of Grade 1 and Grade 2 cashew nuts from the Northern Guinea (NG) and the Southern Sudanian (SS) agro-ecological zones of Benin

NB: The dashed line represents the recommended water content of cashew nuts for export, i.e. 9% (ACi, 2012a).

4.3.3. Occurrence and frequency of fungal contamination

α. Surface disinfected samples

The results from the surface disinfection method showed that in NG, 15 (43%) of the 35 samples in Grade 1 and 11 (31%) of the 35 samples in Grade 2 contained isolates of *A.* section *Flavi* with, respectively, 57 and 37 infected kernels out of a total of 700 plated kernels (**Table 4.1**). On average, 8% of cashew kernels in Grade 1 were contaminated by *A.* section *Flavi* with minimum and maximum values of 1% (Tchaourou) and 20% (Bante). In Grade 2, the average contamination was 5% in the range of 0% (Tchatchou and Ina) to 21% (Bante). *A.* section *Nigri* isolates were found on all 35 samples of both Grade 1 and Grade 2 kernels, giving respectively, 626 and 641 infected kernels over the 700 cultured. The incidence of *A.* section *Nigri* contamination ranged from 83% (Tchaourou + Ina) to 95% (Bante) and 76% (Toui) to 100% (Ina) for Grade 1 and Grade 2, respectively. The mean contamination with *A.* section *Nigri* was 89% for Grade 1 and 92% for Grade 2 (**Table 4.1**).

In SS, 15 (43%) out of the 35 samples were contaminated with *A.* section *Flavi* for both Grade 1 and Grade 2, whereas all the 35 samples were contaminated by *A.* section *Nigri* for both grades. Cashew kernel contamination by *A.* section *Flavi* ranged from 0% (Birni and Pira) to 13% (Patargo) with an average of 5%, and 1% (Birni, Patargo and Pira) to 10% (Penessoulou) with an average of 4% for Grade 1 and Grade 2, respectively. For *A.* section *Nigri*, the incidence of contamination ranged from 58% (Penessoulou) to 97% (Patargo) with an average of 80% for Grade 1 whereas it ranged from 83% (Penessoulou) to 100% (Patargo) with an average contamination of 94% for Grade 2 (**Table 4.1**).

Table 4.1. Fungi contamination of surface disinfected Grade 1 and Grade 2 cashew kernels from different villages of two agro-ecological zones in Benin

Agro-ecological zone[α]	Villages	Contaminated nuts (%)		Number of isolates[β]		% of kernels infected by			
						A. section *Flavi*		*A.* section *Nigri*	
		Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
NG	Alafiarou	100	100	44	41	3 ± 2 **ab**	2 ± 1 **b**	90 ± 5 **ab**	95 ± 5 **ab**
	Bante	99	100	57	66	20 ± 20 **ab**	21 ± 16 **c**	95 ± 3 **b**	97 ± 1 **b**
	Ina	99	100	57	48	18 ± 17 **ab B**	0 **a A**	83 ± 7 **a A**	100 **c B**
	Kilibo	100	100	63	47	9 ± 4 **b B**	1 ± 1 **ab A**	91 ± 5 **ab**	92 ± 6 **a**
	Tchaourou	100	100	64	67	1 ± 1 **a A**	9 ± 6 **bc B**	83 ± 6 **a A**	93 ± 3 **a B**
	Tchatchou	100	100	61	51	4 ± 2 **ab B**	0 **a A**	91 ± 4 **ab**	88 ± 4 **a**
	Toui	100	100	53	53	2 ± 1 **a**	4 ± 2 **bc**	89 ± 6 **ab**	76 ± 16 **a**
					Mean	8 ± 4	5 ± 3	89 ± 2	92 ± 3
					Number of positive/total samples	15/35	11/35	35/35	35/35
					Number of positive/total kernels (%)	57/700	37/700	626/700	641/700
		Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
SS	Birni	99	100	45	49	0 **a**	1 ± 1 **a**	78 ± 10 **ab A**	99 ± 1 **bc B**
	Chabikouma	100	100	65	50	5 ± 3 **b**	4 ± 3 **ab**	95 ± 4 **c**	96 ± 2 **b**
	Kolokonde	85	100	55	66	4 ± 2 **b A**	9 ± 2 **b B**	67 ± 9 **a A**	87 ± 3 **a B**
	Nagayile	99	100	57	49	5 ± 2 **b**	2 ± 2 **a**	78 ± 10 **ab A**	97 ± 2 **b B**
	Patargo	99	100	58	53	13 ± 4 **c B**	1 ± 1 **a A**	97 ± 2 **c A**	100 **c B**
	Penessoulou	100	100	71	68	9 ± 7 **bc**	10 ± 4 **b**	58 ± 10 **a A**	83 ± 4 **a B**
	Pira	100	100	50	52	0 **a**	1 ± 1 **a**	89 ± 9 **bc**	97 ± 2 **b**
					Mean	5 ± 1	4 ± 1	80 ± 4 **A**	94 ± 1 **B**
					Number of positive/total samples	15/35	15/35	35/35	35/35
					Number of positive/ total kernels (%)	38/700	28/700	552/700	659/700

α = NG = Northern Guinea; SS = Southern Sudanian; **β** = on a total number of 40 kernels plated per village.

NB: Means of fungi frequency (± SE) within a column followed by the same low case letter are not significantly different from each other by SNK (*t*-test) at 5%.
Means of fungi frequency (± SE) within a row followed by the same capital letter are not significantly different from each other by SNK (*t*-test) at 5%.

Statistical analysis revealed significant differences between villages across grades, and within grades (**Table 4.1**). Significant differences were found between Grade 1 and Grade 2 for *A.* section *Flavi* contamination in Ina, Kilibo, Tchaourou and Tchatchou in NG, and in Kolokonde and Patargo in SS. For contamination with *A.* section *Nigri*, significant differences were noticed in Ina and Tchaourou in NG, and in Birni, Kolonkonde, Nagayile, Patargo and Penessoulou in SS. There was a significant difference between Grade 1 and Grade 2 for the average contamination by *A.* section *Nigri* in SS.

Pearson correlation coefficients computed for cashew nut counts, water content and frequency of fungi contamination are shown in **Table 4.2**. For Grade 1, the total number of fungi was positively correlated with the presence of *A.* section *Flavi* and the presence of 'other fungi'. Negative correlations were noticed between the presence of 'other fungi' and nut count on the one hand, and on the other hand, the presence of *A.* section *Nigri*. For Grade 2, the same correlations were observed. The presence of *A.* section *Nigri* was negatively correlated with the total number of fungi and the presence of *A.* section *Flavi* (**Table 4.2**).

Table 4.2. Correlations between nut count, water content and the frequency of fungi contamination of surface disinfected Grade 1 and Grade 2 cashew nuts

		Grade 1						Grade 2					
		1	2	3	4	5	6	1	2	3	4	5	6
1	**Nut count**	1						1					
2	**Water content**	0.06	1					0.02	1				
3	**# of isolates**	-0.003	-0.07	1				-0.22	0.07	1			
4	**% of *Flavi***	0.06	0.03	0.50***	1			-0.09	0.05	0.58***	1		
5	**% of *Nigri***	-0.12	0.20	-0.22	-0.004	1		0.19	0.16	-0.35**	-0.26*	1	
6	**% other fungi**	-0.27*	-0.12	0.48***	-0.7	-0.32**	1	-0.33*	-0.06	0.59***	0.11	-0.40***	1

*, **, *** = significant at $p = 0.05$, $p = 0.01$ and $p = 0.001$, respectively.

β. Non-disinfected samples

The results from the non-disinfection method showed that 15 (43%) and 6 (17%) of the 35 samples cultured from NG were positive to *A*. section *Flavi* contamination for Grade 1 and Grade 2, respectively (**Table 4.3**). *A*. section *Flavi* contamination ranged from 3% (Tchatchou) to 28% (Bante) for Grade 1, giving a mean value of 13%, whereas it ranged from 0% to 38% (Bante) for Grade 2 with a mean of 7%. Thirty five (100%) and 34 (97%) out of the 35 samples from Grade 1 and Grade 2, respectively, were contaminated by strains of *A*. section *Nigri*. *A*. section *Nigri* contamination ranged from 80% (Kilibo) to 100% (Tchatchou and Alafiarou) for Grade 1, and ranged from 63% (Toui) to 100% (Bante and Ina) for Grade 2. The average contamination levels were 95% and 85% for Grade 1 and Grade 2, respectively (**Table 4.3**).

In zone SS, 18 (51%) of the 35 samples from Grade 1 and 17 (49%) of the 35 samples from Grade 2 contained strains of *A*. section *Flavi* with 14% and 18% infected kernels, respectively, out of the 280 plated (**Table 4.3**). *A*. section *Nigri* contaminated all the 35 samples from both grades with an average of 92% and 95% for Grade 1 and Grade 2, respectively. *A*. section *Flavi* contamination ranged from 0% (Pira) to 28% (Birni and Penessoulou) for Grade 1 and from 0% (Pira and Nagayile) to 43% (Penessoulou) for Grade 2, while *A*. section *Nigri* contamination ranged from 75% (Penessoulou) to 100% (Patargo) for Grade 1 and from 90% (Pira) to 100% (Patargo) for Grade 2 (**Table 4.3**).

Statistical analysis revealed significant differences between fungi contamination for Grade 1 and Grade 2 in NG and SS (**Table 4.3**). In NG, a *t*-test between Grade 1 and Grade 2 showed significant differences for *A*. section *Flavi* contamination in Alafiarou, Kilibo Tchatchou and Toui. For *A*. section *Nigri* contamination, significant differences between Grade 1 and Grade 2 were noticed in Tchaourou, Tchatchou and Toui. In SS, significant difference within grades was noticed for *A*. section *Flavi* contamination in Nagayile, whereas it was in Penessoulou for *A*. section *Nigri* contamination (**Table 4.3**).

Table 4.3. Fungi contamination of non-disinfected Grade 1 and Grade 2 cashew kernels from different villages of two agro-ecological zones in Benin

Agro-ecological zone[α]	Villages	Contaminated nuts (%)		Number of isolates[β]		% of kernels infected by			
						A. section *Flavi*		*A.* section *Nigri*	
		Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
NG	Alafiarou	100	100	23	18	13 ± 10 **ab B**	0 **a A**	100 **c**	90 ± 10 **bc**
	Bante	100	100	33	29	28 ± 19 **ab**	38 ± 19 **c**	93 ± 7 **ab**	100 **c**
	Ina	100	100	23	20	18 ± 18 **ab**	2 ± 2 **ab**	98 ± 2 **bc**	100 **c**
	Kilibo	100	100	25	18	20 ± 5 **b B**	0 **a A**	80 ± 10 **a**	70 ± 16 **ab**
	Tchaourou	100	100	29	29	5 ± 3 **a**	8 ± 5 **b**	98 ± 2 **c B**	85 ± 5 **b A**
	Tchatchou	100	100	26	26	3 ± 2 **a B**	0 **a A**	100 **c B**	88 ± 13 **b A**
	Toui	100	100	23	17	8 ± 5 **a B**	0 **a A**	95 ± 3 **b B**	63 ± 2 **a A**
					Mean	13 ± 4	7 ± 3	95 ± 2 **B**	85 ± 5 **A**
					Number of positive/total samples	15/35	6/35	35/35	34/35
					Number of positive/total kernels	37/280	21/280	265/280	238/280
		Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
SS	Birni	100	100	29	27	28 ± 8 **c**	23 ± 11 **b**	93 ± 5 **b**	95 ± 2 **a**
	Chabikouma	100	100	26	30	10 ± 5 **bc**	18 ± 8 **b**	93 ± 5 **b**	95 ± 5 **ab**
	Kolokonde	100	100	32	30	13 ± 10 **bc**	23 ± 7 **b**	93 ± 5 **b**	93 ± 5 **a**
	Nagayile	100	100	27	24	15 ± 6 **c B**	0 **a A**	98 ± 2 **bc**	98 ± 2 **ab**
	Patargo	100	100	22	22	5 ± 3 **b**	18 ± 11 **b**	100 **c**	100 **b**
	Penessoulou	100	100	35	30	28 ± 10 **c**	43 ± 24 **b**	75 ± 6 **a A**	93 ± 3 **a B**
	Pira	100	100	21	21	0 **a**	0 **a**	95 ± 5 **bc**	90 ± 7 **a**
					Mean	14 ± 3	18 ± 5	92 ± 2	95 ± 2
					Number of positive/total samples	18/35	17/35	35/35	35/35
					Number of positive/ total kernels	38/280	49/280	258/280	265/280

[α] = NG = Northern Guinea; SS = Southern Sudanian; [β] = on a total number of 40 kernels plated per village.

NB: Means of fungi frequency (± SE) within a column followed by the same low case letter are not significantly different from each other by SNK (*t*-test) at 5%. Means of fungi frequency (± SE) within a row followed by the same capital letter are not significantly different from each other by SNK (*t*-test) at 5%.

Correlation analysis revealed that *A*. section *Flavi* contamination was positively correlated with the total number of strains isolated from both Grade 1 and Grade 2 (**Table 4.4**). The 'other fungi' were negatively correlated with nut count and presence of *A*. section *Nigri*. There was also a positive correlation between the presence of *A*. section *Nigri* and the nut count (**Table 4.4**).

Table 4.4. Correlations between nut count, water content and the frequency of fungi contamination of non-disinfected Grade 1 and Grade 2 cashew nuts

		Grade 1						Grade 2					
		1	2	3	4	5	6	1	2	3	4	5	6
1	**Nut count**	1						1					
2	**Water content**	0.06	1					0.02	1				
3	**# of isolates**	0.19	0.16	1				0.11	-0.01	1			
4	**% of *Flavi***	0.14	0.09	0.64***	1			-0.05	0.03	0.60***	1		
5	**% of *Nigri***	-0.10	0.14	-0.23	-0.11	1		0.31*	0.03	0.19	-0.20	1	
6	**% other fungi**	-0.34**	-0.06	0.13	-0.004	-0.26*	1	-0.31*	0.02	0.20	0.05	-0.33*	1

*, **, *** = significant at $p = 0.05$, $p = 0.01$ and $p = 0.001$, respectively.

4.4. Discussion

In several West African countries, cashew nuts contribute greatly to the revenues obtained from agricultural export. More than 95% of cashew nuts produced in Benin are exported to India due to lack of processing facilities (USAID, 2007). The exported raw cashew nuts have to meet certain regulations that are related to nut count, water content, out-turn and aflatoxin content that results from nut contamination by *Aspergillus* spp. (ACi, 2012a).

The parameters in our study are easily measurable at farmer level using low cost approaches (Cashew Handbook, 2014). Raw cashew nuts are graded from '*excellent'* to '*poor'* according to the nut count (NAIP, 2013), and the accepted range of good cashew nuts for export is 170 to 190 nuts/kg (ACi, 2012a). In our study, the average nut count within grades per agro-ecological zone are suitable for export but may hide some outliers. In some villages, the low nut count can be a consequence of high water content that can alter the quality of the nut. In NG, the villages Kilibo and Bante delivered lower nut counts for Grade 1 compared to Grade 2. These Grade 1 nuts were classified as '*very good*' and '*excellent'* for trading. However, these batches of nuts showed water content above the regulation limit, for both grades. Similar results were noticed in Chabikouma, Patargo and Pira, in SS. These results were obtained at the farmer level and may change along the value chain as a consequence of water loss. In fact, at exporter level, nut count from Benin was reported to be 195 nuts/kg (ITC, 2013). More, the average individual nut weight is reported to be correlated to the variety and age of the cashew tree as well as the ecology of the orchard (DANIDA, 2003; NAIP, 2013).

One of the most important quality parameters for raw cashew nut is its water content. Our results showed an average water content within the required range for export while some villages showed significant differences between both grades. The high variability in water contents of cashew nut from NG zone could be related to its climatic characteristics, compared to the SS zone. Benin is

divided in four agro-ecological zones from southern guinea to northern sudanian (Hell *et al.*, 2000). The NG has an unimodal rainfall distribution, but its proximity with the southern guinea, with 2 rainfall seasons, may lead to some unexpected rains during the cashew nut harvesting period. This could negatively affect the water content of the nuts.

Sorting and grading improved the quality of raw cashew nut with regard to water content in 5 villages in NG zone and 1 village in SS zone. Although there was no significant difference between the water content of the two grades, this could be useful information for cashew nuts traders for selecting villages with raw cashew nut with low water content. Indeed, cashew nut with low water content at farmer gate are easy to handle with regard to fungal spoilage, and this is an advantage for the buyers (Gyedu-Atoko *et al.*, 2014; Kader, 2013; NAIP, 2013). High water content noticed on Grade 1 nuts compared to Grade 2 nuts in some villages, could be explained by the fact that Grade 2 included sometimes shrunken, spoiled and immature nuts that mostly had a low water content.

Moreover, the overall results showed more fungal contamination on Grade 2 nuts than Grade 1 nuts with a significant difference in *A.* section *Nigri* contamination in both agro-ecological zones. The grading had more effect on the contamination of cashew nuts by *A.* section *Nigri* than *A.* section *Flavi*. *A.* section *Nigri* is able to grow at a water activity as low as 0.77 compared to 0.84 for *A.* section *Flavi* at a temperature of 25°C, the average temperature in the tropical dry and wet regions (Fontana, 2008; Moussa *et al.*, 2013). This may explain the high frequency of *A.* section *Nigri* on both non-disinfected and surface disinfected cashew nuts, although the overall results showed that there was less fungal contamination on surface disinfected nuts than non-disinfected nuts. Water activity and temperature are regarded to be the main factors determining the potential for fungal growth on foodstuffs (Plaza *et al.*, 2003).

The effect of sorting on aflatoxin reduction, based on different physical properties of tree nuts such as size, shape, colour and visible fungal growth, has been reported by several authors (Battilani,

2010; Pacheco and Martins, 2013). Garcia-Cela *et al*. (2012) mentioned aflatoxin reduction of 40-80% by hand sorting of pistachios. Using the bright greenish yellow fluorescence system, Lundadei *et al*., (2013) reported up to 92% and 82% of aflatoxin reduction on sorted pistachio and cashew, respectively. In contrast, in a previous study, we did not detect aflatoxin in cashew nuts (Lamboni *et al*., 2016). Looking at the harvesting method for cashew, the contamination by *A. flavus* may occur mostly after the nuts have fallen from the tree on the ground. So, for aflatoxin production, depending on the temperature and water activity, a period of contact (lag phase) between the fungus and the substrate is needed. Bennett *et al*., (1979) demonstrated that aflatoxin production only occurs after the exponential phase of fungal growth, implying that the length of storage at the optimal range of water activity is critical (Vargas *et al*., 2011).

4.5. Conclusion

Sorting and grading contributed to improve substantially the nut count, partially the fungal contamination of cashew nuts intended for export. In a context where no grading is applied, like in Benin, added to the fact that regulations tend to become more strict over time to decrease the risk of contaminants with regard to human health, this study showed a non-biological and low cost approach applicable by small scale farmers to control the quality of cashew nut. Nevertheless, the discrepancy observed in some locations between water content and fungal contamination requires further investigations to ensure that the nut quality is kept constant along the value chain.

4.6. Acknowledgements

This work was financially supported by the International Institute of Tropical Agriculture in Benin, and Wageningen University. Jens Christian Frisvad and Kristian fog Nielsen from the Department of System Biology of the Technical University of Denmark are gratefully acknowledged for providing the infrastructure of laboratories for mycological analyses.

Chapter 5

Impact of small-scale farmers' practices on the quality of raw cashew nuts (*Anacardium occidentale* L.)

This chapter has been submitted for publication:

Yendouban Lamboni, Kerstin Hell, Martinus JR Nout, Eddy J Smid, Manuele Tamo, Martinus AJS van Boekel, Anita R Linnemann*. Impact of small-scale farmers' practices on the quality of raw cashew nuts (*Anacardium occidentale* L.)

Abstract

Background: In order to evaluate the impact of farm management practices on the quality of raw cashew nuts, one hundred and forty small-scale farmers were interviewed in the Northern Guinea (NG) and Southern Sudanian (SS) zones of Benin.

Results: Eighty percent of the farmers reported to select their seedlings from uncertified sources and 59% never asked nor received advice from extension services about cashew farming practices. Farmers reported neither to dry (74% in NG and 94% in SS) nor to sort (80% in NG and 94% in SS) harvested nuts. Cashew revenue contributes significantly to the household income in NG (44% of farmers) and SS (54%). Sorting results in lower nut count (heavier nuts), in both agro-ecological zones, while drying results in higher nut count in NG. When farmers had received advice on farming practices through extension services, a significant positive impact on the estimated yield in the SS zone was observed. Orchard area, orchard age, nut storage duration and origin of seedlings all had a significant impact on the household income.

Conclusion: The farmer's education level, limited access to extension services, lack of sorting, drying and farm management are major parameters that affect the quality of raw cashew nuts. Improvement of these parameters can positively affect nut quality and therefore contribute to better revenue from cashew production.

Keywords: *Anacardium occidentale*, small-scale farmer practices, quality, raw cashew nuts, agro-ecological zone, Benin.

