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Detection, identification, and quantification of aflatoxin producing fungi in food raw materials using loop-mediated isothermal amplification (LAMP) assays

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LIST OF ABBREVIATIONS

A.	*Aspergillus*
Ab-Ag	antibody-antigen
AF	aflatoxin
APS	adenosine 5´-O-phosphosulfate
BBA	Bundesforschungsinstitut für Kulturpflanzen
BFE	Bundesforschungsanstalt für Ernährung
BIP	backward inner primer
BLAST	basic local alignment search tool
CAC	Codex Alimentarius Commission
CBS	Centraalbureau vor Schimmelcultures
CDC	Centers for Disease Control and Prevention
CTAB	Hexadecyltrimethy Ammonium Bromide
CYA	Czapek yeast extract agar
d	day
DG-18	Dichloran 18% glycerol agar
DNA	desoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
ELIDA	enzymatic luminometric inorganic pyrophosphate detection assay
ELISA	enzyme-linked immunosorbent assays
EU	European Union
F.	*Fusarium*
FIP	forward inner primer
FSA	Food Standards Agency
g	g-force, gram
GC	gas chromatography
h	hour

LIST OF ABBREVIATIONS

ha	hectares
HACCP	Hazard Analysis and Critical Control Point
HNB	hydroxynaphtol blue
HPLC	High-Performance Liquid Chromatography
IBT	Technical University of Denmark
ITEM	Istituto Tossine e Micotossine da Parassiti Vegetali
kg	kilogram
l	liter
LAMP	loop-mediated isothermal amplification
LC	Liquid Chromatography
LFIAs	lateral flow immunoassays
LOD	limit of detection
m	milli (10^{-3}), meter
M	mol
MBP	maltose binding protein
MEA	Malt Extract Agar
min	minute
MOPS	3-Morpholinopropanesulfonic acid
MRC	South African Medical Research Council
NCBI	National center for Biotechnology Information
NIRS	near infrared spectroscopy
NRRL	Northern Regional Research Laboratory
OD	optical density
P2	methyl-4,6-bis(4-A',A'-dimethylaminophenyl)pyrylium iodide
p.a.	pro analysi
PCR	polymerase chain reaction
ppb	parts per billion
ppt	parts per trillion
s	second
SEM	Scanning electron microscope
SNA	synthetischer nährstoffarmer agar

LIST OF ABBREVIATIONS

SYBR	Synergy Brands
TLC	Thin Layer Chromatography
Tm	melting temperatures
TMW	Technische Mikrobiologie Weihenstephan
Tris	tris (hydroxymethyl) aminomethan
T_t	time to threshold
UV	ultraviolet
v/v	volume / volume
w/v	mass / volume
µ	micro

1 Introduction

1.1 Aflatoxins and associated hazards

Aflatoxins are a group of secondary fungal metabolites so far known to be produced by nine different species of *Aspergillus* and two different *Emericella* species (Frisvad et al., 2006). Due to their high toxicity and carcinogenic potential they are of high concern for the safety of food world wide (Ellis et al., 1991). Based on chromatographic and fluorescence characteristics, all aflatoxins known to date can be classified into 18 different types. The major ones are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2), as well as M_1 (AFM_1) and M_2 (AFM_2) (Fig. 1) (Lerda, 2010). Other aflatoxins have less commonly been found in nature since they are metabolic derivatives mostly found in pure cultures (Franco et al., 1998). The order of acute and chronic toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$, reflecting the role played by epoxidation of the 8,9-double bond and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-membered lactone ring of the G series. Among these compounds, AFB_1 is normally predominant in concentrations in cultures as well as in food products (Oliveira and Germano, 1997). AFM_1 and AFM_2 are hydroxylated forms of AFB_1 and AFB_2 (Dors, 2011). When AFB_1 in contaminated feed or foodstuffs is ingested by domestic animals, such as dairy cows, the toxin undergoes liver biotransformation and is converted into aflatoxin M_1 (AFM_1), becoming the hydroxylated form of AFB_1. AFM_1 is excreted in milk, tissues and biological fluids of these animals (Oatley et al., 2000; Peltonem et al., 2001; Murphy et al., 2006) and in this form can be taken up by consumers. A linear relationship between the concentration of AFM_1 in milk and the concentration of AFB_1 in contaminated feeds consumed by the animals has been reported. It was found that about 0.3% to 6.2% of AFB_1 ingested with feed is transformed into AFM_1 in milk (Creppy, 2002; Bakirci, 2001).

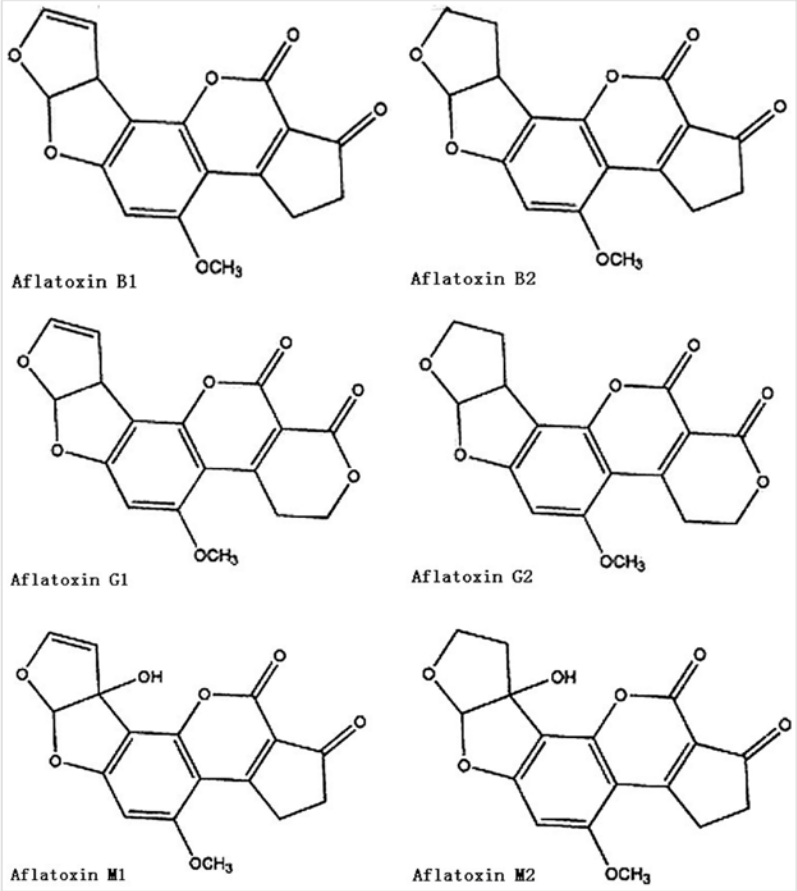

Figure 1 Structures of the aflatoxins (redrawn from Lerda, 2010)

Human and animals are exposed to aflatoxins through their diet (Chulze, 2010; Bandyopadhyay and Cotty, 2011). Therefore, both humans and animals may undergo acute or chronic intoxication caused by mycotoxin ingestion. The pathological condition resulting from this ingestion is called mycotoxicosis (Nierman et al., 2008). Acute toxicity, caused by ingestion of large amounts of aflatoxin from heavily contaminated food, causes decreased liver function and could lead to blood clotting, jaundice, a decrease in serum proteins that are synthesized by the liver, edema, abdominal pain, vomiting and death of the affected person. In 2004, a case occurred in Kenya, in which there were 317 cases and 125 deaths reported due to consumption of aflatoxin contaminated maize (CAC, 2005; Probst et al., 2007 and 2010). The liver is adversely affected by aflatoxin that causes necrosis of liver cells and death (Chao et al., 1991). Beside its acute toxicity, aflatoxin has a high cancerogenic potential. It was estimated that about 25 200 – 155 000 people worldwide, 40% of which in Africa suffer from liver cancer induced by aflatoxin (Liu and Wu, 2010). Epidemiological, clinical and experimental studies have indicated that exposure to large

doses of aflatoxin causes acute toxicity and exposures to small doses for prolonged periods of time are carcinogenic. Chronic toxicity, of which the main symptoms are decreased growth rate that leads to stunted growth, is due to long time exposure to low aflatoxin concentration. In Togo and Benin, children suffering from a condition called Kwashiorkor are underweight as a result of aflatoxin consumption, and also at higher risk for acute hepatitis, Reye's syndrome and diarrhea. Aflatoxin-albumin adducts (32.8 pg/mg) were detected in 99% of children aged 9 months to 5 years. As a result of ingestion of aflatoxin, domestic animals may have lowered production of milk or eggs, and immune suppression caused by reactivity of aflatoxin with T-cells and a decrease in vitamin K activities including a decrease in phagocytosis in macrophages (Robens and Richard, 1992). Aflatoxin has also been linked to immune suppression (Turner et al., 2005) and higher prevalence of hepatocellular cancer has been reported in Africa (Strosnider et al., 2006), especially among people carrying with Hepatitis B and Hepatitis C infections (William et al., 2003).

In addition, there are also economic losses that result from contamination of crops and animal feeds with aflatoxin (Nigam, 2009; Bandyopadhyay and Cotty, 2011). At the global level, aflatoxin contamination is an everlasting concern between the 35N and 35S latitude. Most of the countries in the belt of concern are developing countries which makes the situation even worse since people there frequently rely on highly susceptible crops for their daily nutrition and income and mostly do not have access to proper post harvest handling of commodities, e.g. drying and proper storage.

It has been estimated that 25% of the world’s crops are affected by mould or fungal growth that may result contamination of toxic fungal secondary metabolites known as mycotoxins (Mannon and Johnson, 1985). Aflatoxigenic fungi are common soil habitants all over the world and they frequently contaminate agricultural crops. Aflatoxins, the toxic metabolites produced by different species of toxigenic fungi, can contaminate human food at various stages in the food chain. With the development of world markets for agricultural products and more attention paid to food safety, aflatoxin more and more becomes a problem in countries that previously did not have to worry about aflatoxin contamination. As a result, numerous countries have established or proposed regulations for controlling aflatoxin in food and feeds (Haumann, 1995). The tolerance levels for total aflatoxins (sum of aflatoxins B_1, B_2, G_1 and G_2) in different countries may range from 1 to 35 µg/kg for foods, with an average of 10 µg/kg and from zero to 50 µg/kg for animal feed, with

an average of 20 µg/kg. Tolerance levels for AFM_1 in milk have been set between 0.05 and 0.5 µg/kg, with a threshold of 0.05 µg/kg adopted in most countries (Abbas, 2005). The maximum limit of contamination with aflatoxin in peanuts in Brazil and USA is 20 µg/kg while Canada and the European Union have imposed a limit of 15 µg/kg (Fonseca, 2011). As for animal feeds, the European Commission has set a maximum level for aflatoxin at 0.02 mg/kg (European Commission, 2003).