5.1. Introduction

Cashew (*Anacardium occidentale* L.) is the second largest agricultural export product in Benin, after cotton. Benin is ranked 8th on the world market and 3rd in West Africa in terms of cashew nuts exports (ACi, 2010a). The production in 2015 was estimated at 125.000 tonnes from a total of 468.000 hectares which generated a turnover of 150 million US dollars (FAOSTAT, 2016). Cashew production in Benin accounts for 8% of the national export revenue and 25% of the agricultural export revenue (ACi, 2010a). Benin exports 95% of its production as raw (unshelled) nuts (Cashew Handbook, 2014). A raw nut consist of a kernel surrounded by a hard shell. The trading period is from February to June, corresponding to the harvesting period that could last for several weeks, depending mainly on the weather conditions (ITC, 2013). In Benin, cashew nut trading represents the main employment and major source of income for many collectors, traders and exporters (Degla, 2012).

Cashew nut pricing is determined by quality parameters such as nut count (*i.e.* the number of raw nuts per kg), water content and insect and or fungal damage of the shell (ACi, 2012a, b). The latter is related to the infestation of the kernel by, among others, post-harvest fungi of the *Aspergillus* group (Adebajo and Diyaolu, 2003; Gyedu-Akoto *et al.*, 2014). Post-harvest fungal contamination may lead to the production of mycotoxins and render the nuts unfit for consumption and export (Milhome *et al.*, 2014). In West and East Africa, a maximum water content of 9% with a raw nut count ranging from 170 to 190 nuts/kg is recommended for export (ACi, 2012a; UNIDO, 2011). According to Kashani-Nejad *et al.* (2003) proper harvesting and post-harvest handling practices are key operations in obtaining a maximum yield of good quality nuts and therefore the best price. Harvesting cashew nuts as soon as the mature fruits fall on the ground minimises fungal attack and infestation by insects, and subsequently reduces quality loss (Azam-Ali *et al.*, 2001; Kader, 2013).

The quantity and the quality of the end product are not only affected by management and handling, but also by the socio- economic characteristics of the farmers (Brush, 1995). The farmer's age and education, land holding size, farmer's family size, gender and marital status were associated with crop production and technical efficiency (Chepng'etich *et al.*, 2015; Msuya *et al.*, 2008). Cromwell and van Oosterhout (2000) found that: a larger farm size; the importance of the crop to the farming family; and the age of the head of the household, all contributed positively to crop production.

Most studies available on cashew nuts have focused on the cashew value chain and its economic aspects (ACi, 2010a; Dendena and Corsi, 2014; Krepl *et al.*, 2016). Reports on farmer's practices and the constraints to produce good raw quality nuts are lacking. The purpose of this study was to characterize the small-scale cashew nut farmers and to identify management practices that affect the quality of the raw nuts. Recommendations on quality management are offered to farmers, Governmental agencies, NGO's and extension services.

5.2. Materials and Methods

5.2.1. Study zones, questionnaire and cashew nuts sampling

The survey and sampling were conducted from March to June 2013, in 2 agro-ecological zones of Benin, covering the main production area of cashew nut in Benin: the Northern Guinea zone (NG) and the Southern Sudanian zone (SS). The NG lies within latitudes 8°1' and 10°6'N, whereas the SS lies within latitudes 9°4' and 12°3'N. Both zones have an unimodal rainfall distribution, averaging 1000 mm annually, with maximum temperatures varying from 28°C to 40°C.

The interviews were conducted in 14 villages. Ten farmers were selected per village (**Fig. 2.1**). Farmers were randomly selected among those present at information meetings, and who had cashew nuts in their store for sampling. Information was collected about the farmer, the cashew

farm, the management practices before, during and post-harvest up to the sales (**Table 5.1**). Interview aimed also to estimate the harvest quantity and the contribution of the cashew nut revenues to the total household income. The estimation of the production was used to calculate the yield of the farmer's orchard. Except for the yield, closed-response questions were used to categorize farmers according to the answers given.

After the interviews, raw cashew nuts were sampled. A total of 140 samples were collected. The available nuts were thoroughly mixed and 10 different subsamples were purchased to obtain approximately 3 kg from each farmer. The samples were then placed in paper bags, labelled and transported to the laboratory to assess nut count, water content and pest and microbial incidence.

Table 5.1. Questionnaire on farm management and post-harvest practices of cashew nuts

Information about the farmer	
Q_1	Gender of the farmer? Male____; Female____
Q_2	What is your age? Less 18 years____; 18 to 35 years____; 35-55 years____; over 55 years____
Q_3	What is your education level? Never____; less than 6 years____; 6-13 years____; over 13 years____
Cashew nut farm and its management	
Q_4	What is your orchard age? < 5 years____; 5-15 years____; 15-25 years____; > 25 years____
Q_5	What is your orchard area? < 5 ha____; 5-10 ha____; > 10 ha____
Q_6	What is the source of your seedlings? Form a seedling agency____; for others sources____
Q_7	How do you manage your cashew trees? Never____; pruning____; mowing/weeding____; both____
Q_8	Did you got training on cashew farming? No____; yes____
Q_9	Which types of pest and diseases do you encounter in your farm? No pest and diseases ____; insects____; moulds____; both____
Post-harvest practices	
Q_{10}	How do your collect your nuts? From the ground____; from the tree____; both____
Q_{11}	How do you dry your nuts? No drying____; on the ground____; on plastic or metal sheet____
Q_{12}	Do you sort your nuts? No____; yes____
Q_{13}	How do you store your nuts? No storage____; on the ground____; in jute bag____; in others ____
Q_{14}	How long to you store your nuts? < 1 month____; 1-3 months____; > 3months____
Q_{15}	Which types of pest and diseases do you encounter in the harvest? No pest and diseases ____; insects____; moulds____; both____
Q_{16}	Can you estimate your annual cashew nut production? (per 100 kg jute bags) List_____
Q_{17}	What is the cashew contribution to household income? < 25%___; 25-50%___; 50-75%___; > 75%___

5.2.2. Assessment of nut count, moisture content and fungal contamination

Nut count was determined by mixing each sample and counting 3 replicates of 1 kg. The water content and the fungal contamination were evaluated on cashew kernels extracted from the shell. The extraction of cashew kernels was done according to the method described in Lamboni *et al.* (2016) The water content of the kernels was determined using the oven-drying method prescribed by ISO- 665-2000 (UNECE, 2002). To elucidate fungal contamination, cashew kernels were plated as described in Lamboni *et al.* (2016) The number of isolates belonging to *Aspergillus* section *Flavi* and those belonging to *A.* section *Nigri* were recorded. For the identification of the isolates, the taxonomic schemes and illustrations in Samson *et al.* (2014) and Varga *et al.* (2011) were used. Fungal contamination was computed as percentage of cashew kernels contaminated by a specific isolate.

5.2.3. Statistical analysis

Simple descriptive analysis was used to categorize the population of interviewees, the information collected about the cashew orchards, the post-harvest management practices and the estimated contribution to the household income. The answers to 'yes or no' questions were entered as binomial values. Answers to categorical questions were entered as numbers or percentages. Fungal infestation was calculated as a percentage of contaminated cashew cotyledons. Yield was estimated by computing the estimated production over the orchard area. Graphs and boxplots of measured parameters were made with untransformed data in Excel 2013. Stepwise linear regression was used to identify the factors that affected nut count, water content, fungal contamination, the estimated yield and the contribution of cashew revenues to the household income ($p \leq 0.05$). The statistical package used was SAS/STAT version 9.3 (SAS Institute Inc., 2011).

5.3. Results

5.3.1. Characteristics of the interviewees

Five percent of the cashew nut farmers from the 2 agro-ecological zones were female and 95% were male (**Table 5.2**). Overall, 23%, 55% and 22% of the farmers were 18-35, 36-55 and over 55 years old, respectively. A similar age distribution was observed in both agro-ecological zones. In NG, 50% of the farmers were uneducated against 36% in SS. Twenty nine percent and 39% of the farmers in NG and SS, respectively, had received primary education. Farmers with secondary education level were 20% and 26% in NG and SS, respectively. None of the farmers in SS had a high school level compared to only 1 farmer in NG (**Table 5.2**).

Table 5.2. Main characteristics of cashew nut farmers interviewed in the Northern Guinea and Southern Sudanian zones in Benin

		NG (N=70)	SS (N=70)	Total (N=140)
Gender	Female	04 (5.7%)	03 (4.3%)	07 (5.0%)
	Male	66 (94.3%)	67 (95.7)	133 (95%)
Age	18-35 years	15 (21.4%)	17 (24.3%)	32 (22.9%)
	35-55 years	38 (54.3%)	39 (55.7%)	77 (55.0%)
	Over 55 years	17 (24.3%)	14 (20.0%)	31 (22.1%)
Education level	Never	35 (50.0%)	25 (35.7%)	60 (42.8%)
	Primary (0-6 years)	20 (28.6%)	27 (38.6%)	47 (33.6%)
	College (7-13 years)	14 (20.0%)	18 (25.7%)	32 (22.9%)
	High (over 13 years)	01 (1.4%)	00	01 (0.7%)

NG = Northern Guinea; SS = Southern Sudanian.

5.3.2. Farm characteristics and management practices

Across agro-ecological zones, 93 (66%) respondents had a cashew farm that was between 5 to 10 years old. Ten farmers (7%) had a farm that was less than 5 years old whereas only 4 (3%) had farms that were older than 25 years. Sixty five per cent (91) of the respondents had a farm area that was less than 5 ha compared to 30% (42) with a farm area between 5 and 10 ha. Only 7 respondents (5%) had cashew farms of more than 10 ha.

The majority of the farmers (80%, 112) reported to select their seedlings from relatives and neighbouring farms, whereas 20% (28) buy the seeds from an official seedling agency. The cashew farm management practices consisted either of weeding (67%, 91) or weeding associated with tree pruning (33%, 46). Fifty nine percent (83) of the farmers never received any advice on practices for good cashew tree farming, compared to 57 respondents (41%) who reported to have had such advice. Related to cashew tree farming, the observed pests were insects (87%, 122) followed by fungi (4%, 5). Six percent (9) of the respondents reported no pest in their cashew orchard.

5.3.3. Management practices on harvested cashew nuts

The management practices concerned the harvesting method, drying, sorting, storage method, storage duration and occurrence of storage pests. In both agro ecological zones, the majority of farmers (95% in NG and 100% in SS) reported to collect the cashew nuts from the ground. In NG, only 2 farmers from the 70 interviewed, reported to collect the nuts from both the ground and the tree. Seventy four percent (52/70) and 94% (66/70) of farmers reported to never dry their harvest, in NG and SS, respectively. Drying consisted of drying directly on the ground (14 in NG, and 3 in SS) or on a plastic or metal sheet (4 in NG and 1 in SS). In NG, 80% (56/70) of the farmers do not sort their harvest, compared to 94% (66/70) in SS.

Farmers reported to store the cashew nuts directly on the ground (10/70 in NG and 20/70 in SS) and in jute bags (33/70 in NG and 26/70 in SS). Other storage facilities were plastic/polyethylene

bags and these represented 33% in both agro-ecological zones. The storage duration of cashew nuts was up to 3 months for 43% of farmers in NG, compared to 67% of farmers in SS. The storage pests encountered were insects and moulds and were reported by few farmers: 7/70 in NG and 9/70 in SS.

5.3.4. Yield estimation and contribution of cashew nut revenue to the household income

The estimation of the cashew nut yield is presented in **Fig. 5.1**. On average, the yield was estimated at 3.1 tonnes/ha in NG compared to 3.2 tonnes/ha in SS. The box plot (**Fig. 5.1**) shows a high variability in yield estimation, ranging from 0.5 to 10 tonnes/ha and from 1.0 to 12.5 tonnes/ha in NG and SS, respectively. Across the country, Toui and Chabikouma showed the highest yield estimations, whereas Tchaourou and Penessoulou showed the lowest yield estimations (**Fig. 5.1**). The estimated contribution of cashew nut farming to the farmer's household income was as follows: In NG, 40% (28/70) of farmers reported that cashew nut contributed up to 25% of their household income, compared to 29% (20/70) in SS. The estimation was 44% (31/70) and 54% (38/70) for a contribution between 25 to 50% of household income in NG and SS, respectively. Three respondents in NG assessed the contribution of cashew nut to more than 75% of their household income.

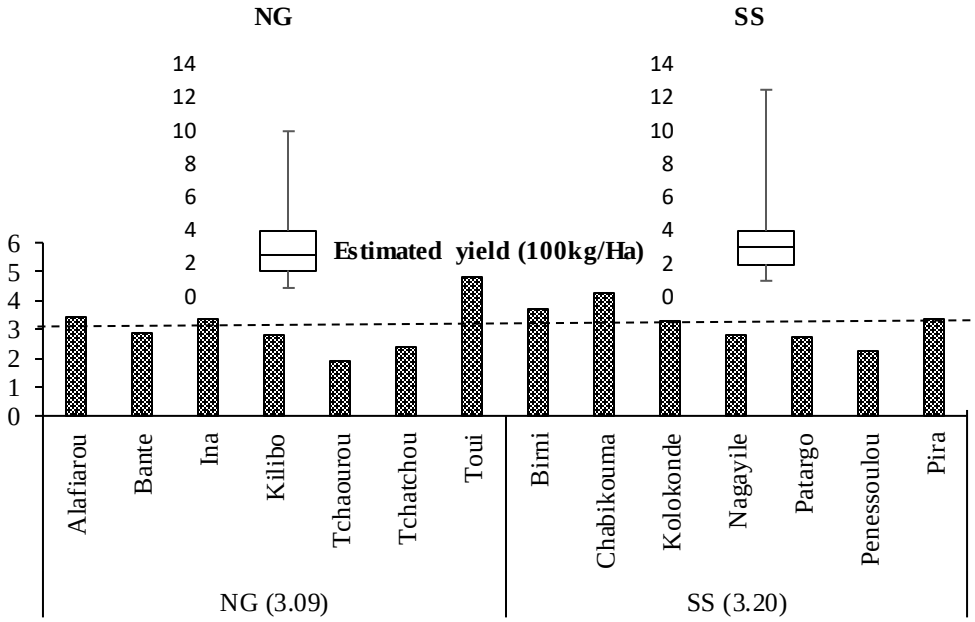


Fig. 5.1. Boxplots and estimation of cashew nut yield in the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

NB: the dashed line represents the average cashew nut yield estimated in Benin.

5.3.5. Nut count, water content and fungal contamination of sampled cashew nuts

The quality parameters measured on cashew nuts samples are presented in boxplots in **Fig. 5.2**. In NG, the average nut count was 172 nuts/kg with some outliers of 130 and 210 nuts/kg. The mean water content was 8.6% with some farmers having nuts with 16.5% water content. The fungal contamination by strains of *Aspergillus* section *Flavi* was low in contrast to the high contamination by strains of *A.* section *Nigri*. Two to 5% of cashew nuts were contaminated by strains of *A.* section *Flavi* compared to 85 to 99% contamination by strains of *A.* section *Nigri* (**Fig. 5.2**). In SS, the nut count averaged 174 nuts/kg with the minimum and maximum being 135 nuts/kg and 215 nuts/kg, respectively. The mean water content was 8.7% with one sample with a water content of 23%. The contamination by strains of *A.* section *Flavi* was between 0 to 18% whereas it was between 85 to 99% for strains of *A.* section *Nigri*. The number of isolates per sample was in the range of 7 to 9 isolates in both NG and SS (**Fig. 5.2**).

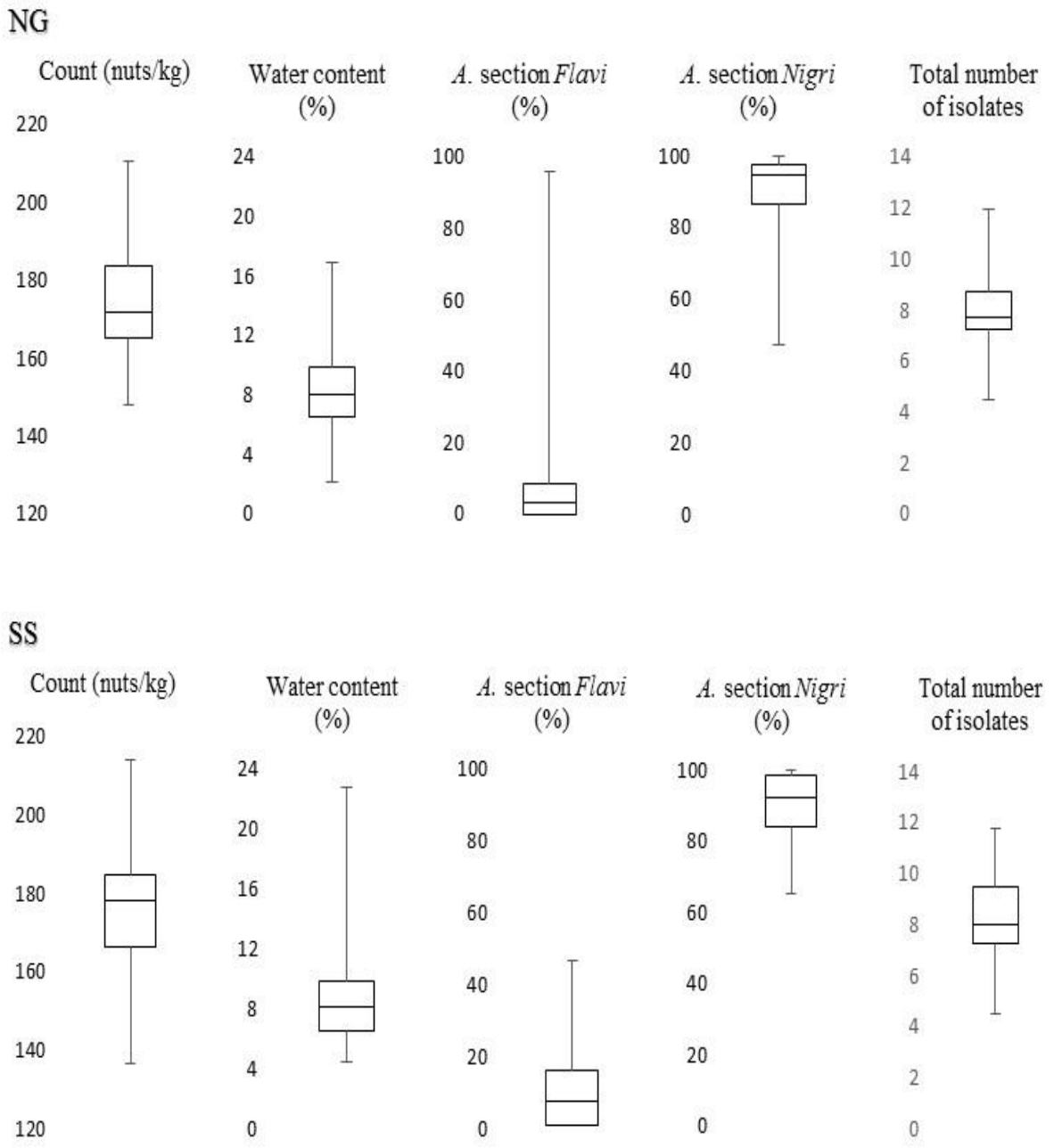


Fig. 5.2. Boxplots[α] of parameters measured on raw cashew nuts in the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

[α] outliers show the maximum and the minimum of each measured parameter.

5.3.6. Relationships between the measured parameters and the survey information

The information about the farmers' orchards and the cashew nut management practices were regressed against nut count, water content, fungal contamination, estimated yield and estimated contribution to household income, see **Table 5.3**, **Table 5.4**, **Table 5.5**, **Table 5.6** and **Table 5.7**, respectively.

Sorting resulted in lower nut count, hence in good quality cashew nuts in both agro-ecological zones (**Table 5.3**). Moreover, In NG, sorting resulted in lower nut count while drying resulted in higher nut count (**Table 5.3**). In both agro-ecological zones, the larger the orchard area the higher the water content was (**Table 5.4**). In NG zone, drying and the presence of storage pest on cashew nuts resulted in low water content of cashew nuts. When regressed with fungal contamination, there was more contamination of species of *A.* section *Flavi* to cashew nuts with farmers with large orchard area, whereas the absence of cashew nut farm management increases the contamination of species *A.* section *Nigri* to cashew nuts (**Table 5.5**).

Table 5.3. Farmers' practices that are significant[a] when regressed against nut count (*Y*) across and within the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

Agro-ecological zone	Regression analysis	R^2	n	*F*-value
Across zones	$Y = 141.44 - 8.58x^1$	0.11	140	1.29*
NG	$Y = 167.13 - 11.91x^2 + 5.54x^3$	0.13	70	5.17**
SS	Not significant			
x^1 Sorting		$t = 2.0$*		
x^2 Sorting		$t = 3.04$**		
x^3 Drying		$t = 2.03$*		

[a] *F* and *t*-tests;

* = significant at < 0.05; ** = significant at < 0.01.

Table 5.4. Farmers' practices that are significant[a] when regressed against nut water content (*Y*) across and within the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

Agro-ecological zone	Regression analysis	R^2	n	*F*-value
Across zones	$Y = 15.34 + 1.18x^1$	0.11	140	1.32**
NG	$Y = 11.75 - 1.30x^2 - 1.2x^3$	0.13	70	4.88**
SS	Not significant			
x^1 Orchard area		$t = 2.52$**		
x^2 Drying		$t = 2.31$*		
x^3 Storage pest		$t = -2.11$*		

[a] *F* and *t*-tests;
* = significant at < 0.05; ** = significant at < 0.01.

Table 5.5. Farmers' practices that are significant[a] when regressed against fungal contamination (*Y*) across and within the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

	Agro-ecological zone	Regression analysis	R^2	n	*F*-value
Flavi	Across zones	Not significant		140	
	NG	$Y = 25.77 + 9.02x^1$	0.13	70	0.73*
	SS	Not significant		70	
	x^1 Orchard area		$t = 2.17$*		
Nigri	Across zones	Not significant		140	
	NG	$Y = 124.72 - 7.90x^1$	0.21	70	1.28*
	SS	Not significant		70	
	x^1 Farm management		$t = -2.39$*		

[a] *F* and *t*-tests;
* = significant at < 0.05; ** = significant at < 0.01.

The absence of farm management had a significant negative effect on the estimated yield of cashew nuts in both agro-ecological zones, while the advice for good cashew nut farming had a significant positive influence on the estimated yield, showing that with farm management and advices for good cashew nuts farming, farmers estimated high yields (**Table 5.6**). The parameters that significantly and positively contributed to the farmer's household income in NG were orchard area and storage duration across both agro-ecological zones. In SS, orchard age and seedlings origin had a significant positive contribution to household income, whereas the presence of pests during production were estimated by farmers to have a significant negative effect on the household income (**Table 5.7**).

Table 5.6. Farmers' practices that are significant[a] when regressed against estimated yield (*Y*) across and within the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

Agro-ecological zone	Regression analysis	R^2	n	*F*-value
Across zones	$Y = 5.50 - 0.71x^1$	0.03	140	4.66**
NG	Not significant			
SS	$Y = 5.73 - 1.21x^2 + 0.98x^3$	0.15	70	5.97**
x^1 No farm management		$t = 2.16$*		
x^2 No farm management		$t = 2.70$**		
x^3 Advice for farming		$t = 2.49$*		

[a] *F* and *t*-tests;
* = significant at < 0.05; ** = significant at < 0.01.

Table 5.7. Farmers' practices that are significant[a] when regressed against estimated contribution to household income (*Y*) across and within the Northern Guinea (NG) and Southern Sudanian (SS) zones in Benin

Agro-ecological zone	Regression analysis	R^2	n	*F*-value
Across zones	$Y = 0.58 + 0.38x^1 + 0.41x^2$	0.27	140	25.43**
NG	$Y = 0.51 + 0.29x^3 + 0.46x^4$	0.26	70	11.64**
SS	$Y = 1.35 + 0.29x^5 + 0.45x^6 - 0.59x^7$	0.52	70	6.39**
x^1 Orchard area		$t = 4.00$**		
x^2 Storage duration		$t = 4.65$**		
x^3 Orchard area		$t = 2.01$*		
x^4 Storage duration		$t = 4.04$**		
x^5 Orchard age		$t = 2.27$*		
x^6 Seeds origin		$t = 2.32$*		
x^7 Pest		$t = 3.11$**		

[a] *F* and *t*-tests;
* = significant at < 0.05; ** = significant at < 0.01.

5.4. Discussion

In this study, information about the farm and the owner, information about the management practices of the orchard and on the harvested cashew nuts were collected. Farmers' socio-economic characteristics appear to be correlated with the productivity and the quality of the crop (Brush, 1995; Cromwell and van Oosterhout, 2000). During our analysis, the description of the cashew nut farmers' population showed that 95% of the cashew farms were owned by men in both agro-ecological zones. Despite the fact that women were active in most economic activities in rural areas, crops with high export revenues, like cashew, were under male control. In its annual report, the Food and Agriculture Organisation of the United Nations reported that cash and export crops are frequently regarded as "men's" crops and subsistence crops as "women's" crops (Heintz and Valodia, 2008). The main raison for this disparity is the fact that on the one hand, access to land is a major constraint for women. In rural areas, land is mostly governed by customary systems, which favour male land ownership. The presence of a land authority continues to be traditional and is more influential in northern regions on Benin. Inheritance laws are more favourable towards men, depriving women of land and consequently of the possibility of starting a plantation (ACi, 2010a). On the other hand, planting and maintenance of cashew farms require much physical effort that is easily furnished by men. Women are mainly involved in harvesting, sorting, drying and transporting the harvest home.

Fifty percent of the cashew nut farmers were aged between 35-55 years. This was previously mentioned by ACi (2010b) where the average age of cashew nut planters was estimated at 40 to 50 years, which is relatively old. This confirms the fact that few youth engaged themselves in cashew nut production activities and prefer to migrate to urban areas. This constitutes one of the constraints of cashew nut production, since the technical efficiency of aged people is reduced due to the reduced labour efficacy (Heintz and Valodia, 2008). Furthermore, we have noticed that more than 75% of the respondents have a limited education level. Education is a key factor that enhances

the ability to derive, decode and evaluate useful information for agricultural production. The low level of education noticed during our study could foster unfavourable attitudes towards the acceptance of improved farming practices (Caswell *et al.*, 2001; Ani, 1998). This could explain the fact that 80% of the respondents reported to obtain the seedlings for their plantation from neighbouring farms or from relatives. The education level limits them to gain support from extension services. Indeed, our results showed that up to 59% of the farmers never benefitted from advice on good cashew nut farming practices. The consequences are noticeable as irregular maintenance of the cashew farms, low quality seedlings for new plantations, poor harvest and poor post-harvest practices (CFC, 2002; ACi, 2010c).

Eighty seven percent of the farmers reported that insects are their main pest during production. *Analeptes trifasciata* (stem girdler), *Selenothrips rubrocinctus* (red-banded thrips), *Pachnoda cordata* (fruit scraper) and *Plocaecderus ferrugineus* (stem and root borer) have been associated with cashew trees and reported as the main challenge that impacts production (Hammed *et al.*, 2008). These insect species have been implicated with economic losses estimated between 52 and 75% of the production level (Ojelade, 1998). Many studies have shown significant increases in yields for farmers who adopt pest management practices (ACi, 2010c; Napit *et al.*, 1988). However, a farmer's decision to adopt a particular pest management strategy will be based on his assessment of the increase in net benefits to be gained by a change in practice. Farmers with no or a poor education level will probably not be receptive to the knowledge on pest management as provided by the extension services. Another constraint is that cashew nut cooperatives in Benin are characterized by an open membership to cashew farmers in contrast to the cotton cooperatives that are characterized by a mandatory membership to all cotton farmers (Mensah *et al.*, 2012). The cotton organisation, SODECO (SOciete pour le DEveloppement du COtton), has its own extension services across the country, and farmers are obliged to follow the guidelines for seedlings and good agricultural cotton practices. In the cashew nut sector, the extensions services that should address the main issues encountered by farmers have often been found to be absent or inefficient (USDA,

2014). Cashew farmers who often also have a cotton field, rely on the cotton extension services with inadequate expertise to support cashew nut farming. Strengthening the cashew nut sector by introducing compulsory membership of cashew nut farmer's association and its related services, mandatory is expected to positively affect the quantity and the quality of the end product.

Drying and sorting are key factors that improve the quality of raw cashew nut (Rosengarten, 2004). Despite the advice to sundry the harvest for 2 to 3 days in order to bring down the water content to 7-8% (Asogwa *et al.*, 2008), only few Beninese cashew farmers use drying of the raw nuts in their practice as noticed during the survey. Farmers also reported not to sort cashew nut before trading. Sorting consists mostly of removing impurities, injured, cracked and immature cashew nuts from the freshly harvested nuts or before storage. The absence of drying and sorting could be explained by the fact that in Benin there is no price difference based on cashew nut grading. In Tanzania, cashew nuts are sorted and sold in 2 grades at different prices, which stimulates farmers to improve the quality of their harvest (UNIDO, 2011).

Cashew nut farmers estimated their yield to be 3.1 and 3.2 tonnes/ha in NG and SS, respectively. Accurate yield data are difficult to gather due to the fact that plantation sizes are often variable and theft is common. Compared to the 0.5 to 0.6 tonnes/ha yield estimated by Catarino *et al.* (2015) in Guinea-Bissau, one could conclude that farmers over-estimated their cashew production in Benin. Moreover, ACi (2010a) estimated the yield in Benin to be 0.3 - 0.6 tonnes/ha. Nevertheless, FAOSTAT reported the yield of 2013 in Benin to be 3.6 tonnes/ha (FAOSTAT, 2016). Despite this high estimate, 59% and 58% of farmers have estimated the cashew revenue in their household income to be less than 50%, in NG and SS, respectively. Nevertheless, as reported by Fitzpatrick (2011), in some African countries, cashew production has a significant contribution to gross domestic product and export exchanges at the country level and represents an essential source for the livelihood of smallholder farmers.

5.5. Conclusions

We conclude that, first of all, the farmer's education level and the limited access to extension services for cashew nut farming are main constraints for good production and high yields. Second, the lack of sorting, drying and farm management significantly and negatively affected nut count, water content, and fungal contamination of cashew nuts, respectively. Farmers agreed that the lack of advice on good cashew production practices negatively influenced the production and, hence their revenues. Good agriculture practices should be strengthened through more supportive extension services to improve production and the quality of raw cashew nuts exported from Benin.

5.6. Acknowledgments

The first author (YL) gratefully thanks the International Institute of Tropical Agriculture in Benin and Wageningen University for financial support and the Department of System Biology of Technical University of Denmark for providing the laboratory infrastructure for mycological analyses. We are grateful to the cashew nut farmers in northern Benin for their cooperation during the surveys.

Chapter 6

General discussion and perspectives

6.1. Introduction

This research project focussed on the occurrence of mycotoxins and other secondary metabolites of *Aspergillus* sp. contamination on raw cashew nuts from Benin and how to improve nut quality. The specific objectives outlined in Chapter 1 were:

(1) - to evaluate the occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels;

(2) - to investigate the diversity in secondary metabolites including mycotoxins of strains of *Aspergillus* section *Nigri* isolated from raw cashew nuts;

(3) - to assess the influence of sorting and grading on quality parameters of raw cashew nuts;

(4) - to study the impact of small-scale farmers' practices on the quality of raw cashew nuts.

The major conclusions of the thesis are:

- Cashew nuts from Benin were found to be contaminated by strains of *A.* section *Flavi* and *A.* section *Nigri,* including toxigenic fungi such as *A. flavus, A. nomius, A. niger* and *A. carbonarius*
- No aflatoxins were detected in the sampled raw cashew nuts from Benin
- Strains from *A.* section *Nigri* were able to produce three different types of mycotoxins in pure culture: fumonisins, ochratoxin A and secalonic acids indicating that these mycotoxins could occur in cashew nuts
- Sorting resulted in Grade 1 cashew nuts with improved nut count and low fungal contamination. Since Grade 1 is sold at premium prices, sorting should be implemented by

small-scale farmers as a non-biological and low cost approach to make cashew harvests more profitable

- Eighty percent of the farmers reported to select the cashew nut seedlings from uncertified sources and 59% never asked or received advice from extension services about cashew farming practices, highlighting the need to promote good agricultural practices through creation of appropriate extension services
- Major parameters that negatively affect the quality of raw cashew nuts were: the low education level of cashew farmers; limited access to extension services; and lack of sorting, drying, and proper farm management. Specific cashew cropping policy must be necessary

The following sections discuss the progress made in achieving the overall objective and provide recommendations for further research.

6.2. Discussion and recommendations

6.2.1. Fungal contamination of raw cashew nuts

The harvesting practice for cashew nuts, i.e. collecting the nuts after they have fallen from the tree on the ground, is the main cause of fungal contamination of nuts via contact with the soil. Therefore, appropriate integrated management practices of cashew nut farming are crucial in controlling the quality and safety and to protect consumers against any direct or indirect consequence of fungal contamination. In Chapter 2, high fungal contamination of cashew kernels was reported. The interior contamination level by *Aspergillus* section *Nigri* (also called black aspergilli) varied from 82.5% to 96.0% in the Northern Guinea (NG) zone, whereas it varied from 70.5% to 98.5% in the Southern Sudanian (SS) zone, with average values of 90.2% and 87.2% in NG and SS, respectively. Moreover, *A.* section *Flavi* contamination varied from 2.0% to 20.5% in NG and from 0.5 to 9.5% in SS with average values of 6.7% and 4.6%, respectively. The same trends were observed for fungal contamination of cashew kernels.

The growth of microorganisms in food products is affected by intrinsic and extrinsic factors. Intrinsic factors include water content, pH and acidity, nutrient content, biological structure, redox potential, naturally occurring antimicrobials and competitive microflora. Extrinsic factors include the type of packaging/atmosphere, time/temperature regime, storage condition and processing (Food and Drug Administration, 2009; Lund *et al*., 2000). Strains of black aspergilli and *A.* section *Flavi* are known as the predominant post-harvest fungi in tropical regions. Their spores are present in the soil, and consequently, in crop residues and storage structures such as Secco, Ago, Ebli-va and clay granary (Donner *et al*., 2009; Hell *et al*., 2000). In our study, fungal contamination was assessed on cashew kernels taken from the shell. The route of contamination, from the outside of the raw cashew nut to the kernel may be investigated further to clarify the presence of fungal spores in the kernel. The cashew nut is an achene with a shell thickness of 2 to 3 mm. The thickness of the shell and the presence of tannins in the shell are considered as barriers for fungal contamination of the kernel (Lund *et al*., 2000; Molyneux *et al*., 2007). Also, during their investigation of post-harvest fungi contamination on tree nuts like almonds, pistachio, hazelnuts, walnuts and peanuts, Deabes and Al-Habib (2011) recorded high post-harvest fungal count on peanuts, pistachio and hazelnuts, compared to cashew nuts. They reported that the cashew nut shell plays a key role as barrier by reducing fungi contamination of the kernels. Nevertheless, the presence of fungal spores in the kernel may be explained by the presence of insect injuries of the cashew shell, which weaken the shell integrity and expose the kernel to fungal contamination (**Fig. 6.1 A**). In addition, the remaining apple flesh after inadequate removal of the cashew apple (**Fig. 6.1 B**) constitutes a way for spores to reach the kernel (Alasalvar and Shahidi, 2008; Barkai-Golan, 2001).

Other tree nuts such as almonds and chestnuts were screened for fungal contamination and showed a different type of mycobiota compared to that found on cashew nut. Although *Botrytis*, *Cladosporium* and *Rhizopus* spp. are reported as major spoilage fungi on variety of nuts, *Aspergillus*, *Eurotium*, *Penicillium* and *Rhizopus* were found to be major fungal genera in almonds

(Bayman, *et al.*, 2002; Purcell *et al.*, 1980; Rodrigues *et al.*, 2012) regardless of the stage of production (field or storage). Moreover, *Aspergillus* and *Penicillium* were found to be associated to chestnuts (Abdel-Gawad and Zohri, 1993; Rodrigues *et al.*, 2012). The wide range of mycobiota observed on tree nuts other than cashew nut was somehow related to the environmental conditions, the stage of production and storage, and the geographic location of the specific nut (Rodrigues *et al.*, 2012). In all cases, when *Aspergillus* is present, it can generally observe that section *Flavi* and *Nigri* seem to increase in number from field to storage (Bayman, *et al.*, 2002).

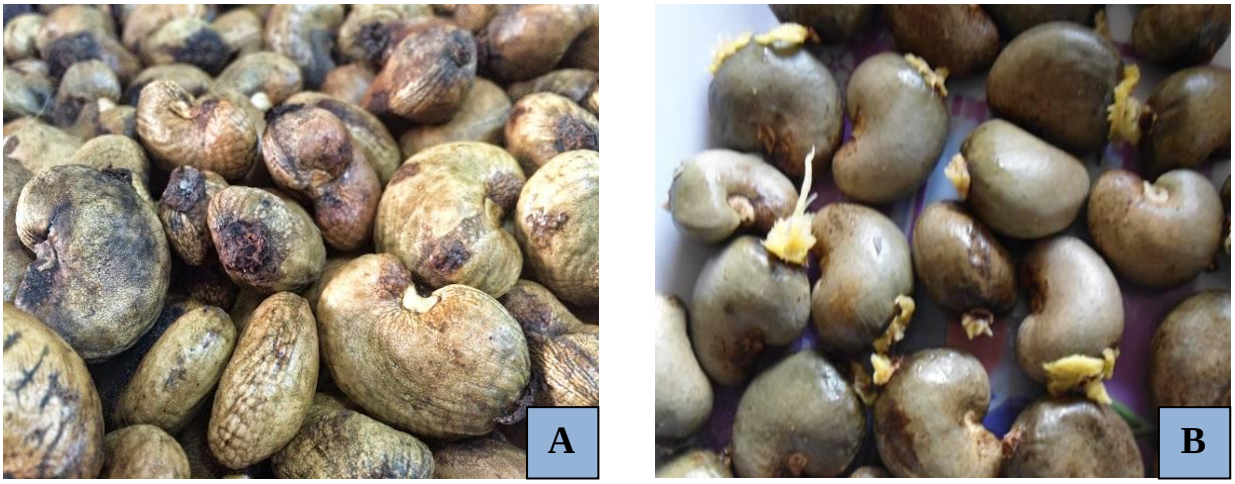


Fig. 6.1. Raw cashew nuts with insect injuries on the shell (A) and with remaining flesh after inadequate removal of the cashew apple (B)

Although the classification of fungi as field or storage fungi has been based on studies done in temperate climates, in which the growing season is unusually hot and dry (CAST, 2003), it was observed that under warm, humid subtropical or tropical climates, *Aspergillus* and *Penicillium* can easily affect crops earlier in the field. Notably, *A. flavus*, a member of section *Flavi*, can infect crops both in the field and storage (Rodrigues *et al.*, 2012). Consequently, appropriate harvesting practices need to be implemented that reduce fungal contamination of harvested cashew nuts. This could be done by using a tarpaulin or fish net suspended beneath the cashew tree to avoid contact of the falling fruit with the soil.

The contamination of cashew nuts by strains of *A.* section *Flavi* was on average 5 to 20 times lower than that by strains of *A.* section *Nigri*. Although the strains of both sections are considered as post-harvest fungi, it has been reported that, in the same environmental conditions, the growth rate of *A. niger* is higher than that of *A. flavus* (Parra and Magan, 2004). This indicates that in contaminated soil, spores of black aspergilli predominate over spores of *A.* section *Flavi*. The ability of some fungal strains to grow rapidly and dominate and suppress other fungi in the same environment is used in biological control of aflatoxigenic strains of *A. flavus* in the presence of non-aflatoxigenic strains. (Atehnkeng *et al.*, 2008; Donner *et al.*, 2010) or in the presence of other fungi in maize fields (Ehrlich, 2014). It has been reported that *A. flavus* strains can be distinguished in 2 groups: the L-morphotype that produces abundant conidia, few, large sclerotia and variable levels of B-aflatoxins, and the S-morphotype that produces numerous small sclerotia, scarce conidiation, and consistently high levels of B- and G-aflatoxins (Cotty, 1989, Cotty and Cardwell, 1999; Donner *et al.*, 2009). A biocontrol agent with trade name "Aflasafe" was developed in Africa (starting from Nigeria, West Africa) consisting of the L-morphotype of *A. flavus* strains.

The L-strains have the ability to displace the S-strains under field conditions: they were first used as atoxigenic biopesticide in the USA under trade names such as Afla-Guard and AF36 (Cotty, 2006; Doster *et al.*, 2014) in maize, pistachio and groundnut fields and in cottonseed. In Africa, based on the strains of L-morphotype that produce no aflatoxins (Atehnkeng *et al.*, 2016; Cotty, 1989; Grubisha and Cotty, 2015), several "Aflasafe-type" products have been developed with geographic specificity: Aflasafe in Nigeria for use in maize fields, Aflasafe KE01 for Kenya for use in maize fields, Aflasafe SN01 for Senegal and Gambia for use in groundnuts fields and Aflasafe BF01 for Burkina Faso for use in groundnuts fields (Atehnkeng *et al.*, 2014; Bandyopadhyay *et al.*, 2016). The corresponding crops are considered as staple food in the specific region and the ability of the L-morphotype of *A. flavus* to displace the S-morphotype could be effective under most of the prevailing field conditions. The manufactured Aflasafe externally looks like cooked sorghum grain, and internally contains very small amount (nanograms) of non-

toxigenic L-morphotype stains of *A. flavus*. Safe product for farmers if properly used, Aflasafe is tossed on field soil by hand 2-3 weeks before flowering at 10-20 kg/ha. Packed in 5 kg boxes and costing about 16 USD /ha at farmer gate, a single application is effective for several years and in several crops. There were reports that farmers achieved 80 to 99% reduction in aflatoxin contamination by applying Aflasafe in maize and groundnut fields and 33% profit on its use (Bandyopadhyay *et al.*, 2016; www.aflasafe.com/aflasafe). The use of Aflasafe developed for Nigeria, a neighbouring country of Benin, should be tested to reduce the presence of toxigenic strains of *A.* section *Flavi* in cashew farms, based on the fact that the farming of cashew mostly starts as a mixture cropping of maize or sorghum with young trees of cashew nuts.

The level of exterior contamination of cashew kernels by strains of *A.* section *Flavi* and *A.* section *Nigri* was correlated to the geographic location of the samples (Chapter 2). The difference noticed could be related to the different climate conditions in the two zones. Both zones have an unimodal rainfall distribution, but the NG zone may benefit from some unexpected rains during the cashew nut harvesting period because of its proximity to the Southern Guinea zone which has two rainfall seasons. Therefore the harvesting period could be monitored to avoid excess humidity on harvested nuts and immediate drying after harvest will lower the water content of the nuts.