1.2 Occurrence of aflatoxins in food

Aflatoxins are toxic secondary metabolites produced by various *Aspergillus* species growing in susceptible agricultural commodities. As elaborated above, they can result in major economic losses and can negatively affect animal and human health. Major food commodities affected are maize, peanuts, Brazil nuts and other tree nuts.

1.2.1 Occurrence of aflatoxins in maize

Maize, one of the principal crops grown for human food consumption and livestock feeding, covers more than 120 million ha of cropland globally per year (Pingali, 2001). It is also one of the crops subject to the most critical mycotoxin problems throughout the world. In a study of 295 persons in Benin, 61% ate maize every day of the week and a further 23% consumed maize five to six times a week (Lutz, 1994), since maize is the most important cereal grown in the Republic of Benin. Maize is generally a staple food throughout the African continent but is highly colonized by aflatoxin producing *Aspergillus* species (Bandyopadhyay and Cotty, 2011) so that the fungal contamination is of great concern. Epidemiologic investigations determined that the outbreak in Kenya, where maize is the primary dietary staple, was the result of aflatoxin poisoning from ingestion of contaminated maize (CDC, 2004). Infection of maize kernels by toxigenic fungi remains a challenging problem despite decades of research progress.

A study by Williams et al. (2004) about aflatoxin contamination of market samples of foods from more than 20 countries showed that the maximum aflatoxin levels found in maize or maize products was 770 ppb in Nigeria, followed by 465 ppb in maize from Mexico. Aflatoxin production on maize grain appeared to be greatly influenced by the environment (Gorman and Kang, 1991). Initially, aflatoxin contamination was thought to be a postharvest problem due to improper storage. Increasing percentage of samples showed high aflatoxin levels from the

beginning of storage to 6 months later. The means detected in these aflatoxin positive samples at the beginning of storage were between 22 and 190 ppb but between 31 and 221 ppb after 6 months (Hell, 1997). Farmers aways leave their maize on the floor in a corner of the room or in the courtyard, with the maize cobs in immediate contact with the floor, which increases the risk of *Aspergillus* development. However, research has indicated that infection by *Aspergillus* and subsequent aflatoxin contamination does also occur prior to harvest in some commodities. Aflatoxin contamination in maize depends on the coincidence of host susceptibility, environmental conditions favorable for infection and, in some cases, vector activity (Munkvold, 2003). In maize, earlier planting dates in temperate areas generally result in a lower risk, but annual fluctuations in weather can jeopardize this advantage. Aflatoxin development in maize can be affected by several cultural practices, partly because of the relationship between drought stress and susceptibility to *A. flavus* and aflatoxin accumulation. Cultural practices that tend to expose plants to greater drought stress will lead to higher levels of aflatoxins (Jones, 1986). Damage to maize kernels by insects, especially the European corn borer, fall armyworm, and corn earworm has been associated with high aflatoxin levels (Widstrom et al. 1975).

1.2.2 Occurrence of aflatoxins in peanut

Peanuts and their products are mainly consumed as snacks as well as ingredients of certain dishes in human daily diet (Princen, 1983). Sargeant et al. (1961) discovered a highly toxic compound as a contaminant of Brazilian peanut meal and it led to the death of thousands ducklings, turkeys, fish and other farm animals. Authors related the substance, which was termed aflatoxin in later research to the presence of *A. flavus* in toxic samples. Today they not only adversely affect the health of consumers and farm animals but also hamper international trade (Nigam et al., 2009). A survey of aflatoxin contamination in peanuts and peanut products from several countries during 1982-1994 including Senegal, Mexico, United States, Philippines, India, UK and Nigeria, indicates that aflatoxin occurrence is extremely variable worldwide, with incidences between 30 to 100%, at levels up to 2 888 μg/kg (Rustom, 1997). From the analysis of 20 peanut butter samples from one company in Turkey, Yentür et al. (2006) found that all samples contained aflatoxin with total aflatoxins (B_1+B_2+G_1) ranging from 8.16 to 75.74 μg/kg. Rodriguez-Amaya and Sabino (2002) revealed that 52% of peanut samples aimed at human consumption were positive for aflatoxins. A determination of the aflatoxin levels in peanut products collected from June 2006 to May 2007 that were traded in the Northeast region of São Paulo, Brazil, showed that

44.2% of samples were positive for aflatoxin at levels between 0.5 to 103.8 μg/kg (Oliveira et al., 2009).

Drought stress and insect damage are two major environmental factors that affect *A. flavus* invasion and aflatoxin contamination of the peanut seeds during growth and development. Pre-harvest aflatoxin contamination of peanuts is associated with late-season drought conditions since peanuts start to dehydrate in the soil under hot and dry environmental conditions (Cole et al. 1989). Several studies have sought to reveal the association of extensive preharvest invasion of peanuts by *A. flavus* and subsequent aflatoxin contamination to severe drought stress and insect damage during the latter part of the growing season (Dickens et al., 1973; McDonald et al., 1967; Pettit et al., 1971). Contamination can also occur after peanuts are dug if they are not quickly harvested, dried, and maintained at a safe moisture level. However, peanuts grown under drought stress may also be predisposed to subsequent aflatoxin contamination during harvest, handling, or storage (Diener, 1960). Less than 0.1% of the population may even carry enough aflatoxin to cause an unacceptable average concentration in the entire population of stock peanuts (Dickens, 1977). Therefore, aflatoxin contamination could have taken place during any of several phases in the production of edible-grade peanuts, e.g. in the field under late-season conditions of drought and heat stress; after peanuts were dug but before being harvested; during transport of peanuts from the field to the point of sale when there could be delays in drying; during storage of farmers' stock or shipment of shelled peanuts when a safe storage moisture content cannot be maintained (Dorner, 2008).

1.2.3 Occurrence of aflatoxins in Brazil nuts and other tree nuts

Credited to the nutritional properties, Brazil nuts are well known for high content of oil, protein, and selenium (Andrade et al., 1999; Chang et al., 1995; Ryan et al., 2006). There are almost 20 000 tons of Brazil nuts harvested every year. The presence of the alfatoxins in Brazil nuts was first observed in some samples offered for entry in 1965 following a general program of investigation of all nuts for aflatoxin by the Vancouver Regional Laboratory of the Canadian Food and Drug Directorate (Stoloff, 1976). Brazil nut production occurs in environments with temperatures of 30-35 ℃ and relative humidities of 80-95%, which influence the level of water activity and moisture in Brazil nuts, and favor aflatoxin production (Johnsson et al., 2008). Similarly with cashew nuts or nutmeg, Brazil nuts are collected manually in indigenous regions

by peasants after the ripe seed pods have fallen down to the ground during the rainy season between December and March. Because of the varying collection frequency, the pods and nuts are in contact with the ground where they may become exposed to fungal infection. In this case, Brazil nuts have been found to be frequently contaminated by aflatoxin in relatively high concentrations (Pacheco and Scussel, 2007). Based on Codex Alimentarius, therefore the EU adopted the maximum levels of aflatoxin B1 and total aflatoxins in Brazil nuts for further processing and ready to eat of 8.0 and 15.0 µg/kg and 5.0 and 10.0 µg/kg, respectively (European Union, 2010). However, the occurrence of aflatoxin in Brazil nuts in a critical constraint for its commercialization has been cited in several reports (Pohland, 1993; FSA, 2004; Pacheco and Scussel, 2007; Olsen et al., 2008; Pacheco and Scussel, 2009; Freitas-Silva and Venâncio, 2011). Almonds, pistachios and walnuts, collectively defined as tree nuts, which are subject to infection by a variety of microorganisms that can induce spoilage or produce metabolites that are toxic to humans, animals and birds. In many cases the sources of infections are not known. However, they are exacerbated by factors such as insect damage, drought and high temperatures. Aflatoxins are a serious concern to exporters of California tree nuts (Molyneux et al., 2007). Almond and pistachio imports to the EU from the US in 2005 were subjected to 41 rapid alert and information notifications (European Commission, 2005), which presented a serious economic threat for producers and exporters.

In addition, among processed infant and adult foods all including nuts, grains and powdered milk, Mushtaq et al. (2012) found that the magnitude of AFB_1 contamination varied widely. However, the levels of aflatoxin in the processed foods intended for infant consumption were found to be higher than the maximum allowable amounts set by the European Union, which can be more hazardous for infants since they are more sensitive and prone to exposure and toxic effects of such highly carcinogenic food contaminants.

1.3 Detection of aflatoxin

Aflatoxin not only has adverse effects on human health but also cause serious economic losses when tons of foods have to be discarded or destroyed as a result of aflatoxin contamination. To ensure food safety, maximum levels for aflatoxins in food and feed have been set by national and international organizations and various approaches have been developed for the determination of aflatoxin concentrations in food and feed commodities.

1.3.1 Chromatography

Chromatography is one of the most common methods for quantifying aflatoxin. In the beginning of aflatoxin analysis and research, Gas Chromatography (GC) was frequently used for detection and quantification of compounds. Lateron, new chromatography-based techniques were developed for aflatoxins. Examples of these improvements are Liquid Chromatography (LC), Thin Layer Chromatography (TLC) (Stroka et al., 2000), and High-Performance Liquid Chromatography (HPLC) (Bacaloni et al., 2008) which nowadays is the most commonly used chromatographic technique for detection of a wide diversity of mycotoxins, especially for aflatoxin dericatives (De Rijk et al., 2011). Frisvad and Thrane (1987) described an HPLC method to identify 182 mycotoxins and other fungal metabolites based on their alkylphenone retention indices and diode array spectra. Coupling of HPLC with mass spectroscopy or tandem mass spectroscopy allows for highly accurate determination of toxin concentrations and compound identification in one analysis (Sobolev, 2007). Alternatively, fluorescence detection of the unmodified aflatoxins is widely used in HPLC applications as well as in Thin Layer Chromatography. Furthermore, there are combinations of the methods above with pre-process techniques, which can detect the concentration of aflatoxin in a solution in a better way. For example, immunoaffinity column sample clean-up followed by a normal or reverse phase of HPLC separation with fluorometric detection is mostly used for quantitative determination of AFM_1 due to the characteristics of specificity, high sensitivity and simplicity of operation (Muscarell et al., 2007).