The quality parameters of raw cashew nuts listed in Chapter 1 are water content, nut outturn, nut count, and the proportion of damaged nuts. Fungal contamination of cashew nuts is not considered as a quality parameter. This could be explained by the fact that the assessment of fungal contamination and identification requires laboratory analysis and is time consuming. Nevertheless, two quality parameters of cashew nuts are closely related to fungal contamination, namely water content and the proportion of damaged nuts. Water content can be routinely assessed at the farm gate with an appropriate device such as portable moisture meter, prior to an accurate assessment in the laboratory, and damaged nuts can be assessed by visual screening of the cashew batch. We recorded average water contents of 8.61 ± 1.8% in NG zone and 8.68 ± 1.5% in SS zone (Chapter

2). At village level, the highest water contents of cashew nut were recorded in Kilibo (10.8%), Bante (10.5%) and Alafiarou (9.2%) in the NG zone, and in Patargo (10.3%), Chabikouma (9.9%) and Pira (9.9%) in the SS zone. For cashew nuts contaminated with *Aspergillus* section *Flavi*, a positive correlation was observed between the water content and the level of fungal contamination in the villages located in the NG zone, while this positive correlation was observed in the villages located in the SS zone for cashew nuts contaminated with *A.* section *Nigri*. This confirms the relation of water content of cashew nuts with their fungal contamination. The parameters that determine the ability of fungi to grow on foodstuffs are water activity (a_w) and temperature (Scott, 1957). These two parameters play a key role in the level of contamination of foodstuffs by strains of both fungal genera investigated during this research. The optimum temperature and minimum a_w for growth of spores of *A.* section *Nigri* are 33°C and 0.77 respectively, while these are 35°C and 0.78 for growth of spores of *A.* section *Flavi*. This difference in fungal contamination may be related to the higher growth rate of germinated spores of the *Nigri* group that is related to the water content of the nut, hence the a_w inside the nut. A slight decrease in a_w of cashew nut may shift the fungal growth from *Flavi* group to *Nigri* group since strains of *Nigri* group have the ability to grow at low water activity and may invade quickly the cashew nut.

Water content defines the amount of water present in a food material and a_w quantifies the part of water available for reaction with other substances, including micro-organisms. Water content influences the mechanical and physical properties of the food material, hence the selling price at a given time, while a_w is the best concept used for product stability and shelf life. Water content is, related to a_w and this relationship can be determined experimentally at constant temperature and pressure resulting is so-called water sorption isotherms (Kaymak-Ertekin and Gedik, 2004). This relationship helps to establish the humidity boundaries to inhibit fungal growth and mycotoxin production during storage (Boente *et al.*, 1996).

Species from *A.* section *Flavi* identified on cashew nuts were *A. flavus, A. tamari, A. costaricaensis, A. minisclerotigenes* and *A. nomius* (Chapter 2) whereas *A. tubingensis, A. niger, A. brasiliensis, A. carbonarius, A. luchuensis, A. aculeatus* and *A. aculeatinus* were identified as species from *A.* section *Nigri* (Chapter 3). *A. flavus* and *A. nomius* are able to produce aflatoxins and *A. niger* and *A. carbonarius* can produce of ochratoxin A and fumonisins (Chiotta *et al.*, 2013; Månsson *et al.*, 2009; Nielsen *et al.*, 2009). The presence of these fungi on cashew nuts may lead, under certain conditions, to the production of mycotoxins in cashew kernels with their subsequent harmful effect on consumer health. This is discussed in the next section.

6.2.2. Production of mycotoxins and other secondary metabolites on cashew nuts

Mycotoxins are secondary metabolites produced by certain filamentous fungi. *Aspergillus* is one of the most important mycotoxigenic genera, including the species *A. flavus* and *A. nomius* which are known to produce aflatoxins. The environmental conditions under which fungi produce mycotoxins mediate crop-fungus interactions and sometimes insect-crop-fungus interactions. Wu *et al.* (2011) state that four climatic factors (temperature, water activity, precipitation and CO_2 concentration) are crucial in determining the extent to which infections with toxigenic fungi will lead to mycotoxins formation. In Chapter 2, no aflatoxin production was recorded on cashew kernels despite the contamination by *A. flavus* and *A. nomius*. It is well known that fungal contamination is not always correlated with the presence of mycotoxins in foodstuffs. Nevertheless, the absence of aflatoxins in the analysed samples does not imply the absence of aflatoxins in each single cashew kernel, even though the sampling procedure was performed according to the European Regulation (EC No. 401/2006) "describing the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs".

The presence of aflatoxins in nuts and nutty products is widely reported. Most reports on aflatoxin contamination concerned processed nuts, namely almonds and hazelnuts (El tawali *et al.*, 2013), pistachios (Dini *et al.*, 2013) and Brazil nuts (Reis *et al.*, 2012). It is remarkable that these ″other

nuts″ are always reported to be more contaminated with aflatoxins than cashew nuts. This suggests again the role of the thick shell of cashew nuts in lowering the contamination with aflatoxigenic fungi and preventing toxin formation. Moreover, authors who evaluated the presence of aflatoxins on raw cashew nuts reported aflatoxin level below the World Health Organisation and the EU limits of 4 µg/kg (Gyedu-Akoto *et al.*, 2014). The method of toxin quantification used in this study, namely "ultra-high performance liquid chromatography tandem mass spectrometry", is one of the most accurate methods for toxin detection (Nielsen *et al.*, 2015). This detection method, associated with the EC No. 401/2006 sampling method, underpins the conclusion of this study that aflatoxins on cashew nuts from Benin were below the detection level, and put Beninese cashew nuts in the range of good quality commodities for export. Nevertheless, ways to reduce contamination with fungi known to produce aflatoxins should be strengthened to minimize the risk of aflatoxins in cashew kernels. Next to good harvesting practices, drying of cashew nuts just after harvesting will lower the water content and subsequently the water activity and prevent growth of fungi.

Other harmful mycotoxins such as ochratoxin A and fumonisins are produced by *A. niger*, whereas only ochratoxin A is produced by *A. carbonarius* (Frisvad *et al.*, 2011; Nielsen *et al.*, 2009). Ochratoxin A is by far the most important toxin produced by *A. niger* and its concentration can be up to 100 fold higher than that of fumonisins (Frisvad *et al.*, 2011). High levels of contamination by strains of *A.* section *Nigri* were recorded in all villages in both agro-ecological-zones of Benin. The presence of *A. niger* and *A. carbonarius* is an indicator of possible production of ochratoxin A (Frisvad *et al.*, 2011; Gerez *et al.*, 2014). We recorded that 14.6% of the *A. niger* strains were able to produce ochratoxin A in cashew nuts, compared to 100% of *A. carbonarius* strains (Chapter 3). The ability of black aspergilli to grow in a wide range of temperatures, and to better adapt to hot and humid environments (Battilani *et al.*, 2006) explains its high level of contamination on nuts from Benin. Moreover, the absence of regulations regarding the contamination with ochratoxin A and fumonisins for raw cashew nuts limits the evaluation of the exact contribution of these toxins to the total level of mycotoxins in cashew nuts. Health issues due to consumption

of processed cashew nuts contaminated by ochratoxin A and fumonisins may occur. More investigations need to evaluate the exact contamination level of *A. niger* and *A. carbonarius* and to test their ability to produce ochratoxin A and fumonisins in cashew nuts. However, immediate action such as drying will lower the contamination of black aspergilli in the harvested nuts and reduce the risk on contamination with ochratoxin A and fumonisins.

6.2.3. Mycotoxins in other tree nuts: economic and health aspects

The negative effect on human health due to the ingestion of mycotoxins is well known, either in acute or chronic mycotoxicosis. The acute effect was mostly specific to the consumption of staple food is the area of occurrence. Aflatoxicosis have been reported repeatedly in Kenya (in 2001, 2000 and 2005) and in India due to consumption of maize and in Malaysia due to consumption of Chinese noodle (Shephard, 2004, Lewis *et al.*, 2005, Reddy and Raghavender, 2007). Wich respect to chronic mycotoxicosis, aflatoxin B_1 has been extensively linked to human primary liver cancer in which it acts synergistically with hepatitis B virus infection. This combination represents a heavy cancer burden in developing countries (Schmale and Munkvold; 2009; Zain, 2011). Approximately 250.000 deaths are caused by hepatocellular carcinomas in China and Sub-Saharan Africa annually and are attributed to risk factors such as a high daily intake (1.4 μg) of aflatoxin and high incidence of hepatitis B (Wild *et al.*, 1992; Zain, 2011).

Although there are no specific reports of negative effects on human health due to the direct consumption of tree nuts contaminated by mycotoxins, the presence of mycotoxins in tree nuts and dried fruits chain can have a negative economic impact.

The economic losses resulting from mycotoxin-contaminated foods and feeds are difficult to estimate but are undoubtedly large, judging from the widespread occurrence of aflatoxin and ochatoxin A contamination, and the large number of commodities affected (Rico-Sole, 2012). In general, losses arise from several factors: from direct food losses; from human illness and reduced

productivity; from direct cost of systems for control of mycotoxins in foods and feeds; from the reduced value of rejected commodities; from the cost of detoxification to recover an acceptable product; and from loss of export markets (Moss, 1991; Rico-Sole, 2012). In West Africa, especially in Benin, cashew nut as export product enters mostly in the last category of economic loss. In most developing countries exporting tree nuts, the whole economic loss due to mycotoxin contamination is large but its estimation bears relation with the worldwide regulations on mycotoxins. Many countries have adopted laws and regulations that set maximum allowed levels of mycotoxins for the affected commodities, in order to decrease the risk to human health or safety. These regulations include the detention of contaminated goods at the borders to avoid their entrance in the market, reducing the trading of these nuts between countries (Rico-Sole, 2012). Otsuki *et al.* (2001) calculated that a 10% lower maximum allowable level of contamination in the European countries would reduce trade flow from Africa to Europe by 4.3% for nuts, which is a huge economic loss for these countries. Also, Rico-Sole (2012) estimated the worldwide total economic loss for 2008 to be around 87 million USD and for 2009 to be 70 million USD for almonds, hazelnuts, pistachios. This estimate was based on real border rejection of the EU-27 countries plus Norway, Liechtenstein, Switzerland and Iceland, Japan, United States of America and Australia. Specifically in 2010, the total losses for the European Union Rapid Alert System for Food and Feed (EU-RASFF) member countries were estimated at 39 million USD. But the notifications on the EU RASFF member countries decreased notably for the above mentioned nuts when the regulation No 165/2010 (increasing the maximum level of aflatoxins allowed in almonds, Brazil nuts, hazelnuts and pistachios) was adopted and implemented (Rico-Sole, 2012).

6.2.4. Application of sorting and grading of raw cashew nuts as low cost approach for safety

Nut count, defined as the number of cashew nuts per kg, usually ranges between 150 and 240 nuts/kg. For export, cashew quality grades are classified as follows: “excellent” implies a nut count of 170-180 nuts/kg, “very good” stands for 181-190 nuts/kg, “good” is 191-200 nuts/kg, “middle”

201-210 nuts/kg, "low middle" 211-220 nuts/kg, "limit acceptable" 221-230 nuts/kg and "poor" i.e. above 230 nuts/kg. The standard for export quality is a nut count between 170-210 nuts/kg (ACi, 2012a, NAIP, 2013). Nut counts reported in Chapter 2 ranged from 160 to 185 nuts/kg with an average of 175 nuts/kg in the NG zone compared to 152 to 189 nuts/kg with an average of 174 nuts/kg in the SS zone. This means that cashew nuts from Benin usually are of export quality (Chapter 2). Nevertheless, in the Beninese context where 95% of cashew nuts are exported (USAID, 2007), cashew nuts are bought at a single price since nuts are always a mixture of export grades and under-grades. Prices offered by traders/buyers at the farm gate are low compared to the price launched by the government during the annual cashew campaign. This causes some farmers to cheat on the weight of the produce by adding foreign materials like stones and sand, or skipping the drying process. The final quality of raw cashew nuts is thus affected and tends to decrease over campaigns.

In Chapter 4, cashew nuts were sorted in two grades: Grade 1 with less than 50% of their shell surface affected by fungi and/or insect injuries and Grade 2 that consisted of all the remaining produce. The sorting and grading process classified raw cashew nuts from 5 villages in the NG zone and 2 villages in the SS zone as being of a higher quality (Grade 1) with regard to their nut count. This process puts the nut quality of most villages in the range of "excellent" for trading (see **Fig 4.1**). Sorting of nuts in different grades has been reported to positively impact the overall quality of nuts, including Brazil nuts (Pacheco and Martins, 2013) and pistachios (Shakerardekani *et al.*, 2012). Moreover, Garcia-Cela *et al.*, (2012) reported aflatoxin reduction of 40-80% by hand sorting of pistachios. In Tanzania, the cashew board implemented sorting into 2 grades with different prices. This resulted in first grade cashew nuts with a low content of foreign materials (UNIDO, 2011). Implementing sorting and grading as a low cost technology at the farmer gate could effectively result in standard grade of good quality of raw cashew nuts and consequently increase the farmer income.

6.2.5. Cashew nut quality as influenced by farm management practices

Farm management practices are critical for the final quality of cashew nuts. According to Luning and Marcelis (2006), food quality management deals with both food quality and quality management. Therefore the main concerns are (a) the people system that is realising food quality, and (b) the food production systems.

(a) Luning and Marcelis (2006) described people systems as people with certain individual characteristics making decisions in order to achieve their goal. This human behaviour is then described as the decision-making outcomes of the humans system over time. In cashew farming, the farmer's socioeconomic profile plays an important role in the quality, from the production, harvest, storage up to trading. Cashew nut is an export commodity that is cultivated primarily for its revenues (Achterbosch *et al.*, 2014). The knowledge about the socioeconomic and sociodemographic profile of cashew growers is crucial to understand the quality of the end product. This profile can differ from one individual to another and from one zone to another (ACi, 2010c). As described in chapter 5, 95% of the cashew farm owners are found to be males, giving to cashew farming the name of "men's crops" by the Food and Agriculture Organisation (FAO, 2010). In addition, 55% are between 35 to 55 years old against 45% for all other age groups. The main reasons are, first, the mode of land management in the rural areas of Benin: lands usually belong to a family, which is headed by the father or the oldest member, and, second, the exodus of young people from rural to urban areas. The profile of cashew growers showed also that more than 75% of cashew farmers have not attended school after primary level. The low level of education constitutes a constraint to professionalise the cashew sector. If farmers would organize themselves in associations, then extension services could provide them with a minimum education in order to help them to manage their cashew crop and to share their knowledge and experience with respect to quality control.

The collective action of farmers has the benefit to influence the distribution of the value added to the product in a specific value chain. It implies that similar agents at the specific level of the value chain determine a joined strategy for coordinated negotiations (Ruben *et al.*, 2007). In the cashew farming system, farmer associations and cooperatives may help to strengthen the position of rural households in multiple ways. They may have the potential to benefit the farmer financially by negotiating lower price for cashew inputs and higher price for their harvest. Moreover, organising farmers in associations or cooperatives will be an effective vehicle for knowledge and technology transfers (Groothuis, 2016; Topper, 2002). As demonstrated in the pineapple sector, promoting and strengthening small-scale producers' associations and cooperatives may be an effective way to encourage quality improvement (Arinloye and van Boekel, 2016) and will help them to meet quality and safety standards imposed by the retailers.

(b) The above mentioned human systems are subjected to a management system that is described as a whole of managerial activities and administrative conditions such as organisational relationships and available information that may influence people decision making activities (Luning and Marcelis, 2006). The cashew production systems consist of farm management and post-harvest management of cashew nuts. During the interviews (Chapter 5), cashew farmer practices that can significantly affect the quality of raw nuts were recorded: (a) 80% of cashew farmers reported to select their seedlings from relatives and neighbouring farms, (b) 59% of cashew farmers never had contact with extension services to buy approved seedlings and/or to benefit from advice for good cashew tree farming, (c) 88% of cashew farmers reported that insects were their main concern during cashew farming, without any prevention measures, (d) 74% and 94% of cashew farmers reported to never dry their harvest in NG and SS zone, respectively, (e) up to 29% of cashew farmers reported to store their harvest directly on the ground.

In addition to the above, the lack of sorting and drying showed a significant negative impact on the nut count and water content, hence on the quality of the raw nuts. Moreover, we noticed that

the level of fungal contamination on cashew nuts is correlated to the level of farm management (Chapter 5). In both agro-ecological zones, cashew growers agreed that the lack of farm management and the lack of advice from extension services have significant impact on the yield and subsequently on the contribution of the revenues from cashew nut production to their household income. There is a need to improve agricultural practices and post-harvest practices for cashew nut production. This could be done through a specific extension service for cashew cultivation like the existing extension service for cotton that exists throughout the country.

6.2.6. Cashew apple as source of income for smallholders in Africa

The introduction of the cashew tree in Africa at the end of the 18th century starting from Mozambique had the unique purpose to produce the nuts as a high value cash crop that can contribute significantly to farm household income, in addition to the cultivation of subsistence crops. Later, the cultivation of cashew trees has spread to all countries from East to West Africa including Benin. Unfortunately, the expansion of the cashew nut sector is nowadays facing many constraints that gradually decrease the potential of revenue earned by smallholder cashew farmers. Some constraints are: lack of access to agricultural inputs, weak extension services and farmer associations, low farm productivity, lack of improved planting materials, soil erosion, bush fires, unseized business opportunities, all of these making cashew farmers revenues unstable. The use of cashew apples may be an alternative to sustain the revenue-making for smallholder farmers in the rural areas. The cashew fruit consists of the nut and the false fruit called apple. During the harvest of the nut, most smallholders leave a huge quantity of apples unused in the farm (Cashew Handbook, 2014). However the cashew apples are highly nutritious: the cashew apple contains five times more vitamin C than an orange and contains more calcium, iron and vitamin B1 than other fruit such as citrus avocados and bananas (Contreras-Calderon *et al.*, 2011; DANIDA, 2003) and has potential for additional income for smallholder farmer households.

Cashew apple can be eaten fresh or juiced (Gyedu-Akoto, 2011). In the rural areas in Benin, it is not uncommon to see fresh cashew apples sold by women in markets during the cashew harvesting period, but the revenue earned does not significantly contribute to the farmer household income. Nevertheless, the large potential of processing cashew apple represents a reliable additional source of income for smallholder cashew farmers. The cashew apple can be used to produce juice, jam, vinegar, syrup, wine, brandy, spirit, preserved fruit, pickles and glazed fruit (DANIDA, 2003). There are many reports of revenue-making out of local processing of cashew apples. In Tanzania, cashew apples were used to produce local beer or a spirit (Ibrahim, 2015). In Mozambique, farmers produce alcohol out of the dried apples and the alcohol produced is often used to pay casual labours employed for harvesting. It was reported that on average farmers get 0.125 l of alcohol per kilogramme of nuts harvested (ACi, 2010c). All these products derived from cashew apples can be a source of additional revenue for smallholder cashew farmers in Africa. The lack of adequate knowledge and appropriate processing materials makes this additional revenue-making approach not yet in reach for smallholder farmers in Benin.

6.3. Methodological limitations

During this research, the selection of villages within each agro-ecological zone for cashew nut sampling was the first challenge because the criteria used were the selection of areas accessible by car and located along the main roads from southern to northern Benin. Even though the villages were randomly selected along these roads, the agro-ecological zone may not be completely covered. The villages far from the main road have less and late access to buyers: the storage time of harvested nuts increases, and so increases the risk of fungal contamination.

Farmers present during the sampling period and having cashew nuts available in their stores were selected for sampling and interview. It would be valuable to apply to all cashew farmers a selecting method that covers the whole village and all cashew producers from both genders. This may also

reduce the gender imbalance observed within the interviewees. During the meeting to introduce the research work at village level, women were less represented due to the act that men consider themselves to better represent the household.

Even though farmers stated that they do not sort cashew nuts after harvest (chapter 5), there is a kind of natural sorting according to the harvesting method: picking of cashew nut from the ground. The picking basically leaves some immature and injured nuts on the ground. Also, farmers reported to store their cashew nuts directly on the ground, in jute bags and in plastic or polyethylene bags. To cover the whole range of fungi susceptible to contaminate raw cashew nut just after harvest, it would be useful to sample the nuts either directly from the field or from the same storage structure: this would avoid discrepancies between storage time and in storage structures.

To distinguish fungal contamination on the two grades of cashew nut (Chapter 4), sorting and grading was done based on visual examination of samples: a bias due to the observer could occur during sorting. It would be useful to apply a sorting method that can be replicated by any observer without any bias.

6.4. Concluding remarks

Cashew trees were introduced in Benin in the 1950's with the primary objective to produce raw cashew nuts for the international market. The nut became an important cash crop giving additional revenues to small-scale farmers in rural areas in the Northern Guinea and Southern Sudanian agro-ecological zones of Benin. Health issues related to mycotoxin intake as a consequence of contamination of cashew nut by mycotoxigenic fungi have not been reported so-far. However, a drastic quality control of cashew nuts prior to export and processing is required at the present time. Cashew nuts from Benin, at present, are of good quality for export. But many pre- and post-harvest constraints threaten their quality. Adequate measures should be applied to preserve and strengthen the quality for sustainable production of raw cashew nuts.