1.3.2 Immunoassay

Immunochemical detection for aflatoxins is based on antibody-antigen reactions (Ab-Ag) (Lee et al., 2004). Since different kinds of aflatoxin molecules can be considered as antigens, it is possible to detect them by developing antibodies against the compounds. Most of the immunological methods are based on enzyme-linked immunosorbent assays (ELISA), which have good sensitivity, speed and simplicity. In addition, some lateral flow immunoassays (LFIAs) also are applied for the qualitative and semi-quantitative detection of aflatoxin in food, feed and milk (Ho and Wauchope, 2002; Anfossi et al., 2011; Salter et al., 2006). Even though several reports have been published on the immunochemical determination of aflatoxin in food, only a few validation studies are available to show that the results comply with certain regulations because of the requirement for expensive instrumentation.

1.3.3 Biosensors and other methods

Biosensors, an alternative to improve the disadvantages of the previous methods, are multidisciplinary tools with an enormous potential in detection and quantification of aflatoxin. There are all kinds of biosensors that base their performance on different physical or biochemical principles, such as optical, optoelectronic, electrochemical, piezoelectric, DNA and combined. Thus, such devices have a huge impact in healthcare, food management, agronomical economy and bio-defense (Nayak et al., 2009). Many kinds of biosensors are applied to detect aflatoxin. However, they mainly work in conjunction with immunochemical methods. Such junctions are based on the high affinity of antigen-antibody interaction and have the aim of increasing the sensitivity and shortening the detection time of the toxic element (Dinçkaya, et al., 2011).

Further methods exist which are less common than the previously described methods but have a wide utility as well. The most important are those ones that base their principle on electrochemistry, spectroscopy and fluorescence. Compared with traditional methods for aflatoxin determination, electrochemical techniques offer some advantages such as reliability, low cost, *in-situ* measurements, fast processes, and easier methodology than common chromatography techniques through a similar performance. Especially for measurement of AFM_1, the disposable immunosensors have been applied directly in milk following a simple centrifugation step without dilution or other pretreatment steps. Exhibition of a good working range with linearity between 30 and 240 ng/ml makes this method useful for AFM_1 monitoring in milk (maximum acceptable level of AFM_1 in milk is 50 ppt) (Micheli et al., 2005). Spectroscopy techniques have been popularized due to the characteristics that fast, low-cost and non-destructive analytical methods suitable to work with solid and liquid samples. Among them, near infrared spectroscopy (NIRS) is an excellent method for a rapid and low cost detection of aflatoxin in cereals (Fernández-Ibáñez et al., 2009). When incorporated with a bundle reflectance fiber-optic probe, NIRS was successfully applied to quantify aflatoxin B_1, ocharatoxin A and total aflatoxins in paprika (Hernández-Hierro et al., 2008). Aflatoxins have a native fluorescence due to their oxygenated pentahetherocyclic structure, which is the basis of most analytical and microbiological methods for detection and quantification of aflatoxins (Rojas-Durán et al., 2007; Rasch et al., 2010).

1.4 Aflatoxin producing fungi

To date about 100 000 fungal species have been identified, from which over 400 can be considered potentially toxic. Only 5% of the toxinogenic species are known to produce toxic compounds causing problems in one or more parts of the world (Bata and Lasztity, 1999). Most aflatoxin producing fungi are members of the genus *Aspergillus* classified into the section *Flavi*. In addition, some species have been described as aflatoxin producers in the teleomorphic genus *Emericella* (Frisvad et al., 2004). They are often isolated from areas with hot, humid climates. Among 22 closely related species in *Aspergillus* section *Flavi*, *Aspergillus flavus* and *A. parasiticus* are frequently encountered in a variety of agricultural products. The two species are responsible for the majority of aflatoxin contamination events, with *A. flavus* being by far the most common species (Varga et al., 2011). Numerous studies have shown that the mycotoxigenic potential and profile of *A. paraisiticus* is far more variable. Razzaghi-Abyaneh et al. (2006) report 100% aflatoxigenic *A. parasiticus* isolates and only 27.5% of aflatoxigenic *A. flavus* strains. Siminarly, Rodrigues et al. (2009) found 77% of atoxigenic isolates in *A. flavus* while all *A. parasiticus* isolates were found to be aflatoxigenic. *A. flavus* typically produces AFB_1 and AFB_2 and can be most frequently isolated from the aerial parts of plants (leaves, flowers), while *A. parasiticus* produces AFG_1 and AFG_2 as well as AFB_1 and AFB_2 and is more adapted to soil environments. The latter species has a more limited distribution (EFSA, 2007). *A. parasiticus* occurred at low requencies similar to those previously observed in Israel and Texas (Joffe, 1969; Schroeder and Boller, 1973; Lisker et al., 1993) while higher incidences of *A. parasiticus* are occasionally observed (Angle et al., 1982; Doster and Michailides, 1994). Four other aflatoxins M_1, M_2, B_2A, and G_2A, which may be produced in minor amounts, were isolated from cultures of *A. flavus* and *A. parasiticus*. Some chemically closely related compounds, aflatoxin GM_1, aflatoxin B_3 (parasiticol) and aflatoxicol have been found to be produced by strains of *A. flavus* (Heathcote and Hibbert, 1974). Other species in *Aspergillus* section *Flavi*, such as *A. nomius*, *A. bombycis*, *A. ochraceoroseus*, *A. tamarii* and *A. pseudotamarii* are also aflatoxin producing species, but are less frequently encountered (Alberts et al., 2006). However, they may play a major role in some commodities or environments.

Aspergillus species are able to grow in a wide variety of substrates and under different environmental conditions; nevertheless, toxin formation in agricultural products mainly occurs under hot and humid weather conditions, and in inadequate or deficient storage facilities. The

most important factors that influence aflatoxin production and mould growth are relative humidity, ranging from 88 to 95% in most of the cases (Park and Liang, 1993), and temperature, ranging from 25 to 27 ℃ for maximum toxin production, and 36 to 38 ℃ for maximum mould growth (Abbas, 2005). Other factors may also influence aflatoxin production: substrate composition, water activity, pH, atmosphere (concentration of oxygen and carbon dioxide), microbial competition, mechanical damage to seeds, strain specificity and variation, instability of toxigenic production, plant stress, insect infestation, and use of fungicides or fertilizers (Gonçalez et al. 2001; Hussein and Brasel, 2001; Magan and Olsen, 2006). Furthermore, the moment of harvesting and drying as well as storage conditions may play an important role in aflatoxin production. Since aflatoxin contamination is cumulative, several factors may add up to the toxin concentrations found in a given sample (Prandini et al., 2009).

1.5 Detection and identification of *Aspergillus* spp.

Even non-mouldy foods or raw materials may contain aflatoxin. Spores can be transferred by insects (especially flies, wasps and bees) or by birds to foods where the spores germinate, produce mycelium, and aflatoxin are excreted. Seeds can also contain aflatoxin due to infection of the egg-cells of the flowering plants. The spores of *A. flavus* and *A. parasiticus* can germinate on the stigma surfaces of plants, and then the germ tube penetrates to the developing embryo mimicking pollen germ tubes (Hill et al., 1985). Although the mycelium can establish an endotrophic relationship with no harm in the healthy plant, significant levels of aflatoxin may be produced in the plant tissue during growth in the field if the plant is under drought stress. In this case, food commodities may already be contaminated at harvest and, although the concentrations are never as high as those formed in stored commodities, they can be economically significant (Hansen and Jung, 1973; Moss 2002). Therefore, the detection of aflatoxin producing fungi is very important in the view of food safety. And for the detection of moulds, methods such as the traditional mycological methods (Pitt and Hocking, 2009), enzyme-linked immunosorbent assays (Notermans et al., 1986), PCR and RT-PCR (Shapira et al., 1996; Haugland et al., 2002) are widely applied.

Identification of species of *Aspergillus* has traditionally relied on macroscopic colony characteristics and microscopic morphology. Initially, Raper and Fennell (1965) did not use any chemical, biochemical or physiological characters for classification of the genus. Later on, both

colour and physiological tests were used in addation in taxonomic research as described by Murakami (1976) and Murakami et al. (1979). Characters included pigment production in Czapek agar, growth on nitrite as sole nitrogen source, acid production, extracellular enzyme production and reaction of broth with $FeCl_3$. Moreover, those characters ignored before have been introduced in later taxomomic studies of *Aspergillus* physiological tests (Klich and Pitt 1988). In addition to their use in chemotaxonomy, many secondary metabolites have bioactive properties as well (Frisvad 1989; Frisvad et al. 1998, 2004; Samson et al. 2004). Mycotoxins are of particular interest since *Aspergillus* species produce some of the most important mycotoxins (Frisvad et al. 2007 and 2008).

However, the traditional mycological methods used to assess mould presence in commodities is time-consuming, labor-intensive, requires facilities and mycological expertise. Above all, most media do not readily allow the identificaiton of mycotoxigenic strains. This can only be performed using high fat media such like coconut cream agar and a UV light source for inspection. However, such analysis still requires at least 5 days of incubation. With the invention of primer initiated enzymatic in vitro nucleic acid amplification technologies, protocols for the specific detection and identification of microorganisms directly from food samples were developed (Cocolin et al., 2002; Aymerich et al., 2003; Amagliani et al., 2006). PCR and real-time PCR have been described as more sensitive and specific methods for detection of moulds (Shapira et al., 1996; Haugland et al., 2002). Although providing rapid and specific results, PCR-based methods require dedicated lab equipment, which makes such methods a rather expensive and time-consuming technology for rapid testing. Developed real-time PCR assays are more rapid as compared to conventional PCR and eliminate gel electrophoresis by detecting fluorescence during DNA amplification (Jothikumard and Griffiths, 2002). However, they require sophisticated and even more expensive equipment, which is not readily available for routine detection in processing facilities and small industries.

1.6 Loop-mediated isothermal amplification

One approach to managing the risks associated with aflatoxin contamination is the use of an integrated system based on the Hazard Analysis and Critical Control Point (HACCP) approach, which should involve strategies for prevention, control, good manufacturing practices, and quality control at all stages of production, from the field to the final consumer (Panisello and

Quantick, 2001). Economically affordable and environmentally sustainable methods are available for application pre or post-harvest to reduce the contamination of aflatoxin. In this case, loop-mediated isothermal amplification (LAMP), a relatively novel technology for nucleic acid amplification under isothermal conditions (Notomi et al., 2000) without the need for dedicated equipments can be a good choice.

1.6.1 Principle

LAMP is a novel approach to nucleic acid amplification which relies on auto cycling strand displacement DNA synthesis performed by *Bst* DNA polymerase under isothermal conditions with a set of four specifically designed primers that hybridize to six different parts of the target DNA sequence (Notomi et al., 2000). Figure 2 shows steps in the LAMP reaction that starts from primer FIP. However, it should be remembered that DNA synthesis can also begin from primer BIP (Tomita et al., 2008). The *Bst* DNA polymerase used to run LAMP reactions is based on the *Geobacillus stearothermophilus* enzyme but was genetically engineered to have a 5´-> 3´ polymerase activity but no 5´-> 3´ exonuclease activity. According to the information of the manufacturer (New England Biolabs), the *Bst* DNA polymerase is produced by an *E. coli* strain which contains a genetic fusion of large fragment of the enzyme and the maltose binding protein (MBP) used for affinity purification. The MBP is removed from the fused protein after purification (Kong et al., New England Biolabs, unpublished results). During primer-initiated polymerization, the large fragment of *Bst* DNA polymerase effectively displaces third strand DNA of new DNA to leave a new single stranded matrix DNA for further primer annealing and DNA polymerization. Due to the high activity of *Bst* DNA polymerase, abundant high molecular weight DNA is produced within short time.

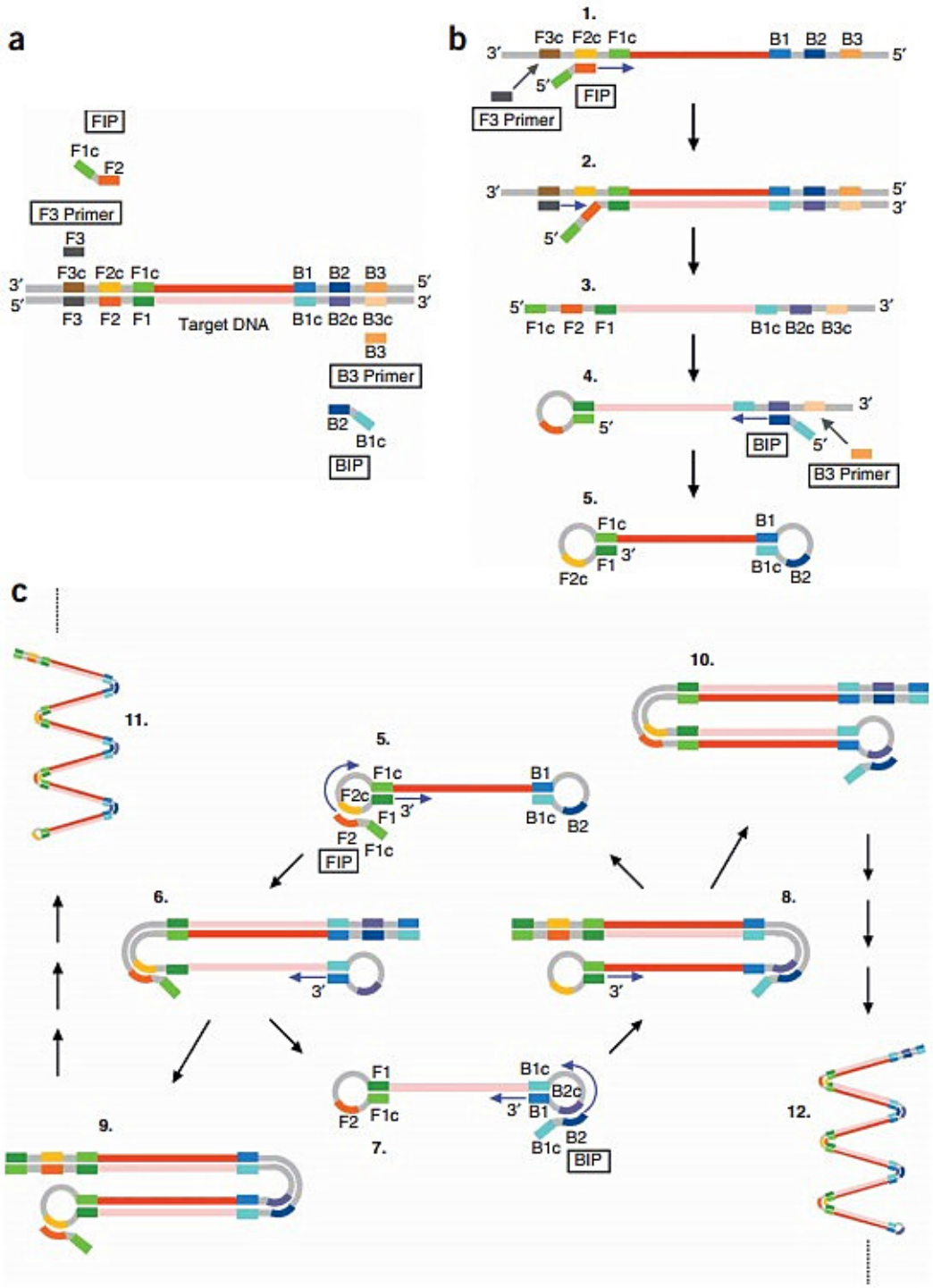

Figure 2 Schematic representation of the LAMP reaction (Tomita et al., 2008)

The exceptionally high specificity of LAMP is because a set of four primers with six binding sites must hybridize correctly to their target sequence before DNA biosynthesis occurs. The outer

primers are known as F3 and B3, while the inner primers are forward inner primer (FIP) and backward inner primer (BIP). Both FIP and BIP contain two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi et al. 2000). All primers are used in the initial steps of the reaction, but in the later cycling steps only the inner primers are used for strand displacement DNA synthesis. Additionally, the concentrations of the inner primers are higher than the concentrations of the outer primers (Notomi et al. 2000). A third pair of primers (loop primers) can be added optionally to the reaction in order to further increase the amount of DNA produced during the LAMP reaction making it faster and more sensitive (Nagamine et al., 2002). The size and sequence of the primers were chosen to result in melting temperatures (Tm) between 60-65 °C, which is the optimal temperature for *Bst* polymerase.

The amplified product has originally been analysed by agarose gel electrophoresis, which typically reveals a ladder like pattern of DNA fragments (figure 3) (Niessen and Vogel, 2010). The smallest (monomer) fragment spans from the 5' end of the F1c part of the forward inner primer (FIP) to the 5'end of the B1c part of the backward inner primer (BIP). Multimers and polymers of that monomeric structure are produced with sizes of a few hundred up to the formation of a smear of high molecular weight DNA of several kilobases in size as exemplified in Notomi et al. (2000). However, mass production of DNA during LAMP bears a high risk of cross contamination of samples by aerosolized product. In order to prevent cross contamination, methods for in-tube detection of DNA amplification were developed. Detection was achieved by direct staining of double stranded DNA using fluorogenic intercalating dyes (Notomi et al., 2000; Iwamoto et al., 2003; Maeda et al., 2005). Substances such like SYBR green (Noble and Fuhrman, 1998), EvaGreen (Wang et al., 2006; Ihrig et al., 2006.), Hoechst 33285 (Latt et al., 1975), ethidium bromide (Higuchi et al., 1992), P2 (Yamamoto and Okamoto, 1995) and SYTO9 (Monis et al., 2005; Njiru et al., 2008) have been used in previous publications. Since most of these substances substantially reduce productivity of the *Bst* DNA polymerase during LAMP, they can be added only after the completion of the LAMP reaction. However, opening of reaction vessels after the reaction is completed will cause heavy spoiling of the lab environment with aerosolized LAMP product, which results in false positive reactions in subsequent LAMP analyses. Precipitation of the DNA produced during LAMP with a fluorescently labeled cationic polymer or the use of a specific fluorescently labelled probe have been shown to be an alternative

for direct in-tube detection (Mori et al., 2006). Indirect in-tube detection of LAMP product was done using reactions related to pyrophosphate which is profusely produced during the reaction as a specific by-product of enzymatic DNA synthesis. By monitoring the turbidity of LAMP reactions resulting from magnesium-pyrophosphate precipitation (Mori et al., 2001), LAMP can be used in real-time mode to produce (semi)quantitative results (Mori et al, 2004). Alternatively, complexometric dyes were used which are present during the LAMP reaction and which change their color upon transfer of a dye-bound bivalent cation to form a pyrophosphate complex. Since color change of a complexometric dye is an indicator for the formation of pyrophosphate during DNA biosynthesis, it can be used as indirect indicator for a positive LAMP reaction. The most widely used compound is calcein, the fluorescence of which can be quenched with manganese cations. The molecule shows a bright green fluorescence as soon as the cation is removed by complexation to pyrophosphate during the *in vitro* synthesis of DNA (Diehl and Ellingboe, 1956; Kepner and Hercules, 1963; Hoelzl-Wallach and Steck, 1963; Demertzis, 1988; Tomita et al., 2008). Hydroxynaphtol blue (HNB) is another complexometric dye and was also verified to be useful for indirect detection of DNA biosynthesis during LAMP (Goto et al., 2009). Moreover, Gandelman et al. (2010) used an indirect luminescence assay in which the pyrophosphate produced during DNA polymerization in LAMP is reacted enzymatically with adenosine 5´-O-phosphosulfate (APS) as the substrate for ATP sulfurylase resulting in the generation of ATP which is simultaneously utilized by firefly luciferase to oxidize luciferin with the emission of light. This so called ELIDA reaction (enzymatic luminometric inorganic pyrophosphate detection assay) has also been used for signal generation in pyrosequencing of nucleic acids (Ronaghi et al., 1998).