The occurrence of fungi and mycotoxins on raw cashew nuts can be minimized or even completely prevented by measures both pre- and post-harvest. The introduction of improved varieties, resistant to fungi and insects may result in healthy nuts and a higher yield. Appropriate harvesting methods, timely harvesting, clean-up, adequate drying and storage practices, management of pests' infestation during storage should be implemented through good agricultural practices. Our results show that sorting and grading are effective in improving quality parameters of cashew nuts. For farmer as well as decision- and policy makers, this represents a low-cost approach that could be implemented throughout the country with an incentive price for first grade cashew nuts. Another constraint for cashew growers to produce cashew nuts of good quality was the lack of adequate education. Low education levels prevent cashew farmers to access basic knowledge on good cashew farming, hence to increase their income. Appropriate education and knowledge sharing policies, through cashew farmers associations may put farming of cashew trees at the level of professional activities.

References

Abarca, M.L., Accensi, F., Bragulat, M.R., Castella, G., Cabanes, F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. Journal of Food Protection 66, 504-506.

Abdel-Gawad, K.M., Zohri, A.A., 1993. Fungal flora and mycotoxins of six kinds of nut seeds for human consumption in Saudi Arabia. Mycopathologia 124, 55–64.

Abdulla, N.Q.F., 2013. Evaluation of fungal flora and mycotoxin in some important nut products in Erbil local markets. Research Journal of Environmental and Earth Sciences 5, 330-336.

Accensi, F., Abarca, M.L, Cabanes, F.J., 2004. Occurrence of *Aspergillus* species in mixed feeds and component raw materials and their ability to produce ochratoxin A. Food Microbiology 21, 623–627.

Acevedo, A., Smith, J., Ana, Y., Villarroel, R., 2011. Incidence of moulds and presence of aflatoxin on toasted cashew nuts (*Anacardium occidentale* L.) in Venezuela. The Annals of the University Dunarea de Jos of Galati Fascicle VI–Food Technology 35, 9-15.

Achterbosch, T.J., van Berkum, S., Meijerink, G.W., 2014. Cash crops and food security; Contributions to income, livelihood risk and agricultural innovation. Wageningen, LEI Wageningen UR (University & Research centre), LEI Report 2014-015, 57p.

ACi (African Cashew initiative), 2010a. Analysis of the Benin cashew sector value chain. GTZ, 64p.

ACi (African Cashew initiative), 2010b. Analysis of the cashew sector value chain in Cote d'Ivoire. https://agriknowledge.org/downloads/5q47rn75s. Accessed 08/02/2017.

ACi (African Cashew initiative), 2010c. Analysis of the cashew value chain in Mozambique. http://www.africancashewinitiative.org/imglib/downloads/ACI_Mozambique_gb_high.pdf.

ACi (African Cashew initiative), 2012a. How to estimate the quality of raw cashew nut (RCN)? http://www.africancashewinitiative.org/imglib/downloads/ACi_GH_CASHEW%20QUALITY_2012.pdf. Accessed 12/02/2016.

ACi (African Cashew initiative), 2012b. Quality of cashew nuts 'Out-turn' and 'Total defective nuts': what you need to know about it. http://africancashewinitiative.com/imglib/downloads/training%20material%20EN/ACI_GH_QUALITY_CASHEW_FLIPCHART_2012.pdf. Accessed 12/02/16.

Adebajo, L.O., Diyaolu, S.A., 2003. Mycology and spoilage of retail cashew nuts. African Journal of Biotechnology 2, 369-373.

Adeniyi, D.O., Adedeji, A.R., 2015. Evaluation of fungal flora and mycotoxin potential associated with postharvest handlings of cashew nut. Archives of Applied Science Research 7, 30-33.

Alasalvar, C., Shahidi, F., 2008. Tree nuts: composition, phytochemicals, and health effects. CRC Press.

Al-Musallam, A., 1980. Revision of the black *Aspergillus* species. Utrecht: Univ Utrecht. 92p.

Andersen, R., Buechi, G., Kobbe, B., Demain, A.L., 1977. Secalonic acids D and F are toxic metabolites of *Aspergillus aculeatus*. Journal of Organic Chemistry 42, 352-353.

Ani, A.O., 1998. Assessment of farmers' extension education needs in Yobe State, Nigeria. Niger Journal of Agricultural Education 5, 152–158.

Arinloye, D.D.A.A., van Boekel, M.A.J.S., 2016. Quality challenges and opportunities in the pineapple supply chain in Benin. In: *Quality and innovation in food chains: Lessons and insights from Africa*. Wageningen Academic Publishers, pp. 271-279.

Asao, T., Buchi, G., Abdel Kader, M.M., Chang, S.B., Wick, E.L., Wogan, G.N., 1965. Structures of aflatoxins B and G1. Journal of American Chemical Society 87, 882–886.

Asogwa, E.U., Hammed, L.A., Ndubuaku, T.C.N., 2008. Integrated production and protection practices of cashew (*Anacardium occidentale*) in Nigeria. African Journal of Biotechnology 7, 4868-4873.

Assunção, R.B., Mercadante, A.Z., 2003. Carotenoids and ascorbic acid from cashew apple (*Anacardium ocidentale* L.) – variety and geographic effects. Food Chemistry 81, 495–502.

Atehnkeng, J., Donner, M., Ojiambo, P.S., Ikotun, B., Augusto, J., Cotty, P.J., Bandyopadhyay, R., 2016. Environmental distribution and genetic diversity of vegetative compatibility groups determine biocontrol strategies to mitigate aflatoxin contamination of maize by *Aspergillus flavus*. Microbial Biotechnology 9, 75-88.

Atehnkeng, J., Ojiambo, P.S., Cotty, P.J., Bandyopadhyay, R., 2014. Field efficacy of a mixture of atoxigenic *Aspergillus flavus* Link: Fr vegetative compatibility groups in preventing aflatoxin contamination in maize (*Zea mays* L.). Biological Control 72, 62-70.

Atehnkeng, J., Ojiambo, P.S., Ikotun, T., Sikora, R.A., Cotty, P.J., Bandyopadhyay, R., 2008. Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Additives and Contaminants 25, 1264-1271.

Azam-Ali S.H., Judge E.C., 2001. Small-scale cashew nut processing. FAO, Rugby, UK, 70p. At http://www.anacardium.info/IMG/pdf/Small-scale_Cashew_Nut_Processing_-_FAO_2001.pdf. [27\11\2016].

Balajee, S.A., Borman, A.M., Brandt, M.E., Cano, J., Cuenca-Estrella, M., Dannaoui, E., Guarro, J., Haase, G., Kibbler, C.C., Meyer, W., O'Donnell, K., Petti, C.A., Rodriguez-Tudela, J.L., Sutton, D, Velegraki, A., Wickes, B.L., 2009. Sequence-Based identification of *Aspergillus*, *Fusarium*, and *Mucorales* species in the clinical mycology laboratory: where are we and where should we go from here? Journal of Clinical Microbiology 47, 877-884.

Bandyopadhyay, R., Ortega-Beltran, A., Akande, A., Mutegi, C., Atehnkeng, J., Kaptoge L., Senghor, A.L., Adhikari, B.N., Cotty, P.J., 2016. Biological control of aflatoxins in Africa: current status and potential challenges in the face of climate change. World Mycotoxin Journal 9, 771-789.

Bankole, S., Schollenberger, M., Drochner, W., 2006. Mycotoxins in food systems in Sub Saharan Africa: A review. Mycotoxin Research 22, 163-169.

Barkai-Golan, R., 2001. Postharvest diseases of fruits and vegetables: development and control. Elsevier.

Battilani, P., 2010. Mycotoxins in nuts. In *XIV GREMPA* Meeting on Pistachios and Almonds. Ed. G. Zakynthinos. Zaragoza: CIHEAM / FAO / AUA / TEI Kalamatas / NAGREF, pp. 167-173.

Battilani, P., Barbano, C., Marin, S., Sanchis, V., Kozakiewicz, Z., Magan, N., 2006. Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. International Journal of Food Microbiology 111, S72-S82.

Bayman, P., Baker, J.L., Mahoney, N.E., 2002. *Aspergillus* on tree nuts: Incidence and associations. Mycopathologia 155, 161–169.

Behrens, R., 1996. Cashew as an Agroforestry Crop: Prospects and Potentials. Margraf Verlag, Weikersheim, Germany.

Bennett, J.W., Horowitz, P.C., Lee, L.S., 1979. Production of sclerotia by aflatoxigenic and nonaflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus*. Mycologia 71, 415-432.

Bennett, J.W., Klich, M., 2011. Mycotoxins. Clinical Microbiology Reviews 66, 737–744.

Blaser, P., Ramstein, H., Schmidt-Lorenz, W., Schlatter, C., 1980. Toxizitat und Mutagenitat der xerophilen Schimmelpilze der Gattung *Eurotium* (*Aspergillus glaucus*-Gruppe). LWT - Food Science and Technology 14, 66-71.

Blumenthal, C.Z., 2004. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology Pharmacology 39, 214-228.

Boente, G., Gonzalez, H.H.L., Martınez, E., Pollio, M.L., Resnik, S.L., 1996. Sorption isotherms of corn – study of mathematical models. Journal of Food Engineering 29, 115–128.

Bonaglia, F., Fukasaku, K., 2003. 'Export Diversification in low income countries: an international challenge after Doha', Working Paper No. 209, OECD Development Centre, Paris.

Brandt, P., 2007. Reports on Food Safety 2005: Food Monitoring. Springer Science & Business Media.

Brush, S.B., 1995. In situ conservation of landraces in centres of crop diversity. Crop Science 35, 346–354.

Carbone, I., Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia, 553-556.

Cardwell, K.F., Cotty, P.J., 2002. Distribution of *Aspergillus* section *Flavi* among field soils from the four agroecological zones of the Republic of Bénin, West Africa. Plant Disease 86, 434- 439.

Cashew Handbook, 2014. Global perspective. A product of www.cashewinfo.com. At http://www.cashewinfo.com/cashewhandbook2014.pdf.

CAST, 2003. Mycotoxins: Risks in plant, animal, and human systems. Task Force Report No. 139. Iowa, USA: Council for Agricultural Science and Technology.

Caswell, M., Fuglie, K., Ingram, C., Jans, S., Kascak, C., 2001. Adoption of Agricultural production practices: Lessons learned from the US. Department of Agriculture area studies project. Washington DC. US. Report No. 792.

Catarino, L., Menezes, Y., Sardinha, R., 2015. Cashew cultivation in Guinea-Bissau–risks and challenges of the success of a cash crop. Scientia Agricola 72, 459-467.

CFC (Common Found for Commodities), 2002. Regional meeting on the development of cashew nuts export from West Africa. CFC Technical Paper N° 3. 381p. At http://common-fund.org/fileadmin/user_upload/Projects/FIGTF/FIGTF_07/Technical_Paper_No._23.pdf.

Chabi Sika, K., Adoukonou-Sagbadja, H., Ahoton, L., Saidou, A., Ahanchede, A., Kefela, T., Gachomo, E.W., Baba-Moussa, L., Kotchoni, S.O., 2015a. Genetic characterization of cashew (*Anacardium occidentale* L.) cultivars from Benin. Journal of Horticulture 2, 153.

Chabi Sika, K., Adoukonou-Sagbadja, H., Ahoton, L.E., Adebo, I., Adigoun, F.A., Saidou, A., Ahanchede, A., Kotchoni, S.O., Baba-Moussa, L., 2015b. Morphological characterization and agronomic performances of cashew (*Anacardium occidentale* L.) accessions from Benin. Journal of Agriculture and Crop Research 3, 27-40.

Chassy, B.M., 2010. Food safety risks and consumer health. New Biotechnology 27, 534–544.

Chepng'etich, E., Nyamwaro, S.O., Bett, E.K., Kizito, K., 2015. Factors that influence technical efficiency of sorghum production: A case of small holder sorghum producers in lower eastern Kenya. Advance in Agriculture 2015. DOI: 10.1155/2015/861919.

Chiotta, M.L., Ponsone, M.L., Sosa, D.M., Combina, M., Chulze, S.N., 2013. Biodiversity of *Aspergillus* section *Nigri* populations in Argentinian vineyards and ochratoxin A contamination. Food Microbiology 36, 182-190.

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A–fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. International Journal of Food Microbiology 149, 171-176.

Commission of the European Communities, 2006. Commission Regulation EC No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union L64, pp 5-24.

Contreras-Calderon, J., Calderon-Jaimes, L., Guerra-Hernandez, E., Garcia-Villanova, B., 2011. Antioxidant capacity, phenolic content and vitamin C in pulp, peel and seed from 24 exotic fruits from Colombia. Food Research International 44, 2047-2053.

Cotty, P.J., 1989. Virulence and cultural characteristic of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79, 808–814.

Cotty, P.J., 2006. Biocompetitive exclusion of toxigenic fungi. In: Barug, D., Bhatnagar, D., Van Egdmond, H.P., Van der Kamp, J.W., Van Osenbruggen, W.A. and Visconti, A. (eds.) *The mycotoxin factbook*. Wageningen Academic Publishers, Wageningen, the Netherlands, pp. 179-197.

Cotty, P.J., Cardwell, K.F., 1999. Divergence of West African and North American communities of *Aspergillus* section *Flavi*. Applied and Environmental Microbiology 65, 2264–2266.

Cromwell, E., van Oosterhout, S., 2000. On-farm conservation of crop diversity: Policy and institutional lessons from Zimbabwe. *Genes in the Field: On-farm Conservation of Crop Diversity*, pp. 217–238.

Dah-Dovonon, J.Z., Idrissou-Yaya, M., 2009. Etude approfondie pour le développement de la filière karité dans la région de l'Atakora et de la Donga. Programme de conversation et de gestion des ressources naturelles, Cotonou, Benin.

DANIDA (Danish International Development Agency), 2003. Information about cashew nut (*Anacardium occidentale*). At http://www.hubrural.org/IMG/pdf/anacarde_danida.pdf.

Darwish, W.S., Ikenaka, Y., Nakayama, S.M., Ishizuka, M., 2014. An overview on mycotoxin contamination of foods in Africa. The Journal of Veterinary Medical Science 76, 789.

Das, I., Arora, A., 2017. Post-harvest processing technology for cashew apple–A review. Journal of Food Engineering 194, 87-98.

De Saeger, S., 2011. Determining mycotoxins and mycotoxigenic fungi in food and feed. Woodhead publishing, Technology and Engineering, 456 p. at: http://app.knovel.com/hotlink/toc/id:kpDMMFFF02/determining-mycotoxins/determining-mycotoxins.

Deabes, M., Al-Habib, R., 2011. Toxigenic fungi and aflatoxin associated to nuts in Saudi Arabia. Journal of American Science 7, 218-225.

Degla, P.K., 2012. Transaction costs in the trading system of cashew nuts in the north of Benin: A field study. American Journal of Economics and Sociology 71, 277–297.

De-Mello, F.R., Scussel, V.M., 2009. Development of physical and optical methods for in-shell Brazil nuts sorting and aflatoxin reduction. Journal of Agricultural Science 1, 3.

Dendena, B., Corsi, S., 2014. Cashew, from seed to market: a review. Agronomy and Sustainable Development 34, 753-772.

Diesing, A.K., Nossol, C., Panther, P., Walk, N., Post, A., Kluess, J., Kreutzmann, P., Danicke, S., Rothkotter, H.J., Kahlert, S., 2011. Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicology Letters 200, 8–18.

Dini, A., Khazeli, P., Roohbakhsh, A., Madadlou, A., Pourenmdari, M., 2013. Aflatoxin contamination level in Iran's pistachio nut during years 2009-2011. Food Control 30, 540-544.

Donner, M., Atehnkeng, J., Sikora, R.A., Bandyopadhyay, R. Cotty, P.J., 2010. Molecular characterization of atoxigenic strains for biological control of aflatoxins in Nigeria. Food Additives and Contaminants 27, 576-590.

Donner, M., Atehnkeng, J., Sikora, R.A., Bandyopadhyay, R., Cotty, P.J., 2009. Distribution of *Aspergillus* section *Flavi* in soils of maize fields in three agroecological zones of Nigeria. Soil Biology and Biochemistry 41, 37-44.

Doster, M.A., Cotty, P.J., Michailides, T.J., 2014. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. Plant Disease 98, 948-956.

EC (Commission Regulation) No 401/2006, 2006. Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union L 70, 12-34.

Ehrlich, K.C., 2014. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations. Frontiers in Microbiology 5.

El tawila, M.M., Neamatallah, A., Serdar, S.A., 2013. Incidence of aflatoxins in commercial nuts in the holy city of Mekkah. Food Control 29, 121-124.

European Commission, 2010. Guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins. Commission regulation (EC) No. 1152/2009 of 27 November 2009 at http://ec.europa.eu/food/food/chemicalsafety/contaminants/guidance-2010.pdf.

FAO (Food and Agriculture Organisation), 2010. Strengthening micro-enterprises in Tanzania: the case of small scale vegetable farmers in Arusha Tanzania, Final Report, Economic and Social Research Foundation.

FAO (Food and Agriculture Organisation), 2015. FAO Statistical Pocketbook 2015. Available at: http://www.fao.org/3/a-i4691e.pdf.

FAOSTAT, 2016. Food and agriculture organisation of the United Nations statistics division. At http://faostat3.fao.org/download/Q/QC/E. Accessed 12/02/16.

FAOSTAT-Food and Agriculture Organization Statistics Division, 2015. [Cited October 2015]. Available from: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor.

Fitzpatrick, J., 2011. Cashew nut processing equipment study: summary. http://www.africancashewalliance.com/sites/default/files/documents/equipment_study_ab_pdf_final_13_9_2011.pdf. [26/08/2016].

Fontana, A.J., 2008. Appendix D: Minimum water activity limits for growth of microorganisms. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt, S.J. and Labuza, T.P. (eds) *Water Activity in Foods: Fundamentals and Applications*. UK, BlacCishing Ltd., Oxford, pp. 405–406.

Food and Drug Administration, 2009. Evaluation and definition of potentially hazardous foods. Chapter 3: Factors that influence microbial growth. Available at https://www.fda.gov/Food/FoodScienceResearch/ucm094145.htm.

Freire, F.C.O., Kozakiewicz, Z., 2005. Filamentous fungi, bacteria and yeasts associated with cashew kernels in Brazil. Revista Ciência Agronômica 36, 249–254.

Freire, F.C.O., Kozakiewicz, Z., Paterson, R.R.M., 1999. Mycoflora and mycotoxins of Brazilian cashew kernels. Mycopathologia 145, 95–103.

Frisvad, J.C., 2011. Rationale for a polyphasic approach in the identification of mycotoxigenic fungi. In: De Saeger S, editor. Determining mycotoxins and mycotoxigenic fungi in food and feed. Oxford: Woodhead Publishing, pp. 279-297.

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson, R.A., Nielsen, K.F., 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS one 6(8), p.e23496.

Frisvad, J.C., Samson, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in Mycology 49, 1-174.

Fung, F., Clark, R.F., 2004. Health effects of mycotoxins: A toxicological overview. Clinical Toxicology 42, 217–234.

García-Cela, E., Ramos, A.J., Sanchis, V., Marin, S., 2012. Emerging risk management metrics in food safety: FSO, PO. How do they apply to the mycotoxin hazard? Food Control 25, 797–808.

Gerez, C.L., Dallagnol, A., Ponsone, L., Chulze, S., Font de Valdez, G., 2014. Ochratoxin A production by *Aspergillus niger*: effect of water activity and a biopreserver formulated with *Lactobacillus plantarum* CRL 778. Food Control 45, 115-119.

Ghosal, S., Biswas, K., Chakrabarti, D.K., 1979. Toxic naphtho-gamma-pyrones from *Aspergillus niger*. Journal of Agricultural and Food Chemistry 27, 1347-1351.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61, 1323-1330.

Greco, M., Kemppainen, M., Pose, G., Pardo, A., 2015. Taxonomic characterization and secondary metabolite profiling of *Aspergillus* section *Aspergillus* contaminating feeds and feedstuffs. Toxins 7, 3512-3537.

Groothuis, R.J., 2016. Decision-making of rural farm households growing cashew or shea.

Grubisha, L.C., Cotty, P.J., 2015. Genetic analysis of the *Aspergillus flavus* vegetative compatibility group to which a biological control agent that limits aflatoxin contamination in USA crops belongs. Applied and Environmental Microbiology 81, 5889-5899.

GTZ, 2006. Fact finding mission on "Support for the development of the cashew sector in Dak Lak". GTZ. EDE Consulting for Coffee available at http://www.value-chains.org/dyn/bds/docs/700/060404_Cashew_study_GTZ_fin_eng.pdf.

Gyedu-Akoto, E., 2011. Utilization of some cashew by-products. Nutrition and Food Science 41, 393-400.

Gyedu-Akoto, E., Lowor, S.T., Assuah, M., Kumi, W., Dwomoh, E.A., 2014. Assessment of post-harvest handling effects on quality of cashew nuts and kernels in Ghana. Journal of Scientific Research and Reports 3, 953-965.

Hammed, L.A., Anikwe, J.C., Adedeji, A.R., 2008. Cashew nuts and production development in Nigeria. American Eurasian Journal of Scientific Research 3, 54-61.

Heintz, J., Valodia, I., 2008. Informality in Africa: A Review. WIEGO Working Paper No.3. http://wiego.org/sites/files/publications/Heintz_WIEGO .WP3.pdf. [15/09/2016].

Hell, K., Cardwell, K.F., Poehling, H.M., 2003. Distribution of fungal species and aflatoxin contamination in stored maize in four agro-ecological zones in Benin, West-Africa. Journal of Phytopathology 151, 690-698.

Hell, K., Cardwell, K.F., Setamou, M., Poehling, H.M., 2000. The influence of storage practices on aflatoxin contamination in maize in four agro-ecological zones of Benin, West Africa. Journal of Stored Products Research 36, 365-382.

Hell, K., Fandohan, P., Bandyopadhyay, R., Cardwell, K., Kiewnick, S., Sikora, R., Cotty, P., 2008. Pre- and postharvest management of aflatoxin in maize. In: Leslie, J.F., Bandyopadhyay, R. and Visconti, A. (eds) *Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade*. CABI Publishing, Wallingford, UK, pp. 210-219.

Hong, S-B., Cho, H-S., Shin, H-D., Frisvad, J.C., Samson, R.A., 2006. Novel *Neosartorya* species isolated from soil in Korea. International Journal of Systematic Evolutionary Microbiology 56, 477-486.

Hong, S-B., Lee, M., Kim, D-H., Varga, J., Frisvad, J.C., Perrone, G., Gomi, K., Yamada, O., Machida, M., Houbraken, J., Samson, R.A., 2013. *Aspergillus luchuensis*, an industrially important black *Aspergillus* in East Asia. PloS one, 8(6):e63769.

IARC (International Agency for Research on Cancer), 1993. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risk to humans, Lyon, IARC 56, 489–521.

Ibouraïman, B., Léonard, A.E., Adam, A., Bonaventure, A.C., Vincent, E., Aliou, S., Daouda, B.O., Guillaume, A.L., Sévérin, B., Daniel, C.C., 2016. Effect of Climatic Factors on Cashew (*Anacardium occidentale* L.) Productivity in Benin (West Africa). Journal of Earth Science and Climatic Change 2016.

Ibrahim, G., 2015. Disseminated cashew-nut production technologies and their effect to cashew-nut productivity in Mkinga district, Tanzania. Doctoral dissertation, Sokoine University of Agriculture.

ITC (International Trade Centre), 2013. Global cashew market - A snapshot overview. At http://www.gambiatradeinfo.org/sites/default/files/Global%20Cashew%20Market%20Overview.pdf.

Johnson, D.V., 1973. The botany, origin and spread of the cashew *Anacardium occidentale* L. Journal of Plantation Crops 1, 1-7.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York: Academic Press, pp. 21-132.

Jurjević, Ž., Peterson, S.W., Stea, G., Solfrizzo, M., Varga, J., Hubka, V., Perrone, G., 2012. Two novel species of *Aspergillus* section *Nigri* from indoor air. IMA Fungus 3, 159-173.

Kader, A.A., 2013. Impact of nut postharvest handling, de-shelling, drying and storage on quality. In: Harris, L.J. (eds) *Improving the safety and quality of nuts*, Cambridge: Woodhead Publishing Ltd, pp. 22-34.

Kashani-Nejad, M., Tabil, L.G., Mortazavi, A.S., Kordi, A.S., 2003. Effect of drying methods on quality of pistachio nuts. Drying Technology 21, 821–838.

Kaymak-Ertekin, F., Gedik, A., 2004. Sorption isotherms and isosteric heat of sorption for grapes, apricots, apples and potatoes. Lebensmittel-Wissenchaft und Technology 37, 429–438.

Klitgaard, A., Iversen, A., Andersen, M.R., Larsen, T.O., Frisvad, J.C., Nielsen, K.F., 2014. Aggressive dereplication using UHPLC–DAD–QTOF: screening extracts for up to 3000 fungal secondary metabolites. Analytical and Bioanalytical Chemistry 406, 1933-1943.

Krepl, V., Kment, P., Rajdlova, G., Kapila, P.F., 2016. African countries' agricultural trade value chain assessment case study: Tanzania (Cashew nut exports). AGRIS on line Paper in Economy Informatics 8, 45.

Lamboni, Y., Frisvad, J.C., Hell, K., Linnemann, A.R., Nout, M.J.R., Tamo, M., Nielsen, K.F., van Boekel, M.A.J.S., Smid, E.J., 2016. Occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels (*Anacardium occidentale* L.) from Benin. LWT-Food Science and Technology 70, 71-77.

Lamboni, Y., Hell, K., 2009. Propagation of mycotoxigenic fungi in maize stores by post-harvest insects. International Journal of Tropical Insect Science 29, 31-39.

Larsen, T.O., Smedsgaard, J., Nielsen, K.F., Hansen, M.E., Frisvad, J.C., 2005. Phenotypic taxonomy and metabolite profiling in microbial drug discovery. Natural Product Report 22, 672-695.

Lawal, O.W., Fagbohun, E.D., 2014. Studies on bio-deterioration, aflatoxin contamination and nutritive values of processed cashew (*Anacardium occidentale* L) nuts during storage. Nature and Science 11, 127-133.

Leong, Y.H., Ismail, N., Latif, A.A., Ahmad, R., 2010. Aflatoxin occurrence in nuts and commercial nutty products in Malaysia. Food Control 21, 334-338.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A.M., Misore, A., Decoct, K., Rubin, C., 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. Environmental Health Perspectives 113, 1763–1767.

Logrieco, A.F., 2010. Mycotoxicological risks of main European food/feed chains. Toxicology Letter 196 (Supplement 1), S24.

Lowor, S., Yabani, D., Winifred, K., Agyente-Badu, C.K., 2016. Production of Wine and Vinegar from Cashew (*Anacardium occidentale*)" Apple". British Biotechnology Journal 12, 1.

Lund, B., Baird-Parker, A.C., Gould, G.W., 2000. Microbiological safety and quality of food. Vol. 1, Springer US. 2024p.

Lundadei, L., Ruiz-Garcia, L., Bodria, L., Guidetti, R., 2013. Image-based screening for the identification of bright greenish yellow fluorescence on pistachio nuts and cashews. Food and Bioprocess Technology 6, 1261–1268.

Luning, P.A., Marcelis, W.J., 2006. A techno-managerial approach in food quality management research. Trends in Food Science and Technology 17, 378-385.

Månsson, M., Klejnstrup, M.L., Phipps, R.K., Nielsen, K.F., Frisvad, J.C., Gotfredsen, C.H., Larsen, T.O., 2009. Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. Journal of Agricultural and Food Chemistry 58, 949-953.

Maresca, M., Fantini, J., 2010. Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon 56, 282–294.

Marín, S., Hodžić, I., Ramos, A.J., Sanchis, V., 2008. Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. Food Microbiology 25, 683-689.

Massi, F.P., Sartori, D., de Souza-Ferranti, L., Iamanaka, B.T., Taniwaki, M.H., Vieira, M.L.C., Fungaro, F.H.P., 2016. Prospecting for the incidence of genes involved in ochratoxin and fumonisin biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*. International Journal of Food Microbiology 221, 19-28.

Medina, A., Mateo, R., López-Ocana, L., Valle-Algarra, F.M., Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Applied Environmental Microbiology 71, 4696-4702.

Mensah, E.R., Karantininis, K., Adégbidi, A., Okello, J.J., 2012. Determinants of commitment to agricultural cooperatives: cashew nuts farmers in Benin. IAAE Triennial Conference, Foz do Iguaçu, Brazil, 18-24 august 2012. [http://purl.umn.edu/125946].

Michodjehoun-Mestres, L., Souquet, J.-M., Fulcrand, H., Bouchut, C., Reynes, M., Brillouet, J.-M., 2009. Monomeric phenols of cashew apple (*Anacardium occidentale* L.). Food Chemistry 112, 851–857.

Milhome, M.A.L., Lima, C.G., Delima, L.K., Lima, F.A.F., Sousa, D.O.B., Nascimento, R.F., 2014. Occurrence of aflatoxins in cashew nuts produced in north-eastern Brazil. Food Control 42, 34–37.

Mitchell, J.D., Mori, S.A., 1987. The cashew and its relations (*Anacardium*: Anacardiaceae). Memoirs of the New York botanical garden, pp. 42-76.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010. Production of fumonisin B-2 and B-4 by *Aspergillus niger* on grapes and raisins. Journal of Agricultural and Food Chemistry 58, 954-958.

Molyneux, R.J., Mahoney, N., Kim, J.H., Campbell, B.C., 2007. Mycotoxins in edible tree nuts. International Journal of Food Microbiology 119, 72–78.

Moss, M.O., 1991. Economic importance of mycotoxins - recent incidence. International Biodeterioration 27, 195–204.

Mosseray, R., 1934. Les *Aspergillus* de la section "*Niger*" Thom & Church. La Cellule 43, 203-285.

Moussa, W., Ghazali, F.M., Jinap, S., Ghazali, H.M., Radu, S., 2013. Modelling growth rate and assessing aflatoxins production by *Aspergillus flavus* as a function of water activity and temperature on polished and brown rice. Journal of Food Science 78, M56-M63.

Msuya, E.E., Hisano, S., Nariu, T., 2008. Explaining productivity variation among smallholder maize farmers in Tanzania. World Congress of Rural Sociology Association, Goyang, Korea. 31p.

NAIP (National Agricultural Innovation Project), 2013. A value chain on cashew for domestic and export markets. India Council of Agricultural Research. Final report. Kerala, India. Available at http://naip.icar.org.in/naip/download/c2-205201.pdf.

Namdeo, N.A., Koulagi, K., Wader, L.K., 2007. Grade development and study of price-quality relationship of cashew nut in north district of Goa. Agricultural Economics Research Review 20, 171-176.

Napit, K.B., Norton, G.W., Kazmierczak, Jr R.F., Rajotte, E.G., 1988. Economic impacts of extension integrated pest management programs in several states. Journal of Economic Entomology 81, 251-256.

Nielsen, K.F., Mogensen, J.M., Johansen, M., Larsen, T.O., Frisvad, J.C., 2009. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Analytical and Bioanalytical Chemistry 395, 1225-42.

Nielsen, K.F., Ngemela, A.F., Jensen, L.B., de Medeiros, L.S., Rasmussen, P.H., 2015. UHPLC-MS/MS determination of ochratoxin A and fumonisins in coffee using QuEChERS extraction combined with mixed-mode SPE purification. Journal of Agriculture and Food Chemistry 63, 1029-1034.

Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C., Samson, R.A., 2009. Fumonisin B2 production by *Aspergillus niger* in Thai coffee beans. Food Additives and Contaminants: Part A 26, 94-100.

Noonim, P., Mahakarnchanakul, W., Varga, J., Frisvad, J.C., Samson, R.A., 2008. Two novel species of *Aspergillus* section *Nigri* from Thai coffee beans. International Journal of Systemic and Evolutionary Microbiology 58, 1727-1734.

Nørholm, M.H., 2010. A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. BMC Biotechnology 10, 21.

O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7, 103-116.

Ojelade, K.T.M., 1998. Review of twenty years of cashew (*A. occidentale*, L.) entomology in Nigeria. Nigeria Journal of Tree Crop Research 2, 80-91.

Oliveri, C., Torta, L., Catara, V., 2008. A polyphasic approach to the identification of ochratoxin A-producing black *Aspergillus* isolates from vineyards in Sicily. International Journal of Food Microbiology 127, 147-154.

Orwa, C., Mutua, A.; Kindt, R., Jamnadass, R., Simons, A., 2009. Agroforestry database: a tree reference and selection guide version 4.0. Available at: http://www.worldagroforestry.org/treedb2/AFTPDFS/Anacardium_occidentale.pdf.

Ostry, V., Malir, F., Ruprich, J., 2013. Producers and important dietary sources of ochratoxin A and citrinin. Toxins 5, 1574–1586.

Otsuki, T., Wilson, J.S., Sewadeh, M., 2001. Saving two in a billion: a case study to quantify the trade effect of European food safety standards on African exports. Development Research Group (DECRG), the World Bank. Food Policy.

Pacheco, A.M., Martins, M., 2013. Brazil nut sorting for aflatoxin prevention: a comparison between automatic and manual shelling methods. Food Science and Technology (Campinas) 33, 369-375.

Palumbo, J.D., O'keeffe, T.L., Ho, Y.S., Santillan, C.J., 2015. Occurrence of ochratoxin A contamination and detection of ochratoxigenic *Aspergillus* species in retail samples of dried fruits and nuts. Journal of Food Protection 78, 836-842.

Papademetriou, M.K., Herath, E.M., 1998. Integrated production practices in cashew in Asia. FAO/RAP Publication: 1998/12. FAO Regional Office for Asia and the Pacific. BANGKOK, THAILAND. At http://www.fao.org/docrep/005/ac451e/ac451e00.htm#Contents.

Pardo, E., Marin, S., Sanchis, V., Ramos, A.J., 2005. Impact of relative humidity and temperature on visible fungal growth and OTA production of ochratoxigenic *Aspergillus ochraceus* isolates on grapes. Food Microbiology 22, 383– 389.

Pařenicová, L., Skouboe, P., Frisvad, J., Samson, R.A., Rossen, L., ten Hoor-Suykerbuyk, M., Visser, J., 2001. Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species. Applied and Environmental Microbiology 67, 521-527.

Parra, R., Magan, N., 2004. Modelling the effect of temperature and water activity on growth of *Aspergillus niger* strains and applications for food spoilage moulds. Journal of Applied Microbiology 97, 429-438.

Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Applied Environmental Microbiology 72, 680-685.

Pitt, J.I., Hocking, A.D., 1997. Fungi and food spoilage. (2nd Ed.). Blackie Academic Press, London.

Pitt, J.I., Hocking, A.D., 2009. Fungi and food spoilage. (3rd Ed.). Springer, USA.

Plaza, P., Usall, J., Teixido, N., Vinas, I., 2003. Effect of water activity and temperature on germination and growth of *Penicillium digitatum*, *P. italicum* and *Geotrichum candidum*. Journal of Applied Microbiology 94, 549–554.

Prabhakaran Nair, K.P., 2010. Cashew nut (*Anacardium occidentale* L.). The Agronomy and Economy of Important Tree Crops of the Developing World, 21-66.

Purcell, S.L., Phillips, D.J., Mackey, B.E., 1980. Distribution of *Aspergillus flavus* and other fungi in several almond-growing areas of California. Phytopathology 70, 926–929.

Raper, K., Fennell, D.I., 1965. The genus *Aspergillus*. Baltimore, MD, USA: Williams and Wilkins. 686p.

Reddy, B.N., Raghavender, C.R., 2007. Outbreaks of aflatoxicoses in India. African Journal of Food, Agriculture, Nutrition and Development 7.

Reis, T.A., Oliveira, T.D., Baquião, A.C., Gonçalves, S.S., Zorzete, P., Corrêa, B., 2012. Mycobiota and mycotoxins in Brazil nut samples from different states of the Brazilian Amazon region. International Journal of Food Microbiology 159, 61–68.

Rico-Sole, R., 2012. Economic impact of mycotoxins in nuts and dried fruit chain. Acta Horticulturae 963, 155-172.

Rodrigues, P., Venancio, A., Lima, N., 2012. Mycobiota and mycotoxins of almonds and chestnuts with special reference to aflatoxins. Food Research International 48, 76–90.

Rosengarten, Jr F., 2004. The book of edible nuts. Courier Corporation. Dover Publications. 416p.

Ruben, R., van Tilburg, A., Trienekens, J., van Boekel, M., 2007. Linking market integration, supply chain governance, quality, and value added in tropical food chains. In: Ruben, R., van Boekel, M., van Tilburg, A. and Trienekens, J. (eds.) T*ropical food chains: governance regimes for quality management*. Wageningen Academic Publishers, Wageningen, the Netherlands, pp. 13-46.

Sakuda, S., Kimura, M., 2010. Toxins of microorganisms. Comprehensive natural products II. Chemistry and Biology 4, 411–455.

Samen, S., 2010. A primer on export diversification: key concepts, theoretical underpinnings and empirical evidence, growth and crisis unit, World Bank, Washington, DC.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O., 2002. Introduction to food and airborne fungi. (6th Ed.). Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M., Frisvad, J.C., 2004. New ochratoxin or *sclerotium* producing species in *Aspergillus* section *Nigri*. Studies in Mycology 50, 45–61.

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black aspergilli. Studies in Mycology 59, 129–145.

Samson, R.A., Visagie, C.M., Houbraken, J., Hong, S.B., Hubka, V., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Susca, A., Tanney, J.B., Varga, J., Kocsube, S., Szigeti, G., Yaguchi, T., Frisvad, J.C., 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Studies in Mycology 78, 141–173.

SAS Institute Inc., 2011. STAT Software, version 9.3.

Schmale, D.G., Munkvold, G.P., 2009. Mycotoxins in crops: A threat to human and domestic animal health. APSnet. DOI: 10.1094/PHI-I-2009-0715-01. Assessed on 03\10\2017 (https://www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/default.aspx).

Schuster, E., Dunn-Coleman, N., Frisvad, J., van Dijck, P., 2002. On the safety of *Aspergillus niger* – a review. Applied Microbiology and Biotechnology 59, 426-435.

Schweiggert, R.M., Vargas, E., Conrad, J., Hempel, J., Gras. C.C., Ziegler, J.U., Mayer, A., Jiménez, V., Esquivel, P., Carle, R., 2016. Carotenoids, carotenoid esters, and anthocyanins of yellow-, orange-, and red-peeled cashew apples (*Anacardium occidentale* L.). Food Chemistry 200, 274-282.

Scott, W.J., 1957. Water relations of food spoilage microorganisms. Advances in Food Research 7, 83-127.

Shakerardekani, A., Karim, R., Mirdamadiha, F., 2012. The effect of sorting on aflatoxin reduction of pistachio nuts. Journal of Food, Agriculture and Environment 10, 459-461.

Shephard, G.S., 2004. Mycotoxins worldwide: current issues in Africa. In: Barug, D., Van Egmond, H., Lopez-Garcia, R., Van Ossenbruggen, T. and Visconti, A. (eds.) *Meeting the Mycotoxin Menace*. Wageningen Academic, Wageningen, pp. 81–88.

Škrbić, B., Živančev, J., Godula, M., 2014. Multimycotoxin analysis of crude extracts of nuts with ultra-high performance liquid chromatography/tandem mass spectrometry. Journal of Food Composition and Analysis 34, 171-177.

Souza, K.O., Viana, R.M., Oliveira, L de S., Moura, C.F.H., Miranda, M.R.A., 2016. Preharvest treatment of growth regulators influences postharvest quality and storage life of cashew apples. Scientia Horticulturae 209, 53-60.

Storari, M., Bigler, L., Gessler, C., Broggini, G., 2012. Assessment of the ochratoxin A production ability of *Aspergillus tubingensis*. Food Additives and Contaminants: Part A 29, 1450-1454.

Suleiman, M.N., 2010. Occurrence and distribution of fungi associated with bio-deterioration of cashew nuts in the eastern senatorial district, Kogi State, Nigeria. Archives of Applied Science Research 2, 462-465.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725-2729.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673-4680.

Tolosa, J., Font, G., Manes, J., Ferrer, E., 2013. Nuts and dried fruits: Natural occurrence of emerging *Fusarium* mycotoxins. Food Control 33, 215-220.

Topper, C.P., 2002. Issues and constraints related to the development of cashew nuts from five selected African countries. International Trade Centre (ITC), Common Fund for Commodities (CFC), Reunion Regionale sur le Developpement des Exportations de noix de Cajou d'Afrique, Cotonou, Bénin, pp.1-24.

Turner, N.W., Subrahmanyam, S., Piletsky, S.A., 2009. Analytical methods for determination of mycotoxins: A review. Analitica Chimica Acta 632**,** 168-180.

UNECE standard DDP-17, 2002. Concerning the marketing and the commercial quality control of cashew kernels. Ed. 2002. New York and Geneva, United Nations.

UNIDO, 2011. Tanzania's cashew value chain: A diagnostic. United Nations Industrial Development Organization (UNIDO). Vienna, Austria.

USAID (United States Agency for International Development), 2007. Cashew processing, marketing and consumption in West Africa. Current status and opportunities. WATH/Accra Technical Report No. 22.

USDA (United States Department of Agriculture), 2014. Benin: Agricultural situation, Global Agricultural Information Network Report. [02 November 2016] at http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Sit...

USDA (United States Department of Agriculture), 2015. National nutrient database for standard reference release 27, Basic Report: 12087, Nut, cashew nuts, raw [Internet]. http://ndb.nal.usda.gov/ndb/foods/show/3677?fgcd=Nut+and+Seed+Products&manu=&lfacet=&format=&count=&max=35&offset=35&sort=&qlookup=.

Vaamonde, G., Patriarca, A., Pinto, V.F., Comerio, R., Degrossi, C., 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *flavi* from different substrates in Argentina. International Journal of Food Microbiology 88, 79-84.

Van Egmond, H.P., Jonker, M.A., 2008. Regulations and limits for mycotoxins in fruits and vegetables. In: Barkai-Golan, R. and Paster, N. (eds) *Mycotoxins in Fruits and Vegetables*. Academic Press Publications, San Diego, USA, pp. 45-74.