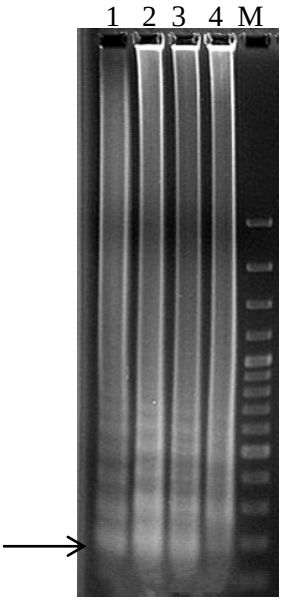

Figure 3 Agarose gel electrophoresis of LAMP reactions for 4 target fungi species. 1. LAMP reaction for *A. parasiticus* with Apara ID153, and size of the smallest LAMP fragment is 145 bp; 2. LAMP reaction for *A. flavus* with Afla ID58, and size of the smallest LAMP fragment is 152 bp; 3. LAMP reaction for *A. caelatus* with Aca ID56, and size of the smallest LAMP fragment is 153 bp; 4. LAMP reaction for A.nomius with Anom ID9, and size of the smallest LAMP fragment is 151 bp; M = size marker 100 bp ladder (MP Biomedicals).

1.6.2 Advantages and applications

The major advantage of the loop-mediated isothermal amplification technique is its simplicity and user-friendliness. No specialized equipment such as a thermal cycler is necessary to perform it. Reactions can be run in any temperature controlled water bath or heating block to provide a constant temperature since the amplification proceeds under isothermal conditions. A further advantage is the high specificity for the target sequence due to the special design of primers which recognize six distinct regions whithin the target DNA. Moreover, *Bst* DNA polymerase is much less prone to inhibitory substances coming from the sample matrix to be analyzed. The effectiveness of the enzyme is therefore less affected by biological substances as compared to *Taq* polymerase used in PCR (Kaneko et al. 2007). As LAMP is less affected by the various sample components than PCR, purification of DNA can be less elaborated or, in some cases, is not necessary (Nagamine et al. 2001). Another advantage is that amplification can be carried out rapidly because of no need for initial heat denaturation of the template DNA, and no requirement of thermal cycling (Nagamine et al. 2001).

During the past decade, the LAMP method has been widely applied in nucleic acid analysis because of the advantages listed above. Fu et al. (2011) briefly summarized applications of the

LAMP method in pathogenic microorganisms, genetically modified ingredients, tumor detection, and embryo sex identification. Recently, Niessen et al. (2013) together with the author of the current thesis as co-author, published a critical review of the application of LAMP-based methods and their usefulness in detecting and identifying food borne bacterial pathogens and toxicants as well as mycotoxin producing food borne fungi as compared to other methods. To be more specific for the application in the detection of mycotoxigenic fungi and spoilage yeasts in food, *F. graminearum* (Niessen and Vogel, 2010; Denschlag et al., 2012), *Fusarium* spp. (Denschlag et al., 2013), *Aspergillus* spp., especially the aflatoxin producing species (Luo et al., 2012; Storari et al., 2013) and yeast (Hayashi et al., 2007) were identified and detected by LAMP assays.

1.7 Real-time loop-mediated isothermal amplification

It is well known that real-time monitoring of some nucleic acid amplification reactions permits the quantification of template nucleic acid present in a sample such as real-time PCR (Heid et al., 1996). In recent years, real-time PCR has been established as a method used for prediction of the potential aflatoxigenic risk in plant derived food such like maize, pepper, and paprika (Mayer et al., 2003; Mideros et al., 2009) or peanuts (Passone et al., 2010). Sardiñas et al. (2011) applied real-time PCR for the quantification of *A. flavus* and *A. parasiticus* with a detection limit at spore concentrations $\geq 10^6$ spores/g in flour samples without prior incubation. The latter assay was demonstrated to showed an even higher sensitivity when a pre-incubation step of samples was integrated in the protocol in order to accomplish a more effective cell disruption of spores at germination. However, this additional step prolonged analysis time considerably.

As turbidity derived from precipitating magnesium pyrophosphate occurs, judging the presence or absence of this white precipitate allows easy distinction of whether or not nucleic acid was amplified by the LAMP method. Since an increase in the turbidity of the reaction mixture upon the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity. Mori et al., (2001 and 2004) employed this effect by continuously measuring the turbidity of multiple samples simultaneously to monitor amplification of DNA in real-time.

Tomita et al. (2008) demonstrated that calcein fluorescence can be quenched by complexation to manganese ions, and then the dissolved complex is conducive to the formation of a

pyrophosphate-manganese complex during DNA synthesis so that the free calcein displays its bright green fluorescence under UV-light. As an alternative to the measurement of magnesium pyrophosphate turbidity, calcein can therefore be used as a fluorescence indicator in indirect measurement of LAMP-based DNA amplification (Denschlag et al., 2013). Direct measurement for quantifying DNA produced during the LAMP reaction can be achieved by adding an intercalating fluorescent stain such like SYBR green 1(Lucchi et al., 2010). Such dyes can also be used in connection with the Tube Scanner device which is a simple portable device combining a heating block as amplification platform with a fluorescent detection unit with the ability to acquire real time data.

The fluorescence or turbidity based methods for the quantification of nucleic acids takes advantages of two important features of the LAMP method, namely the high specificity that eliminates the need to check the amplification product, and the ease to detect the amplification products with the fluorescence method or the turbidity method. Moreover, this method needs neither expensive apparatus nor fluorescence probe, which is commonly used in real-time PCR.

1.8 Objectives of the work

The aim of the current study was to set up a simple, rapid, specific and effective method for the identification, detection and quantification of the most important aflatoxin producing fungi based on the loop-mediated isothermal amplification (LAMP) technology, to apply it to the analysis of pure cultures and food commodities, to follow fungal contamination in commodities as well as to provide guidance for prevention, control, food manufacturing practices, and quality control at all stages of production, from the field to the final consumer.

2 Material and Methods

2.1 Material

2.1.1 Equipment

Table 1 Overview of used equipment

Device	Model	Manufacture
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Camera	IXUS 95	Canon, Beijing, China
Coffee grinder	MKM 6003	Robert Bosch Hausgeräte GmbH, Munich, Germany
Centrifuges	Z216MK	Hermle Labortechnik GmbH, Wehingen, Germany
	Z382K	Hermle Labortechnik GmbH, Wehingen, Germany
Counting chamber	Thoma, depth of 0.1 mm	BRAND GmbH, Wertheim, Germany
DNA-quantification	Nanodrop1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
Cell disruption	Fastprep 24 bead beater	MP Biomedicals, Illkirch, France
Microscope	Axiolab E	Carl Zeiss, Oberkochen, Germany
Heating block	ThermoSTAR 100	Quantifoil Instruments, Jena, Germany
PCR-Cycler	Eppendorf Mastercycler gradient	Eppendorf AG, Hamburg, Germany
pH determination	InLab 412, pH 0-14	Mettler-Toledo, Gießen, Germany
Pipettes	Pipetman (1ml, 200, 100, 20, 10, and 2 µl)	Gilson-Abomed, Langenfeld, Germany
Real-time turbidimeter	Loopamp LA-320C	EIKEN Chemical Co., LTD, Tokyo, Japan
Shaking	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Ultra sonification	UP 200S	Dr. Hielscher GmbH, Teltow, Germany
UV lamp, 365 nm	MiniUVIS	Desaga, Heidelberg, Germany
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany
Water purification system	Euro 25 and RS 90-4/UF pure water system	SG Wasseraufbereitung GmbH, Barsbüttel, Germany

2.1.2 Chemicals

Table 2 Overview about used chemicals

Chemicals	Purity	Manufacturer
Agar	european agar	Difco, BD Sciences, Heidelberg
Bst DNA polymerase	8000 U/ml	New England Biolabs, Frankfurt, Germany
Bst 2.0 DNA polymerase	8000 U/ml	New England Biolabs, Frankfurt, Germany
Calcein	p.a.	Sigma-Aldrich, Taufkirchen, Germany
Chloramphenicol	≥98,5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Chloroform	≥99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
CTAB	≥98 %, for biochemistry	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$CuSO_4 \times 5H_2O$	p.a.	Sigma-Aldrich, Taufkirchen, Germany
Dichloran	for synthesis	Merck, Darmstadt, Germany
dNTP mix (10 mM each A, T, G, C)	≥99%	Fermentas, St. Leon-Rot, Germany
EDTA	for molecular biology	Sigma-Aldrich, Steinheim, Germany
Ethanol, absolute	≥99,8 %	VWR, Prolabo, Foutenay-sous-Bois, France
Formamide	Molecular Biology Grade	Merck, Darmstadt, Germany
$FeSO_4 \times 7H_2O$	p.a.	Sigma-Aldrich, Taufkirchen, Germany
Glucose	for biochemical use	Merck, Darmstadt, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Isoamyl alcohol	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	≥99,5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
KCl	p.a.	Merck, Darmstadt, Germany
KH_2PO_4	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 \times 3H_2O$	p.a.	Merck, Darmstadt, Germany
KNO_3	p.a.	Merck, Darmstadt, Germany
Malt extract	microbiology grade	AppliChem, Darmstadt, Germany
$MgCl_2$	p.a.	Merck, Darmstadt, Germany
$MgSO_4 \times 7H_2O$	p.a.	Merck, Darmstadt, Germany

$MnCl_2$	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MOPS (3-Morpholinopropanesulfonic acid)	p.a.	Merck, Darmstadt, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
$NaNO_3$		
$(NH_4)_2SO_4$	p.a.	Merck, Darmstadt, Germany
Primers	HPSF grade	MWG-BiotechAG, Ebersberg, Germany
Saccharose	p.a.	SIGMA-Aldrich, Steinheim, Germany
Soy peptone	for microbiology	Oxoid, Basingstoke, Hampshire, England
Tris–HCl	p.a.	Merck, Darmstadt, Germany
Tween 20	p.a.	Mallinkrodt Baker B. v., Deventer, NL
Yeast extract	for microbiology	Merck, Darmstadt, Germany
$ZnSO_4 \times 7H_2O$	p.a.	Sigma-Aldrich, Taufkirchen, Germany

2.1.3 Consumables and molecular-biological kits

Table 3 Overview about used consumables

Material	Type	Manufacturer
Cryo vials	2 ml	Sarstedt, Nümbrecht, Germany
Filter pipette tips	200 µl, 100 µl, 10 µl	SafeGuard Filter Tips, PeQlab, Germany
Glass beads	5 mm, 2 mm	Carl Roth, Karlsruhe, Germany
Petri dishes	without cams, 92×16 mm	Sarstedt, Nümbrecht, Germany
Reaction tubes	2 ml, 1.5 ml, 500 µl	Eppendorf, Hamburg, Germany
Multiply µStrip Pro 8-stripPCR tubes	200 µl	Sarstedt, Nümbrecht, Germany
Screw cap tube	1.5 ml	Sarstedt, Nümbrecht, Germany
Sea sand	extra pure	Merck, Darmstadt, Germany
Sterile Falcon tubes	15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
Zirconia/silica beads	0.1 mm, 0.5 mm	Carl Roth, Karlsruhe, Germany
E.Z.N.A.® Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA

2.1.4 Fungal strains

Fungal strains used in this study are listed in table 6 (Page 37), including 39 *Aspergillus* species, 23 *Penicillium* species, 75 *Fusarium* species and 37 isolates representing other fungal species.
A total of 69 fungal isolates listed in table 7 (Page 43) were obtained from Brazil nuts as described by Gonçalves et al. (2012) and Calderari et al. (2013).