Van Egmond, H.P., Schothorst, R.C., Jonker, M.A., 2007. Regulations relating to mycotoxins in food. Analytical and Bioanalytical Chemistry 389, 147-157.

Varga, J., Frisvad, J.C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., Samson, R.A., 2011. New and revisited species in *Aspergillus* section *Nigri*. Studies in Mycology 69, 1-17.

Varga, J., Frisvad, J.C., Samson, R.A., 2011. Two new aflatoxin producing species and an overview of *Aspergillus* section *Flavi*. Studies in Mycology 69, 57-80.

Varga, J., Kevei, F., Hamari, Z., Tóth, B., Téren, J., Croft, J.H., 2000. Genotypic and phenotypic variability among black aspergilli. *Integration of modern taxonomic methods for Penicillium and Aspergillus classification*. Amsterdam, the Netherlands: Harwood Academic, pp. 397-411.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Toth, B., Bartok, T., 2010. Fumonisin contamination and fumonisin producing black aspergilli in dried vine fruits of different origin. International Journal of Food Microbiology 143, 143-149.

Vargas, E.A., dos Santos, E.A., Whitaker, T.B., Slate, A.B., 2011. Determination of aflatoxin risk components for in-shell Brazil nuts. Food Additives and Contaminants 28, 1242-1260.

Varoglu, M., Corbett, T.H., Valeriote, F.A., Crews, P., 1997. Asperazine, a selective cytotoxic alkaloid from a sponge-derived culture of *Aspergillus niger*. Journal of Organic Chemistry 62, 7078-7079.

Wang, J., Jiang, Z., Lam, W., Gullen, E.A., Yu, Z., Wei, Y., Wang, L., Zeiss, C., Beck, A., Cheng, E.C., Wu, C., 2015. Study of malformin C, a fungal source cyclic pentapeptide, as an anti-cancer drug. PloS one 10:e0140069.

Wezel, A., Bohlinger, B., Bocker, R., 1999. Vegetation zones in Niger and Benin: present and past zonation. In: Herrmann, K., Vennemann, K., Strahr, K. and Von Oppen, M. (eds.) *Agricultural Atlas of natural and agronomic resources of Benin and Niger*. Hohenheim. SFB308. at: https://www.uni-hohenheim.de/atlas308/a_overview/a3_1/html/english/nframe.htm) (14\03\2014).

Whitaker, T.B., Slate, A.B., Doko, B., Maestroni, B., Cannavan, A., 2010. Sampling procedures to detect mycotoxins in agricultural commodities. Springer, New York, NY, USA.

Wild, C.P., Hudson, G.J., Sabbioni, G., Chapot, B., Hall, A.J., Wogan, G.N., Whittle, H., Montesano, R., Groopman, J.D., 1992. Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. Cancer Epidemiology Biomarkers and Prevention 1, 229-234.

Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R., Munkvold, G., Paul, P., Payne, G., Takle, E., 2011. Climate change impacts on mycotoxin risks in US maize. World Mycotoxin Journal 4, 79-93.

Yang, J., Li, J., Jiang, Y., Duan X., Qu, H., Yang, B., Chen, F., Sivakumar, D., 2014. Natural occurrence, analysis, and prevention of mycotoxins in fruits and their processed products. Critical Reviews in Food Science and Nutrition 54, 64-83.

Zaied, C., Abid, S., Bouaziz, C., Chouchane, S., Jomaa, M., Bacha, H., 2010. Ochratoxin A levels in spices and dried nuts consumed in Tunisia. Food Additives and Contaminants: Part B 3, 52-57.

Zain, M.E., 2011. Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society 15, 129-144.

Summaries

in English and French

Summary

Cashew nut is a cash crop in tropical regions of Africa including Benin, providing substantial revenues to small-scale farmers and contributing significantly to the national gross domestic product. At present, most African countries producing raw cashew nuts represent up to 35% of raw material for most processing companies located is Asia. In Beninese context, about 95% of raw cashew nuts are exported. Therefore, the production of raw cashew nuts of good quality is mandatory to enter the international market. Moreover, health issues related to the consumption of nuts contaminated with mycotoxins are of high concern for human being. The objective of this research was to investigate the occurrence of mycotoxins and other secondary metabolites of *Aspergillus* sp. on raw cashew nuts and to evaluate farmers' practices that affect the quality of raw nuts. The specific objectives were: (i) to evaluate the occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels; (ii) to investigate the diversity in secondary metabolites including mycotoxins from strains of *A.* section *Nigri* (black aspergilli) isolated from raw cashew nuts; (iii) to assess the influence of sorting and grading on quality parameters of raw cashew nuts and (iv) to study the impact of small-scale farmers' practices on the quality of raw cashew nuts.

Chapter 1 formulated the justification and the relevance of the research, its objectives and outline. It provided general information about the cashew tree, the cashew nut, the economic importance of cashew worldwide and in Benin, and the parameters used to assess the quality of cashew nuts intended for the international market. Furthermore, a summary on the mycotoxins associated with cashew nuts namely aflatoxins and ochratoxin A was provided together with the regulations on quality and safety of cashew nuts.

Chapter 2 describes the investigation of the occurrence of fungal contamination on raw cashew kernels focusing on two main sections of the genus *Aspergillus*: *Flavi* and *Nigri*. Also the amounts of aflatoxin in raw kernels and some quality parameters applicable at the small-scale farmer's gate such as the nut count and the water content, were evaluated. Two culturing methods were used to evaluate the occurrence of *A.* section *Flavi* and *A.* section *Nigri* on raw cashew kernels from the two agro-ecological zones of Benin: the northern guinea (NG) and the southern sudanian (SS) zones. In surface disinfected samples 90.2% of raw kernels were contaminated by strains of *A.* section *Nigri* in the NG zone compared to 87.2% in the SS zone. The level of contamination of raw cashew kernels by strains of *A.* section *Flavi* was 6.7% in the NG zone whereas it was 4.6% in the SS zone. When non disinfected kernels were plated, *A.* section *Nigri* was predominant in both NG and SS zones, with percentages of 89.7% and 93.4%, respectively. Ten and 15.7% of raw cashew kernels were contaminated by strains of *A.* section *Flavi* in NG and SS zones, respectively. *A. flavus, A. tamari, A. costaricaensis, A. minisclerotigenes* and *A. nomius* were identified as strains in *A.* section *Flavi*.

The average water content and the cashew nut count were respectively 8.6% and 172 nuts/kg in the NG zone and 8.7% and 174 nuts/kg in the SS zone. Significant differences between villages in both zones were found for both water content and nuts count. The occurrence of aflatoxins, analysed by ultra-high performance liquid chromatography tandem mass spectrometry, showed that none of the analysed samples was positive for natural occurrence of aflatoxins with a detection limit of 0.05 - 0.2 µg/kg. All the above results showed that, at present, cashew nuts from Benin were in the range of good quality for export.

The level of contamination by strains of *A.* section *Nigri* recorded in raw cashew nuts underlined the need to investigate on possible mycotoxins and other secondary metabolites produce by these strains (**Chapter 3**). This investigation was possible with the use of polyphasic taxonomic approach including (i) morphological description, (ii) physiological and biochemical

characteristics of the isolates using the liquid chromatography high resolution mass spectrometry, (iii) and confirmation by DNA sequences analysis based on β-tubulin and calmodulin genes. Seven species of black aspergilli were isolated based on morphological and chemical identification namely *A. tubingensis* (44% of total strains), *A. niger* (with a chemical profile similar to *A. welwitschiae*) (32%), *A. brasiliensis* (10%), *A. carbonarius* (8.7%), *A. luchuensis* (synonym to *A. kawachi* or *A. acidus*) (2.7%), *A. aculeatus* (2%) and *A. aculeatinus* (0.7%). Forty five different metabolites and their isomers were identified and classified in 2 groups. Group A of known mycotoxins: fumonisin B_2, fumonisin B_4, ochartoxin B, ochratoxin A and secalonic acid A, secalonic acid D and secalonic acid F. Group B included other secondary metabolites like nigragillin, pyranonigrin A, nigerazine A, nigerazine B, nigerapyrone E, tensyuic acid A, tensyuic acid F, pyranonigrin B, pyranonigrin C, pyranonigrin D, fonsecin, tensidol A, pyrophen, atromentin, tensyuic acid B, funalenone, rubrofusarin, orlandin, asperazine, tensyuic acid C, tensyuic acid D, nigerasperone A, tensidol B (pestalamide A), fonsecin B, malformin A_2, tubingensin A, tubingensin B, malformin C, kotanin, nominine, antafumicin A, antafumicin B, aurasperone C, aurasperone F, aurasperone E, aurasperone B, flavasperone and nafuredin. DNA analysis resulted in a phylogenetic trees based on combined sequences data of β-tubulin and calmodulin of 27 strains of *A.* section *Nigri* that clustered in 5 clades. Also, the chemical profile clustering showed also 5 groups confirming the species specific metabolites production. Even though the presence of fungi has not always meant the presence of mycotoxins, the production of ochratoxin A and fumonisins by isolates on *A.* section *Nigri* could constitute an additional and hidden problem in term of mycotoxins content, and can negatively affect the safety and nutritional quality of cashew nuts.

Chapter 4 assessed the influence of sorting and grading of raw cashew nuts as low cost technology applicable at small-scale famers' level to augment the score of customary quality parameters. Sorting and grading resulted in two grades. First (good) grade (Grade 1) consisted of nuts with less than 50% of their shell surface affected by fungi and/or insect injuries, whereas second (bad) grade

(Grade 2) were nuts that had 50% or more of their shell surface covered by fungi and/or insect symptoms. Nut counts in Grade 1 were from 151 to 174 nuts/kg and from 142 to 182 nuts/kg in NG and SS, respectively. In Grade 2, nut counts were from 168 to 202 nuts/kg in NG and from 171 to 197 nuts/kg in SS. We recorded water content of 8.6% for both Grade 1 and Grade 2 in NG and significant difference was noticed in average water content in SS where it was 9.7% and 6.4% for Grade 1 and Grade 2, respectively. For fungal contamination, 42.9% and 50.0% of samples showed significant differences between Grade 1 and Grade 2 for contamination by *A.* section *Flavi* and *A.* section *Nigri*, respectively, under surface disinfection method. Non-disinfected samples showed the same significant difference for 35.7% of *A.* section *Flavi* contaminated nuts and 28.6% of *A.* section *Nigri* contaminated nuts. These results indicated that sorting and grading resulted in Grade 1 of better quality with high nut count, and low fungal contamination. These quality parameters are improved and therefore can have a positive impact on the marketability of raw cashew nuts, increasing the potential profit of small-scale farmers.

The research described in **Chapter 5** evaluated the impact of farm management practices on the quality of raw cashew nuts, in order to enable the implementation of good agricultural and good harvesting practices that may improve the quality of the raw cashew nut. Interviews revealed the dominance of men (95%) in cashew nut cultivation indicating that it as a gendered activity. Cashew nut farming is labour intensive with 55% of farmers in the range of 35 to 55 years old. Unfortunately, most cashew farmers were less educated with up to 76% not exceeding primary educational level, making the educational level of farmers to be the main constrain for the production of cashew nut of good quality. This resulted of 80% of cashew farmers to select their seedlings from uncertified sources and 59% to never ask or received advice from extension services about cashew farming practices. Cashew nut farmers reported that cashew revenue contributed significantly to the household income for 44% and 54% of farmers in the NG and the SS zone, respectively. When farmers had received advice on farming practices through extension services, a significant positive impact on the estimated yield in the SS zone was observed. Other

constrains for good cashew nut production were lack of sorting, drying and farm management. Farmers reported neither to dry (74% in NG and 94% in SS) nor to sort (80% in NG and 94% in SS) their harvested nuts. Orchard area, orchard age, nut storage duration and origin of seedlings all had a significant impact on the household income of cashew nut farmer. The farmer's education level, limited access to extension services, lack of sorting, drying and farm management are major parameters that affect the quality of raw cashew nuts. Improvement of these parameters can positively affect raw nut quality and therefore contribute to better revenue from raw cashew production.

Chapter 6 presents a general discussion of results, the methodological limits together with concluding remarks on how far the thesis has realised its objectives. Furthermore, recommendations are given for production of better raw cashew nuts. Together with good farming and post-harvest management practices that, the implementation of specific extension service for cashew growers and an incentive for sorting and grading as low cost technology, will impact the quality parameters of raw cashew nuts intended for export from Benin.

Résumé

La noix de cajou est une culture des régions tropicales d'Afrique, y compris le Benin, fournissant de revenus substantiels aux petits producteurs et contribuant de manière significative au produit intérieur brut du pays. De nos jours, la plupart des pays africains producteurs de noix de cajou brutes, fournissent jusqu'à 35% de la matière première aux entreprises de transformation des noix de cajou situées en Asie. Au Benin, environ 95% des noix de cajou brutes sont exportés vers l'extérieur. Par conséquent, une production de noix de cajou de bonne qualité est indispensable pour que la noix de cajou du Benin puisse être mise sur le marché international. De plus, les problèmes liés à la consommation des denrées contaminées par les mycotoxines est une préoccupation majeure pour la sante publique. L'objectif de cette étude a été d'évaluer la présence des mycotoxines et d'autres métabolites secondaires produites par les *Aspergilli* sur les noix de cajou brutes et d'évaluer les pratiques agricoles qui affectent la qualité des noix. Les objectifs spécifiques ont été de (i) évaluer la présence des souches de *Aspergillus* section *Flavi* et *A.* section *Nigri* et des aflatoxines dans les noix de cajou brutes, (ii) étudier la diversité des métabolites secondaires, y compris les mycotoxines provenant des souches de *A.* section *Nigri* isolées a partir des noix de cajou brutes, (iii) évaluer l'influence du triage et du calibrage des noix de cajou sur les paramètres de qualité des noix brutes, (iv) évaluer l'impact des pratiques agricoles des petits producteurs sur la qualité des noix de cajou.

Le **Chapitre 1** formule la justification et la pertinence, les objectifs et les grandes lignes de la recherche. Les informations d'ordre général sur l'arbre de cajou et la noix de cajou, l'importance économique de la noix de cajou dans le monde et au Benin, et les paramètres utilisés pour évaluer la qualité des noix de cajou destinées au marché international ont été évoqués. En outre, un aperçu général sur les mycotoxines associées aux noix de cajou, en particulier les aflatoxines et l'ochratoxine A a été fourni ensemble avec la règlementation internationale sur la qualité et la sécurité des noix de cajou.

Dans le **Chapitre 2**, la contamination par les champignons des noix de cajou brutes a été déterminée en se focalisant sur deux sections principales du genre *Aspergillus* à savoir les sections *Flavi* et *Nigri*. De même, le niveau d'aflatoxine dans les noix brutes et certains paramètres de qualité applicables au niveau des petits producteurs de noix de cajou, a savoir le comptage des noix et le taux d'humidité ont été évalués. Les noix de cajou ont été échantillonnées dans deux zones agro-écologiques du Benin: la savane Guinéenne du Nord (NG) et la savane Soudanienne du Sud (SS). Deux méthodes de culture à savoir la désinfection des noix et la culture directe sur milieu de culture ont été utilisées au laboratoire pour évaluer la présence des souches de *A.* section *Flavi* et *A.* section *Nigri* sur les noix de cajou. Dans le cadre de la désinfection des noix, 90,2% des échantillons de noix de cajou de la zone NG ont été contaminés par les souches de *A.* section *Nigri* comparés à 87,2% des échantillons de noix de cajou de la zone SS. Les niveaux de contamination des noix de cajou par les souches de *A.* section *Flavi* ont été de 6,7% et 4,6% respectivement dans les zones NG et SS. En culture directe des noix de cajou sur un milieu de culture, les souches de *A.* section *Nigri* ont été prédominantes dans les deux zones agro-écologiques NG et SS, avec respectivement 89,7% et 93,4% de noix contaminées. Dix et 15,7% des noix brutes ont été contaminées par les souches de *A.* section *Flavi* dans les zones NG et SS, respectivement. *A. flavus, A. tamari, A. costaricaensis, A. minisclerotigenes* et *A. nomius* ont été identifiés comme des espèces dans le groupe de *A.* section *Flavi*.

Le taux d'humidité moyen des noix et le comptage moyen des noix ont donné respectivement 8,6% et 172 noix/kg dans la zone NG et 8,7% et 174 noix/kg dans la zone SS. Des différences significatives ont été observées entre les villages dans les 2 zones pour ces 2 paramètres mesurés. L'analyse des aflatoxines a été réalisée par la chromatographie en phase liquide ultra haute performance couplée avec une double spectrométrie de masse. Cette analyse a révélé l'absence des aflatoxines dans les noix de cajou avec une limite de détection de 0,05 - 0,2 µg/kg. Tous les résultats susmentionnés ont montré que, à l'heure actuelle, les noix de cajou du Benin sont de bonne qualité et propice à l'exportation.

Les niveaux de contamination par les souches de *A.* section *Nigri* enregistrés sur les noix de cajou ont révélé la nécessité de rechercher la présence éventuelle des mycotoxines et d'autres métabolites secondaires produits par ces souches (**Chapitre 3**). Ces recherches ont été possible grace à l'utilisation de l'approche taxonomique polyphasique incluant (i) la description morphologique des souches, (ii) les caractéristiques physiologiques et biochimiques des isolats utilisant la chromatographie liquide en haute résolution couplée à une spectrométrie de masse, (iii) et la confirmation par l'analyse des séquences des ADN sur la base des gènes β-tubuline et calmoduline. Sept espèces de *A.* section *Nigri* ont été isolées sur la base de l'identification morphologique et biochimique: notamment *A. tubingensis* (44% des souches de *A.* section *Nigri* utilisées), *A. niger* (avec un profil chimique similaire à *A. welwitschiae*) (32%), *A. brasiliensis* (10%), *A. carbonarius* (8,7%), *A. luchuensis* (synonyme de *A. kawachi* ou *A. acidus*) (2,7%), *A. aculeatus* (2%) et *A. aculeatinus* (0,7%). Quarante-cinq différents métabolites et leurs isomères ont été identifiés et classés en 2 groupes: Le Groupe A comprenant les mycotoxines connues telles que fumonisine B_2, fumonisine B_4, ochartoxine B, ochratoxine A, acide sécalonique A, acide sécalonique D et acide sécalonique F; le Groupe B comprenant d'autres métabolites telles que nigragilline, pyranonigrine A, nigerazine A, nigerazine B, nigerapyrone E, acide tensyuic A, acide tensyuic F, pyranonigrine B, pyranonigrine C, pyranonigrine D, fonsecine, tensidol A, pyrophen, atromentine, acide tensyuic B, funalenone, rubrofusarine, orlandine, asperazine, acide tensyuic C, acide tensyuic D, nigerasperone A, tensidol B (pestalamide A), fonsecine B, malformine A_2, tubingensine A, tubingensine B, malformine C, kotanine, nominine, antafumicine A, antafumicine B, aurasperone C, aurasperone F, aurasperone E, aurasperone B, flavasperone et nafuredine. L'analyse des séquences d'ADN a révélé un arbre phylogénétique basé sur les données séquentielles combinées de β-tubuline et calmoduline de 27 isolats de *A.* section *Nigri* regroupées en 5 clades ou groupes. De même, le regroupement par profil chimique de ses isolats a montré aussi 5 groupes confirmant ainsi l'effet de production spécifique des métabolites des souches. Même si la présence de champignon ne signifie pas toujours la présence de mycotoxine, la production de ochratoxine A et

des fumonisines par les souches de *A.* section *Nigri* peut constituer un problème supplémentaire et non révélé en terme de teneur en mycotoxine, ce qui pourrait négativement impacter sur la sécurité et la qualité nutritionnelle des noix de cajou.

L'effet du triage et du calibrage des noix de cajou en tant que technologie à faible coût applicable au niveau des petits producteurs, a été évalué dans le **Chapitre 4,** dans le but d'élever le niveau des paramètres habituels de qualité. La méthode de triage et de calibrage a distingué 2 grades de noix de cajou: Le premier (bon) grade (appelé Grade 1) a regroupé les noix de cajou ayant moins de 50% de la surface de leur coque couverte des dommages des champignons et/ou des blessures d'insectes, alors que le second (mauvais) grade (Grade 2) a regroupé les noix avec 50% ou plus de la surface de leur coque couverte par des dommages des champignons et/ou des blessures d'insectes. Le comptage des noix du Grade 1 s'est situé dans une marge de 151 à 174 noix/kg et de 142 à 182 noix/kg respectivement pour les zones NG et SS. Dans le Grade 2, le comptage des noix s'est situé dans une marge de 168 à 202 noix/kg pour la zone NG et de 171 à 197 noix/kg pour la zone SS. Un taux moyen d'humidité de 8,6% a été enregistré sur les noix de cajou aussi bien de Grade 1 que de Grade 2 de la zone NG. Dans la zone SS une différence significative a été observée entre les taux moyens d'humidité des noix de Grade 1 (9,7%) et les noix de Grade 2 (6,4%). Pour la contamination par des champignons, 42,9% et 50% des échantillons de noix de cajou ont montré une différence significative entre les Grade 1 et Grade 2 pour la contamination par les souches de *A.* section *Flavi* et *A.* section *Nigri*, respectivement avec la méthode de désinfection des noix. Avec la méthode de culture directe sur milieu de culture, les mêmes différences significatives ont été observées pour 35,7% des noix contaminées par *A.* section *Flavi* et 28,6% des noix contaminées par *A.* section *Nigri*. Ces résultats indiquent que le triage et le calibrage de noix de cajou ont révélé des noix de Grade 1 de bonne qualité avec un nombre élevé de noix par kg, et un taux réduit de contamination par les champignons. Ces paramètres de qualité de noix de cajou ont été améliorés et par conséquent peuvent avoir un impact positif sur la valeur

marchande des noix brutes, valorisant de ce fait le potentiel du profit pour les petits producteurs des noix de cajou.

Le travail décrit au **Chapitre 5** a eu pour objectif d'évaluer l'impact des pratiques agricoles sur la qualité des noix de cajou, dans le but de permettre la mise en œuvre des bonnes pratiques agricoles et des bonnes pratiques post-récoltes susceptibles d'améliorer la qualité des noix de cajou brutes. L'interview a révélé la domination des hommes (95%) dans les cultures de noix de cajou indiquant de ce fait que c'est une activité liée au genre. La culture des noix de cajou est une activité exigeant une main-d'œuvre importante, 55% des producteurs ont entre 35 et 55 ans. Malheureusement, la majorité des producteurs de noix de cajou avaient un niveau d'éducation bas, avec jusqu'à 76% n'ayant jamais dépassé le niveau d'éducation primaire; ceci fait du niveau d'éducation des agriculteurs la principale contrainte pour la production de noix de cajou de bonne qualité. Par conséquent, 80% des producteurs s'orientent vers des semences non certifiées pour leur culture et 59% n'ont jamais eu recours aux conseils des services de vulgarisation sur les bonnes méthodes de culture des noix de cajou.

Les producteurs de noix de cajou ont rapporté que les revenus provenant de la noix de cajou ont contribué de manière significative au revenu total de leur ménage pour 44% dans la zone NG et pour 54% la zone SS. Lorsque les producteurs de noix de cajou rapportent avoir reçu des conseils sur les bonnes pratiques agricoles à travers les services de vulgarisation, un impact positif significatif sur les rendements estimés a été observé dans la zone SS.

D'autres contraintes pour une bonne production de noix de cajou ont été l'absence du triage, l'absence de séchage et l'absence d'une bonne gestion agricole. Les producteurs ont déclaré ne pas sécher (74% dans la zone NG et 94% dans la zone SS), ni trier (80% dans la zone NG et 94% dans la zone SS) leur récoltes de noix de cajou. La superficie des vergers, l'âge des vergers, la durée de stockage des noix de cajou et l'origine des semences ont tous eu une incidence significative sur le revenu des producteurs de noix de cajou. Le niveau d'éducation des producteurs,

l'accès limité aux services de vulgarisation, l'absence de triage, l'absence de séchage et l'absence d'une bonne gestion agricole ont été des paramètres importants ayant affecté la qualité des noix de cajou brutes. L'amélioration de ces paramètres peut entrainer une incidence positive sur la qualité des noix brutes et contribuer ainsi à l'amélioration des revenus provenant de la production de noix de cajou.

Enfin, le **Chapitre 6** présente une discussion générale des résultats, les limites de la recherche ainsi que des remarques sur comment la thèse a atteint ses objectifs. En outre, des recommandations ont été faites dans le but de la production de noix de cajou de meilleure qualité. Ajouté aux bonnes pratiques agricoles et de post-récolte, la mise en place des services de vulgarisation spécifiques à la production de noix de cajou et une incitation au triage et au calibrage comme technologie à bas coût accessible à tous les producteurs, impacteront significativement les paramètres de qualité des noix de cajou du Benin, destinées à l'exportation.

Acknowledgements

Acknowledgements

A PhD research project is a journey that is sometimes very hectic but full of experiences. This journey would not be possible to accomplish without guidance, help and support from many people. First and foremost, my utmost gratitude is extended to my promotor, co-promotors and supervisors, namely: Prof. Dr. Tiny van Boekel, Dr. Anita Linnemann, Dr. Rob Nout, Prof. Dr. Eddy Smid, Dr. Kerstin Hell and Dr. Manuele Tamo. Your scientific rigor, constructive criticisms, deepest comments, our fruitful discussions both in Wageningen and in Benin were very valuable. Individually or as coaching team, I really appreciated your professional guidance, intellectual input, encouragement, patience and moral support.

Dear Kerstin, you were the first who believed in this project, and I really appreciated your initiative to travel to Wageningen University and to introduce and discuss the concept note that I've written about this research. Special thanks.

Prof. Kristian fog Nielsen, I really appreciated your enthusiastic collaboration on this cashew nut project through MycoRed sponsorship. It gave me the opportunity, to discover the Denmark and its warm-hearted people and to learn important techniquess and useful software to perform part of this research. I appreciated the coaching of Prof. Jens Christian Frisvad for the identification of fungi. My sincere gratitude is extended to Dr. Jakob Blæsbjerg Nielsen and Dr. Yuksel Gezgin for their useful coaching during the DNA sequencing.

I would like to express my greatest gratitude to some institutions/departments that, in one way or another contributed in the accomplishment of this research project:

- The International Institute of Tropical Agriculture, especially its branch of Cotonou, Benin;

- The Wageningen University & Research, especially the Food Quality and Design group (FQD) and the Laboratory of Food Microbiology (FHM);

- The Technical University of Denmark, especially the Institute for System Biology and its Metabolomics Platform;

- The European Commission project called MycoRed (EC-KBBE-2007-222690-2);

- Landbouw Export Bureau (LEB) Foundation, the Netherlands (2012-0190B).

I am thankful to Marion Rodenburg (WUR), Ina Dombeek, Lysanne Hoksbergen and Kimberly Boss (Secretaries of FQD, WUR), Andre Hessouh (IITA-Benin) for providing administrative support.

During this research, sampling and data collection were possible with the help of the local population from Benin: Districts of Alafiarou, Bante, Birni, Chabikouma, Ina, Kilibo, Kolokonde, Nagayile, Patargo, Penessoulou, Pira, Tchaourou, Tchatchou and Toui. I acknowledge here your valuable collaboration. I am thankful to Claude Gande who was of great assistance during the laboratory work in Benin.

To my colleagues and friends at FQD/DTU and the "Beninese family": Daylan, Liya, Radhika, Grace, Marine, Probo, Fahui, Ita, Lesley, Augustine, Andreas, Jesper, Yann, Harold, Fernande, Nicodeme, Djalal, Sylvain, Folachode and Guillaume. Thanks for being there at some nice and hardest moments of my PhD. Most of all, thank you for the great moments and remarkable experience I had with some of you during our PhD trip to United Kingdom.

Special thanks go to my paranymphs Ayusta Fitriyono and Mostapha Zahir for your support during my preparation for PhD defence. Thank you for your great job.

Scientists, colleagues and friends from IITA-Benin: Peter Neuenschwander, Manuele Tamo, Georg Goergen, Ousmane Coulibaly, Muaka Toko, Desiré Gnanvossou, Georges Negloh, Djana Mignouna, Albert Tchamba, Rousseau Djouaka, Razack Adeoti, Brice Gbaguidi, Raymond Allomasso, Sounkoura Adetonah, Simon N'cho, Elie Dannon, Benjamin Datinon, Joelle Toffa.

Cyriaque Agboton, Maurille Elegbede, Miriam Karlsson, Djibril Aboubakar, Antonio Ognakossan, Claude Gande, Wilfried Allogni, Francois Onikpo, Mohamed Akadiri, Ben Azoma and Remy Ahoyo. I do remember each of your words of encouragement. Thanks.

My sincere gratitude is extended to the members of families Lamboni, Sodzi, Ketoglo, Kolani and Teby. Thanks for your encouragement.

I owe my greatest gratitude, with all my respect to my late parents: Mr. Damenang Lamboni (my father) and Mrs. Dembien Poguemin Siangou (my mother). You drove me to school and showed me the way to success. I am also thankful to my late uncle Lucien Yendouname Lamboni. Your support for my education and your encouragement for this PhD were effective. Unfortunately you passed away before the end. Father, mother, uncle, be proud of this achievement.

I would like to thank my brothers, sisters, cousins and friends: Lazare, Evelyne, Tanti, Nana, Sista, Emmanuel, Togbe, Severin, Totine, Victor and Blaise. Thank you all for your moral support and encouragement.

Last but not least, I owe my deepest gratitude to my lovely wife Therese Kouamba Sodzi. Dear Thea, this journey was also yours. You were present every single minute, during all lows and highs of this journey Thanks for your love, patience, courage, understanding, care and support. You deserve to this PhD. It is also yours.

To all, Thanks, Merci, Dank u wel, Akpé, Awànŭ, N'faa.

Leo Yendouban

About the Author:
Bibliography and Biography

Publications

Full papers

1- Kerstin Hell, **Yendouban Lamboni**, Thomas Houndékon and Guirguissou Maboudou Alidou, **2006**. Augmented release of *Teretrius nigrescens* Lewis (Coleoptera: Histeridae) for the control of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) in stored cassava chips. *Journal of Stored Products Research* 42, 367-376.

2- Samapundo, S., Devlieghere, F., De Meulenaer, B., **Lamboni, Y.**, Osei-Nimoh, D. and Debevere, J. M. **2007**. Interaction of water activity and bicarbonate salts in the inhibition of growth and mycotoxin production by *Fusarium* and *Aspergillus* species of importance to corn. *International Journal of Food Microbiology* 116, 266–274.

3- Samapundo, S., Devlieghere, F., De Meulenaer, B., Atukwase, A., **Lamboni, Y.** and Debevere, J. **2007**. Sorption isotherms and isosteric heats of sorption of whole yellow dent corn. *Journal of Food Engineering*, 79, 168-175.

4- Samapundo, S., De Meulenaer, B., Osei-Nimoh, D., **Lamboni, Y.**, Debevere, J. and Devlieghere, F. **2007**. Can phenolic compounds be used for the protection of corn from fungal invasion and mycotoxin contamination during storage? *Food Microbiology*, 24, 465–473.

5- M.T. Gueye, G. Goergen, D. Badiane, K. Hell & **L. Y. Lamboni 2008**. First report on occurrence of the larger grain borer *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) in Senegal. *African Entomology* 16(2): 309–311.

6- K. Hell, B.G.J. Gnonlonfin, G. Kodjogbe, **Y. Lamboni**, and I. K. Abdourhamane **2009**. Mycoflora and occurrence of aflatoxin in dried vegetables in Benin, Mali and Togo, West Africa. *International Journal of Food Microbiology* 135, 99-104.

7- **Lamboni Yendouban** and Kerstin Hell **2009**. Propagation of mycotoxigenic fungi in maize stores by post-harvest insects. *International Journal of Tropical Insect Science* 29 (1), 31–39.

8- Kukom Edoh Ognakossan, Agbéko Kodjo Tounou, **Yendouban Lamboni** and Kerstin Hell **2013**. Post-harvest insect infestation in maize grain stored in woven polypropylene and in hermetic bags. *International Journal of Tropical Insect Science*, 33 (01), 71-81.

9- Hell, K., K., Edoh Ognakossan, **Y., Lamboni 2014**. PICS hermetic storage bags ineffective in controlling infestations of *Prostephanus truncatus* and *Dinoderus* spp. in traditional cassava chips. *Journal of Stored Products Research*, 58, 53-58.

10- **Yendouban Lamboni**, Jens C. Frisvad, Kerstin Hell, Anita R. Linnemann, Eddy J. Smid, Rob M. J. Nout, Manuele Tamo, Kristian F. Nielsen, Martinus A. J. S. van Boekel **2016**. Occurrence of *Aspergillus* section *Flavi* and section *Nigri* and aflatoxins in raw cashew kernels (*Anacarduim occidentale* L.) from Benin. *LWT - Food Science and Technology*, 70, 71–77.

11- **Lamboni, Y.**, Nielsen, K. F., Linnemann, A. R., Gezgin, Y., Hell, K., Nout, M. J. R., Smid, E. J., Tamo, M., van Boekel, M. A. J. S., Hoof, J. B., & Frisvad, J. C. **(2016).** Diversity in Secondary Metabolites Including Mycotoxins from Strains of *Aspergillus* Section *Nigri* Isolated from Raw Cashew Nuts from Benin, West Africa. *PloS one*, *11*(10), e0164310.

Submitted papers

12- **Yendouban Lamboni**, Martinus J. R. Nout, Eddy J. Smid, Kerstin Hell, Manuele Tamo, Martinus A. J. S. van Boekel, Anita R. Linnemann. Influence of sorting and grading on nut count, water content and fungal contamination of raw cashew nuts (*Anacardium occidentale* L.).

13- **Yendouban Lamboni,** Kerstin Hell, Martinus JR Nout, Eddy J Smid, Manuele Tamo, Martinus AJS van Boekel Anita R Linnemann**.** Impact of Small-Scale Farmers' Practices on the Quality of Raw Cashew Nuts (*Anacardium occidentale* L.).

Proceedings

14- Hell, K., **Lamboni, Y.** and Cardwell, K. **2003**: Role of insects in the propagation of mycotoxigenic fungi in stores in Benin. In: Credland, P.F., D.M. Armitage, C.H. Bell, P.M. Cogan and E. Highly (eds): Advances in Stored Product Protection. *Proceedings of the 8th International Working Conference on Stored Product Protection, 22-26 July 2002, York, UK. CAB International, Oxon UK. 330-338.*

15- Mukandila K.P., Hell, K., Hauser, S., **Lamboni, Y**., Masimango, J.T. **2010**. Qualité des produits dérivés du manioc prélevés au niveau des sites de fabrication et dans les marchés de Kinshasa, RD Congo. *Proceedings. 11th ISTRC-AB Symp. Kinshasa, DR Congo 4-8 October, 2010.*

16- K. Hell, K. Edoh Ognakossan, A.K. Tonou, **Y. Lamboni**, K.E. Adabe, and O. Coulibaly **2012**. Maize stored pests control by PICS-bags: Technological and economic evaluation:

in: Innovative research along the cowpea value chain. 2012. *Proceedings of the Fifth World Cowpea Conference on Improving livelihoods in the cowpea value chain through advancement in science, held in Saly, Senegal, 27 September–1 October 2010,* edited by O. Boukar, O. Coulibaly, C.A. Fatokun, K. Lopez, and M. Tamò. IITA, Nigeria. 432 pp.

Books and thesis

17- **Yendouban Lamboni 2001**. Etude de la dynamique des populations des principaux coléoptères ravageurs des stocks de maïs (*Zea mays* L.) et estimation du rôle de ces ravageurs comme vecteurs des champignons nuisibles au maïs stocké dans les greniers. *Mémoire d'ingénieur agronome. ESA/UL, Lomé, Togo. 108p. (Agric. Engineer thesis)*

18- **Lamboni, Y.**, Fandohan, P., Hell, K., and Georgen, G. **2003**. Petit Manuel d'Identification des Principaux Ravageurs des Denrées Stockées en Afrique de l'Ouest. *IITA / INRAB / PTAA, Cotonou Bénin, 46p*.

19- **Yendouban Lamboni 2005**. Inhibition of growth and fumonisin production of *Fusarium verticillioides* and *F. proliferatum* on maize by bicarbonate salts and phenolic compounds. *Master's dissertation for the degree of Master of Science in Food Science and Technology; Gent University/KULeuven, Belgium. 92p.*

Posters

20- Hell, K., **Lamboni, Y**. and Cardwell, K. F. **2002**. Role of insects in the propagation of mycotoxigenic fungi in stores in Benin. *Poster presented during the 8th International conference on Stored Product Protection. 22nd - 26th July 2002. York, UK.*

21- Ognakossan, K.E., K.E. Adabe, K. Hell, **Y. Lamboni**, and O. Coulibaly **2010**. Use of PICS bags for the control of *P. truncatus* and *Dinoderus* spp. on stored cassava chips: First results. *Poster presented during the 5th World Cowpea Conference, 27 September to 1 October 2010, Saly, Senegal.*

22- Kukom Edoh Ognakossan, **Yendouban Lamboni** and Kerstin Hell **2012**. Control of *Prostephanus truncatus* and *Dinoderus* spp. In Stored Cassava Chips in Benin with Purdue Improved Cowpea Storage (PICS) – Bags. *Poster presented during Purdue Improved Crop Storage Workshop, 10 – 12 April 2012, Accra, Ghana.*

23- Klitgaard, A., Månsson, M., Gezgin, Y., **Lamboni, Y.**, and Nielsen, K. F. 2014. Study of the plasticity of secondary metabolites in the black Aspergilli using UHPLC-qTOF molecular networking. *Poster presentation at the 10th International Conference of the Metabolomics Society, Tsuruoka, Japon, June 23-26, 2014*.

Curriculum vitae

Leo Yendouban Lamboni is a Togolese, born on November 6th, 1972 in Lomé, Togo. He attended primary and secondary school in Togo and graduated from secondary school in 1994. At University of Lomé, he started his training at "Ecole Supérieure d'Agronomie" (ESA/UL) and graduated in November 2001 as Agricultural Engineer with a specialisation in Crop Protection and Production. His engineer dissertation was entitled "Study of the population dynamic of main Coleoptera of maize and estimation of the role of these pests as vectors of harmful fungi on maize stored in granaries". The research was performed at International Institute of Tropical Agriculture (IITA) located in Benin. From December 2001 to August 2013, he stayed as research assistant in Aflatoxin Laboratory, IITA-Benin. In September 2003, he was awarded a scholarship by the Flemish Interuniversity Council (VLIR-UOS) for an M.Sc. in Food Science and Technology jointly organised buy Ghent University (UGent) and Catholic University of Leuven (KU Leuven) in Belgium. The M.Sc. thesis was entitled "Inhibition of growth and fumonisin production of *Fusarium verticillioides* and *F. proliferatum* on maize by bicarbonate salts and phenolic compounds". Back to IITA-Benin, he worked as Research Assistant in the Department of Post-Harvest and Food Safety from 2005 to 2007 and since then as Research Associate in the Department of Agriculture and Health. In 2010 Leo Yendouban started his sandwich PhD degree with the Food Quality and Design group (FQD) and the Laboratory of Food Microbiology, Wageningen University, the Netherlands. The title of his project was "Raw cashew nut quality as function of contamination by mycotoxins and other secondary metabolites of *Aspergillus* spp. and farmer practices". His PhD was sponsored by Wageningen University & Research, Grant number P2688. The field work was partly sponsored by IITA-Benin. In 2012, he won a visiting scientist fellowship from MycoRed, an European commission project, for 3 months stay at the Institute for System Biology Centre of Technical University of Denmark (DTU). Also, in 2012, He was awarded a LEB foundation sponsorship for a trip to Bari, Italy. Again from October 2013 to July 2014, a joint collaboration with FQD allows him to stay at DTU System Biology for laboratory work. The current dissertation reports the results of his PhD research, which have also been published in peer-reviewed journals and presented at national and international scientific meetings.

Overview of Completed Training Activities

Overview of completed training activities

Activity	Graduate school / Institute	Year
DISCIPLINE SPECIFIC ACTIVITIES		
Course		
Advanced food analysis	VLAG, Wageningen	2010
Genetics and physiology of food associates microorganisms	VLAG. Wageningen	2010
Food fermentation	VLAG, Wageningen	2012
Detection techniques for mycotoxins in the food chain	ISM-Mycored-ISPA, Bari, Italy	2012
Reaction kinetics in food science	VLAG, Wageningen	2012
Food safety, quality assurances systems and risk analysis	Ghent University, VLIR-UOS, Belgium	2012
Analytical microbial natural product chemistry	DTU System Biology, Denmark	2014
Workshops, symposia, summer schools etc.		
Amélioration de la qualité des produits agricoles au Benin	IITA-Benin, Cotonou	2010
Etat des lieux des recherches récentes sur les filières ananas, karité, anacarde et crevette au Bénin	Project Nuffic/NTP 263, Benin	2011
GENERAL COURSES AND WORKSHOPS		
24th VLAG PhD Week	VLAG, Wageningen	2010
Information literacy including EndNote introduction	WUR, Wageningen	2010
Techniques of writing and presenting scientific paper	WUR, Wageningen	2012
Advanced course guide to scientific artwork	WUR, Wageningen	2012
Introduction to R for statistical analysis	CT de Wit and PE&RC, Wageningen	2012
Scientific publishing	WUR. Wageningen	2012
Project and time management	WGS, Wageningen	2012
OPTIONALS COURSES AND ACTIVITIES		
Writing VLAG research proposal	FQD, Wageningen	2010
PhD excursion of FQD to UK	FQD, Wageningen	2012

Raw cashew nut quality as function of contamination by mycotoxins and other secondary metabolites of *Aspergillus* spp. and farmer practices

Leo Yendouban Lamboni

PhD Thesis

ISBN: 978-94-6343-752-3
DOI: 10.18174/442674

Cover, design and layout

Leo Yendouban Lamboni

Cover images modified from:
http://europroxima.com/blog/ochratoxin-a-in-wine/
https://www.linkedin.com/pulse/raw-cashew-nuts-beninmadagascar-monali-dighe/
http://bioweb.uwlax.edu/bio203/s2013/ernst_ale2/
https://microbewiki.kenyon.edu/index.php/Aspergillus_niger
https://commons.wikimedia.org/wiki/File:Cashew_Brazil_nut_cut.jpg

Printing

Proefschriftmaken, Vianen (NL) (www.proefschriftmaken.nl)