2.1.5 Naturally infected Brazil nuts

A total of 32 Brazil nut samples selected among the 288 Brazil nut samples analyzed by Calderari et al. (2013) were available for the current study. Samples had been stored at -20 °C prior to analysis. There were 5 samples obtained from the state of Amazonas, 19 from the state of Pará, and 8 from the state of São Paulo. Samples had been collected from the Amazonian rainforest, at local markets, supermarkets and during processing in different Brazilian states (see table 8, Page 47).

2.2 Methods

2.2.1 Media and growth conditions

All fungal cultures were maintained on MEA plates or SNA plates (synthetischer nährstoffarmer agar, Nirenberg, 1976), and kept at 4 °C after incubation. The growth conditions differed according to the utilization of the cultures as follows. For long term storage, cultures were grown on porous clay granules in 3% (w/v) malt extract broth amended with 0.3% (w/v) soy peptone, at ambient temperature for five days. Then the granules were transferred into cryo vials in 1 ml sterile 80% glycerol and kept frozen at -80 °C. For morphological examination and conidium harvest, fungi were cultivated on MEA plates at ambient temperature until sporulation occurred. For direct testing for mycelium of *Aspergillus* strains with the LAMP assays cultures were grown on SNA plates at ambient temperature in the dark for two days and analyzed before sporulation occurred as described under the DNA preparation section. As for the isolates from Brazil nuts, Dichloran 18% glycerol (DG-18) agar and Czapek yeast extract agar were applied for isolation and morphological analysis. All media were sterilized by autoclaving at 121 °C for 20 min. The composition of all media used during the current study is listed in table 4.

Table 4 Composition of media used in this study

Medium	Compound	Concentration (g/l)
CYA pH 6.2 ± 0.2	$NaNO_3$	3
	KCl	0.5
	$MgSO_4 \times 7H_2O$	0.5
	$ZnSO_4 \times 7H_2O$	0.01
	$CuSO_4 \times 5H_2O$	0.005
	$FeSO_4 \times 7H_2O$	0.01
	$K_2HPO_4 \times 3H_2O$	1.3
	Yeast extract	5
	Saccharose	30
	Agar	15
DG-18 pH 5.6 ± 0.2	Peptone	5
	KH_2PO_4	1
	$MgSO_4 \times 7H_2O$	0.5
	Chloramphenicol	0.1
	Glycerol 100%	220
	Dichloran	0.002
	Glucose	10
	Agar	15
MEA pH 5.4 ± 0.2	Malt extract	30
	soy peptone	3
	Agar	15
SNA	KH_2PO_4	1
	KNO_3	1
	KCl	0.5
	$MgSO_4 \times 7H_2O$	0.5
	Glucose	0.4
	Saccharose	0.4
	Agar	20

2.2.2 DNA preparation

2.2.2.1 Preparation of highly purified fungal DNA

Highly purified fungal genomic DNA for specificity testing was extracted from mycelia grown in 50 ml malt extract broth for 5-7 days at ambient temperature. Mycelia were vacuum filtered and freeze dried after two rinses with 50 ml of sterile tap water. Lyophilized mycelia were finely ground with a mortar and pestle after adding a spoonful of sterile sea sand until no intact hyphae were seen under the microscope. Ground mycelia were subjected to DNA extraction according to the method described by Niessen and Vogel (2010).

2.2.2.2 Rapid DNA preparation from mycelia

A different protocol was used for the rapid preparation of small amounts of genomic DNA from mycelia of fungi grown on SNA plates for two days in the dark at ambient temperature. Two circles were cut out of the colonies with an inverted sterile pipette tip (5 mm in diameter) and

without the lower portions containing no mycelia. The portions containing the mycelia were collected in a sterile 1.5 ml conical screw cap tube and washed twice with sterile water with spinning at 6 000 × g for 1 min after each washing cycle. Supernatants were discarded before addition of 50 mg of 2 mm diameter glass beads, 100 mg of sterile sea sand, and 60µl ultrapure water. Mechanical lysis of the cells was done by two cycles of 40 s at 5 m/s in a Fastprep 24 bead-beater. Following mechanical lysis, samples were spun at 6 000 × g for 1 min at ambient temperature and 5µl of the supernatant was added as template to LAMP reaction. Template DNA from artificially contaminated Brazil nuts, peanuts, and coffee beans for the LAMP assays was also prepared by using this method from pellet resuspended in 0.5 ml of ultrapure water after centrifugation of the collected washing fluid from the samples.

2.2.2.3 Rapid DNA preparation from conida

Before DNA preparation, conidia of *A. nomius*, *A. flavus*, *A. parasiticus* and *A. caelatus* were harvested from the corresponding colonies grown on MEA plates at ambient temperature until abundant conidiation occurred. Conidia were harvested by two repetitive cycles of adding sterile 5 mm glass beads and 3 ml of sterile tap water per plate before shaking for 1 min and collecting the solution in a sterile 15 ml tube. Conidia were spun and washed twice with 2 ml of sterile deionized water. Finally, washed conidia were re-suspended in 2 ml of sterile deionized water and concentrations were assessed by counting in a Thoma type counting chamber (depth 0.1 mm). Conidial suspensions used for direct LAMP analysis of plate grown pure cultures were obtained using a sterile toothpick to take conidia from cultures by touching the culture surface before immersing into 100 µl of ultrapure water. The conidium suspension was disrupted by vigorous shaking together with 0.3 g zirconia/silica beads (diameter 0.1 mm : diameter 0.5 mm = 1:1) for 10 min in a vortex at maximum speed followed by boiling for 10min. Five µl of the supernatant were used as template for LAMP reactions after centrifugation at 16 000 × g for 5 min at ambient temperature. Similarly, DNA of fungal material collected by surface washing from naturally infected Brazil nuts was extracted with the bead-beater method described above.

2.2.2.4 DNA preparation from samples

Samples of Brazil nuts, peanuts and maize were finely ground using coffee grinder before DNA preparation. DNA of samples, with and without artificial inoculation with a 10-fold serial dilution of conidia of the three reference species, was extracted according to the CTAB method described by Alary et al. (2002) with some modifications. Five microliter of CTAB extraction buffer (20 g/l

CTAB, 1.4 M NaCl, 100 mM Tris HCl, 20 mM EDTA, pH 8.0) and 1 g of finely ground sample were added in a 50 ml Falcon tube, mixed well by vortexing 30 s at highest speed and treated with ultrasonic for 3 min at 50 % intensity with maximum amplitude. Following ultrasonication, samples were incubated in a water bath at 65 ℃ for 30 min and the tubes were mixed every 5 min by inversion. The supernatant of the solution after centrifuged at 15 000 × g for 15 min at 20 ℃ was transfered to a new sterile 15 ml Falcon tube and then an equal volume of chloroform-isoamyl alcohol (24:1) was added. The mixture was homogenized by vortexing for 30 s and phases were separated by centrifugation at 12 000 × g for 15 min at 20 ℃. The upper aqueous phase was transferred to a new 15 ml Falcon tube and 2 volumes of CTAB precipitation buffer (5 g/l CTAB, 40 mM NaCl, pH 8.0) were added. After temperate inversion, the mixture was spun at 12 000 × g for 15 min at 20 ℃ after standing for 1 h at room temperature. The supernatant was discarded and the pellet was dissolved in 1 ml of 1.2 M NaCl with addition of 1 ml of chloroform-isoamyl alcohol (24:1). The mixture was homogenized by vortexing for 30 s before centrifugation at 12 000 × g for 10 min at 20 ℃. The upper phase was transferred to a new 2 ml reaction tube, 0.6 vol of isopropanol at ambient temperature was added and the mixture was mixed by thoroughly inverting before centrifugation at 17 000 × g for 15 min at 20 ℃. The DNA pellet was washed twice with ice cold 70% ethanol. The DNA pellet was dried under a fume cabinet and re-dissolved in 30 µl of sterile deionized water.

2.2.3 Primers design

Primers used in the LAMP assays for *A. nomius*, *A. parasiticus*, *A. flavus* or *A. caelatus* were designed using the Primer Explorer V. 4 software tool available on the Eiken Genome site (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). The primer sets Anom ID9 (*A. nomius*) and Apara ID153 (*A. parasiticus*) were designed according to an 1140 bp partial sequence of the alpha amylase 1 (*amy*1) gene of *A. nomius* KS2 (GenBank accession no. DQ467925) and an 1178 bp partial sequence of the *amy*1 gene of *A. parasiticus* 2999 (GenBank accession no. DQ467918), respectively, while the primer set Afla ID58 (*A. flavus*) was generated from a 2485 bp fragment of the ATP citrate lyase subunit 1 (*acl*1) gene of *A. oryzae* RIB40 (GenBank accession no. XM_001820729). In order to increase the concentration of DNA produced during the LAMP reaction, a pair of loop primers were designed manually for each primer set according to the instructions given in Nagamine et al. (2002). In addition, since *A.*

caelatus is a common non-aflatoxin producing species in Brazil nuts, a set of primers for LAMP assay of *A. caelatus* was designed based on the sequence of *acl*1 gene fragment (see figure 4) amplified from genomic DNA of *A. caelatus* IBT 29700 by PCR with primers ACL1-615f (5'-GGY ATG ATG GAC AAC ATY GT-3') and ACL1-1386r (5'-GCU ATU ARC ATA GRA CCA TC-3'). All the sequences of the designed primers are given in table 5.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          10        20        30        40        50        60
GGACCATCTC CAAGAACTTG CTAGCGTAGG CGGGAAGGCG GCGGCGGAAC CAAAGAAGAG
ACATGACACC ACCAATGCCA ATGTCCTCCC GGAAGACATC GGAGATGGGC ATACCGGCGT
AGAGAAGCTC CTGGCCACGG TCGTCGGAGA TAGTGGAGAT GAAGGCAGCG GGCTTACGGA
CGAGACCGAG CTCCTGGGCC CAGGAGTAGT CGATAGGATC TTAGGGGGAA CAGGCTCGGG
CTGAGGCTGG ATGTTGCCCT TGCTGACCTC CTGCTCGTAG ACCTCCTTGA GCACCTTGGG
CATGTCCTCA AAGGTCTCGG GAACGTGGAT ACCAGCCTCC CTCATGGCCT TGTTCTTAGC
AACAGCGGTT TCCAGGTCAG AGTTGGCGGA AGCACCAGCG TGACCGAACT GGACCTCGGT
CTTGAACATG CTGGCGCAGG TACCAATAGC CCAAGCAACA ATGGGCTTGG TGATGGTGCC
GTTCTTGACG GCCTCGATGA CACGGTACTC CTCAACACCA CCGACTTCAC CGAGCAACAC
AAGGATCTTG CACTCCGGCT CGTTCTGGTA ACGCAGCAAG TGGTCGATGA AAGTGGTACC
AGGGTAACGA TCACCACCAA TGGCAACACC CTCGTAGACA CCATCGGTAT TTTGAGAGAC
AATGTTGTTC AATTCGTTGG ACATACCACC GGACTTGGAG ACGTAGCCAA CAGAGCCCTT
GCGGTAGAGC TTGGAAGCCA CAATGT
```

Figure 4 The sequence of amplified *acl*1 gene of *A. caelatus*

Table 5 LAMP reaction conditions and sequences of LAMP primers for *A. nomius*, *A. flavus*, *A. parasiticus* and *A. caelatus* respectively.

Target sequence source	**LAMP condition**	**Primer designation***	**5'-3' Oligonucleotide sequence**
*amy*1 *A. nomius* (DQ467925)	61 ℃ 4%（v/v）formamide	FIP-Anom ID9	CCG GGT CAC CGT TGA GGA CTT GGC CTG GAT ACA ACA AAG C
		BIP-Anom ID9	TGT CCC TAC CAG GAC GTC ATG GGG GTG AGA CTG CAA GAA GAG
		F3-Anom ID9	AAC ACG TCC AGA AGG ACT TC
		B3-Anom ID9	ACT GGT TTT CAT CCG GCT TG

		loopF-Anom ID9	CCG ATG CAG TAC ACG CCT G
		loopB-Anom ID9	CGG CGT ACT GAA CTA CCC AA
*acl*1 *A. oryzae* (XM_001820729)	64 ℃ 2.5%（v/v）formamide	FIP-Afla ID58	TAG ACC TGC TTG AGC ACG CCA TGA GGG AGG CTG GTA TCC
		BIP-Afla ID58	AGG TCA GCA AGG GCA ACA TCC GGC CCA GGA GTA GTC GAT AG
		F3-Afla ID58	ACC GCT GTT GCT AAG AAC AA
		B3-Afla ID58	TTA CGG ACG AGA CCG AGC
		loopF-Afla ID58	ATG TCC TCA AAG GTC TCG GG
		loopB-Afla ID58	GAG CCT GTT CCC CCT AAG AT
*amy*1 *A. parasiticus* (DQ467918)	64 ℃ 2%（v/v）formamide	FIP-Apara ID153	CCT GGG TCT GAT CCT CAT AGT CCA GTT CCC AAG ACT ACT TCC
		BIP-Apara ID153	TTG AGA ATT GCT GGC TAG GAG ATG TAC CAT TCA TTT TTG ACC TCA TC
		F3-Apara ID153	TTA CAG TGT GTT TAA ACC GTT
		B3-Apara ID153	GTA GTT CGA TAC CAA TGT TCC
		loopF-ApaID153	TTG AAT GAG ACA GAA CGA GT
		loopB-ApaID153	TTG CCT GAT CTT GAT ACC A
*acl*1 *A. caelatus* (PCR product)	64 ℃ 3%（v/v）formamide	FIP-Aca ID56	CCA ACT CTG ACC TGG AAA CCG CGT GGA TAC CAG CCT CCC T
		BIP- Aca ID56	CAG CGT GAC CGA ACT GGA CCC ATC ACC AAG CCC ATT GTT G
		F3- Aca ID56	TTG GGC ATG TCC TCA AAG G
		B3- Aca ID56	CAT CGA GGC CGT CAA GAA C
		loopF- Aca ID56	TGT TGC TAA GAA CAA CGC CAT G
		loopB- Aca ID56	TGG CGC AGG TAC CAA TAG C

2.2.4 DNA amplification

Preparation of master mixes for LAMP was done according to the protocol described by Niessen and Vogel (2010). In order to prevent cross contamination, two different sets of pipettes were used for preparation steps involving DNA and for steps involving no DNA. Moreover, preparation of master mixes and preparation and addition of template DNA/incubation of reactions were done in separate rooms. Specific LAMP assays were carried out using separate primer sets according to table 5 for *A. nomius* (Anom ID9), *A. flavus* (Afla ID58), *A. parasiticus* (Apara ID153) and *A. caelatus* (Aca ID56) and run in 200µl Multiply-µStrip Pro 8-strip PCR tubes which were placed in a heating block at a constant temperature to run LAMP reactions. Master mixes were prepared for all LAMP reactions according to the following protocol. Per 25 µl reaction add 2.5 µl 10 × LAMP buffer (200 mM MOPs, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, pH 8.8), 1 µl $MgCl_2$ (200 mM), 3.5 µl dNTP mix (10 mM each nucleotide), 2.6 µl primer mix (1.6 µM FIP, 1.6 µM BIP, 0.8 µM Loop B, 0.8 µM Loop F, 0.2 µM F3 and 0.2 µM B3), 1.0 µl *Bst* or *Bst* 2.0 DNA polymerase (8U from 8000 U/ml stock solution in glycerin), 1µl calcein reagent. The preparation of this reagent was by mixing a 2× LAMP buffer in which $MgSO_4$ was substituted by 25 mM $MnCl_2$ with calcein to result in a 2.5 mM solution which was diluted 1:1 with 80% glycerol and filter sterilized through a 0.2 µm filter cartridge. Aliquots were stored at -20 °C. To increase the specificity, optimum levels of formamide were added to the master mix. Ultrapure water was added to make up 25 µl, including template DNA. Highly purified genomic DNA of *A. nomius* CBS 260.86, *A. flavus* CBS 113.32, *A. parasiticus* CBS 126.62 and *A. caelatus* IBT 29700 was used as positive controls throughout the current study, respectively. Water was added instead of DNA as negative control in all reactions. The specific conditions for the three LAMP reactions are shown in table 5. For visualization of results in assays using indirect calcein fluorescence, reaction tubes were placed on a black photographic cardboard under a 365 nm UV lamp. Positive reactions showed a bright green fluorescence. Results were documented as digital images using a hand held digital camera. For result documentation of LAMP assays run in a real-time turbidimeter, OD 600nm data were recorded per time in a Microsoft Excel® file for further analysis of data.

2.3 LAMP assays

2.3.1 Sensitivity and specificity

Highly purified genomic DNA of *A. nomius*, *A. flavus*, *A. parasiticus* and *A. caelatus* was serially 10-fold-diluted from 10^1 to 10^7 with original concentrations being 3.8 µg/µl, 0.12 µg/µl, 1.0 µg/µl and 2.29 µg/µl, respectively. Sensitivity of reactions was tested by addition of 2 µl of the serially diluted DNA to the LAMP master mix before incubation. The reaction conditions for the four LAMP assays are listed in table 5.

Specificities of the primers sets in LAMP assays for *A. nomius*, *A. flavus*, *A. parasiticus* and *A. caelatus* were analyzed using highly purified genomic DNA extracted from pure cultures of 173 fungal strains as listed in table 6. Reactions with primer sets Anom ID9, Afla ID58, Apara ID153 and Aca ID56 were run for 1 h at 61 °C, 64 °C, 64 °C, and 64 °C(as listed in table 5), respectively, using 200 ng of purified DNA per reactuion as template. Ultrapure water was added as negative control.

2.3.2 Identification of pure cultures

Seventeen fungal species, including 15 fungal species closely related to the respective target organisms and two unrelated species, were selected from the 173 fungal strains listed in table 6 for LAMP assays with pure cultures. The tested fungi were grown on SNA plates and incubated in the dark for 2 days before sporulation. Cell lysate of mycelia from cultures was analyzed directly in the LAMP assays after simple mechanical pre-treatment using Fastprep as described previously (see 2.2.2.2).

2.3.3 Identification of fungal from Brazil nuts

Sixty-nine fungal strains isolated from surface disinfected seeds and shells of Amazonian Brazil nuts as given in table 7 were inoculated on MEA plates and incubated for 5-7 d until sporulation occurred. Conidia were collected from cultures by touching the culture surface with a sterile toothpick. DNA for LAMP assays was released from the conidia on immersed toothpick into 100 µl of ultrapure water by using the bead-beater treatment described above (see 2.2.2.3).

2.3.4 Detection of artificially contaminated food

Peanuts, Brazil nuts, and unroasted coffee beans were infected by spraying 10 ml of the respective fungal conidial suspensions (containing 10^6 conidial/ml) on the surface of the samples.

Among them, peanuts were separated into shells and kernels and subsequently inoculated by spraying with conidial suspensions of *A. flavus* and *A. parasiticus* in separate experiments. Similarly, unroasted coffee beans as well as salted in shell Brazil nuts from a Chinese retail were spray-inoculated with a conidial suspension of *A. nomius*. Also whole shelled Brazil nuts and Brazil nut fragments from a German retail market were spray-infected with the same fungal conidial suspension. Inoculated samples were incubated for 1-2 weeks at room temperature.

Template DNA for the LAMP assays to detect *A. flavus* and *A. nomius* in artificially contaminated peanuts and shelled Brazil nuts, respectively, was prepared as follows: 5 g of intact sample was washed with 10 ml sterile tap water containing 0.1% Tween 20 by mixing for 10 min on a vertical shaker at ambient temperature in sterile 50 ml plastic tubes. After draining the washing fluid into a fresh tube, samples were spun at 6 000 × g for 5 min at ambient temperature. The remaining pellet was washed three times with 5 ml ultrapure water after discarding the supernatants with intermediate spinning under the previous conditions. The clean pellet was resuspended in 0.5 ml of ultrapure water and DNA was extracted using the Fastprep method as previously described for mycelia (see 2.2.2.2). On the other hand, extraction of DNA from *A. nomius* contaminated coffee beans and salted Brazil nuts and *A. parasiticus* contaminated samples of peanuts was performed with the E.Z.N.A.® Bacterial DNA Kit according to the manufacturer's recommendations to prepare LAMP ready template DNA.

2.3.5 Detection of naturally infected Brazil nuts

The samples of naturally contaminated Brazil nuts were analyzed during the current study are listed in table 8. For analysis, ten shelled Brazil nuts were washed three times with sterile deionized water containing 0.1% Tween 20. Washing solutions were collected and pooled for each sample. After centrifugation of the pooled solutions, a clean pellet containing fungal material and debris was obtained by washing three times as described for the artificially contaminated samples under 2.3.4. Template DNA used in LAMP assays of *A. nomius* and *A. flavus* was 10-fold diluted supernatant from the resuspended pellet after bead-beater treatment as described previously (see 2.2.2.3).

2.4 Real-time LAMP assays

2.4.1 Optimized reaction conditions

Three sets of LAMP primers targeting the *A. flavus acl*1 gene, the *A. parasiticus amy*1 gene, and the *A. nomius amy*1 gene, and ingredients of master mix used for previous LAMP assays were adopted for real-time LAMP assays with the following modifications of the previously used protocol: *Bst* polymerase 2.0 was used instead of *Bst* polymerase; master mixes were prepared with no addition of calcein but with higher formamide contents of 5.5% (v/v), 5.5% and 3.7% for the *A. nomius*, *A. flavus* and *A. parasiticus* real-time LAMP assays, respectively. Reactions were incubated at a constant temperature of 65.5 °C for 60 min for Afla ID58, 67 °C for 80 min for Apara ID153, and 66.5 °C for 60 min for Anom ID9. Reactions were run in a Loopamp LA-320C real-time turbidimeter. The LA-320CE software package (EIKEN Chemical Co., LTD, Tokyo, Japan) was used for control of the turbidimeter and real-time turbidity measurement done in intervals of 6 s. Turbidity was monitored at 600 nm in a maximum of 32 parallel reactions in 4 independent heating blocks. A threshold of 0.05 for the first derivative of turbidity against time was set to measure time to threshold (T_t) throughout all experiments.

2.4.2 Analysis of the influence of background DNA

To determine the influence of background sample DNA on the real-time LAMP reaction, 5µl of nontarget DNA extracted from non-infected peanuts, maize and Brazil nuts were mixed with the LAMP reaction mixture after 1, 2, 5 and 10-fold dilution in ultrapure water with the initial concentration was 5.90 µg/µl, 1.57 µg/µl and 1.08 µg/µl, respectively. Two µl highly purified DNA from reference strains with DNA concentrations of 1.6×10^{-4} µg/µl for *A. flavus*, 2.6×10^{-4} µg/µl for *A. nomius*, and 1.0×10^{-4} µg/µl for *A. parasiticus*, respectively, were then added to the reaction mixtures containing background DNA. Samples were then measured by real-time LAMP assays to compare with reactions containing only reference DNA without addition of background DNA.

2.4.3 Quantification of conidia and definition of the contamination level

In order to analyze the sensitivity of the assays on the turbidimeter platform, DNA used as amplification target for real-time LAMP assays was extracted from a 10-fold serial dilution of pure conidial suspensions of *A. flavus* strain CBS 113.32, *A. parasiticus* strain CBS 126.62 and *A. nomius* strain CBS 260.86 using the bead-beater method as described above (see 2.2.2.3). Each

cnidial concentration was analyzed 3 times and the average T_t value was used for further calculations. The time after which the deviation of turbidity (OD600 nm) as a function of time (dturb./dt) reached a threshold level of 0.05 (time to threshold, T_t) was plotted against log conidial number per reaction. Plotting of x versus y resulted in a standard curve which was used to determine the conidium concentrations of unknown samples from their T_t value.

In order to analyze correlations between T_t values and the corresponding contamination levels of sample materials, 5 µl of DNA extracted from artificially contaminated peanuts, maize, and Brazil nuts (10^6 to 10 conidia/g in 10-fold serial conidia dilution) immediately after inoculation using the modificated CTAB method described previously (see 2.2.2.4).

3 Results

3.1 Design of primers

Primers used for the LAMP and real-time LAMP assays of *A. nomius*, *A. parasiticus*, *A. flavus* or *A. caelatus* were designed using the PrimerExplorer V. 4 software tool available on the Eiken Genome site (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd.(Tokyo, Japan). The software generated about five primer sets for each target gene. *In silico* testing of all primers was performed by using the nucleotide BLAST search tool on the NCBI sequence database. All the sequences of the selected primers which showed that the value of dG (dimmer) was about -2.0 and had no significant hits to non-target species in the *in silico* tests are listed in table 5 (Page 29). Figure 5 shows the positions of each selected primer within the nucleotide sequence of the *amy*1 genes of *A. parasiticus* and *A. nomius*, respectively, and the *acl*1gene of *A. flavus* and *A. caelatus*, respectively. The smallest possible LAMP product is marked in gray for each primer set. As showed in figure 3 (Page 17), reactions of assays for *A. nomius*, *A. flavus*, *A. parasiticus* and *A. caelatus* resulted in the production of high amounts of DNA with the smallest fragment having the predicted size of 151 bp, 152bp, 145 bp and 153 bp, respectively, which was from the 5’ end of the F1c part of the forward inner primer (FIP) to the 5’end of the B1c part of the backward inner primer (BIP).

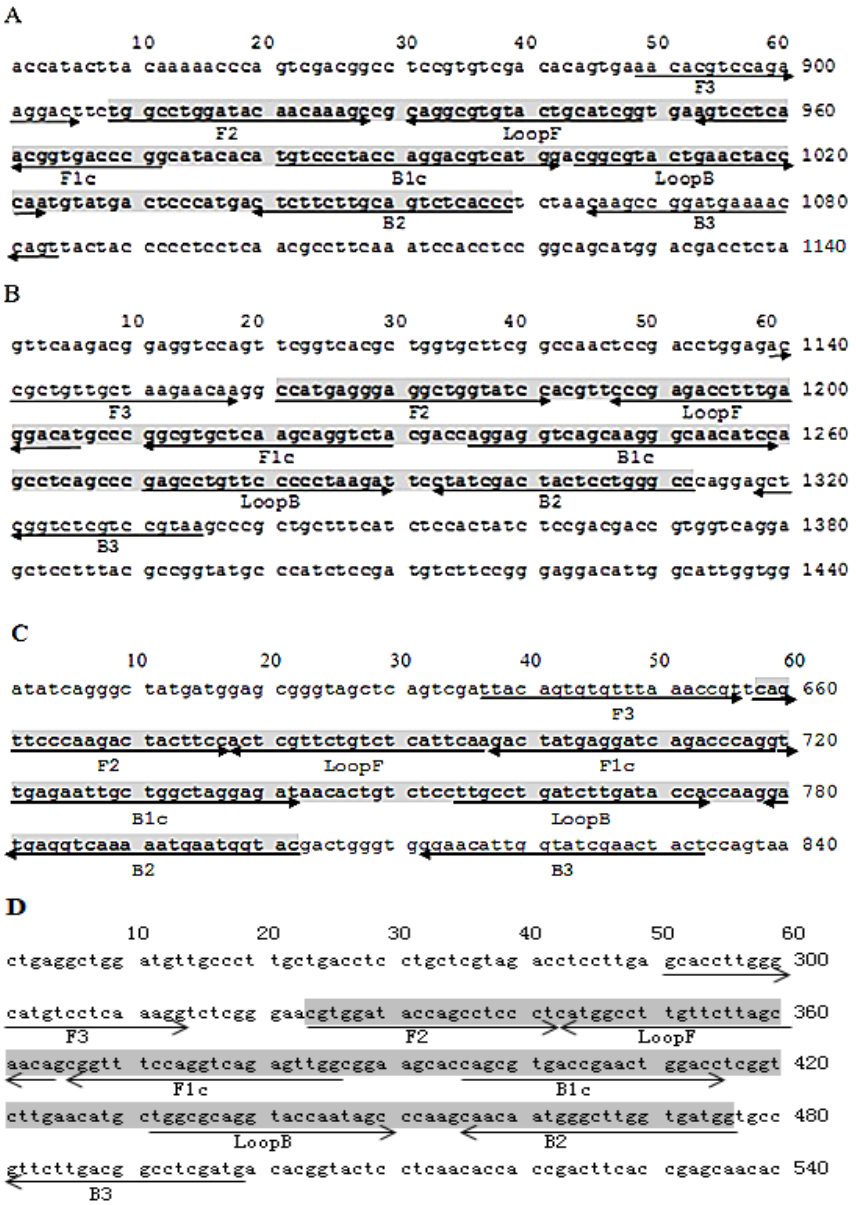

Figure 5 Positioning and orientation of four target fungi specific LAMP primers. A. *A. nomius* specific LAMP primers, Anom ID9; B. *A. flavus* specific LAMP primers Afla ID58; C. *A. parasiticus* specific LAMP primers Apara ID153; D. *A. caelatus* specific LAMP primers Aca ID56.The smallest LAMP fragments (151 bp, 152 bp, 145 bp and 153 bp, respectively) are marked in gray.

3.2 Sensitivity of LAMP assays

Addition of calcein to the LAMP master mix led to a bright green fluorescence visible against a background of black photographic cardboard under UV 366 nm light and indicated positive LAMP reactions as shown in figure 6. However, the fluorescence of the calcein indicator was still quenched with manganese in the negative control reaction since no complexation of the metal with pyrophosphate, a byproduct of enzymatic DNA synthesis, appeared. LAMP reactions were

positive after addition of purified target DNA until dilutions of 10^{-5}, 10^{-6}, 10^{-5} and 10^{-4} for *A. flavus*, *A. nomius*, *A. parasiticus* and *A.caelatus*, respectively. From the results obtained, detection limits of 2.4, 7.6, 20 and 458 pg DNA/reaction were calculated for *A. flavus*, *A. nomius*, *A. parasiticus* and *A.caelatus*, respectively. No difference in fluorescence intensity of the LAMP signal was found between dilutions of target DNA in none of the assays. No fluorescence appeared in samples with DNA concentrations below the detection limits of the respective LAMP assays. Negative controls set up with water instead of DNA showed no fluorescence under the same conditions.

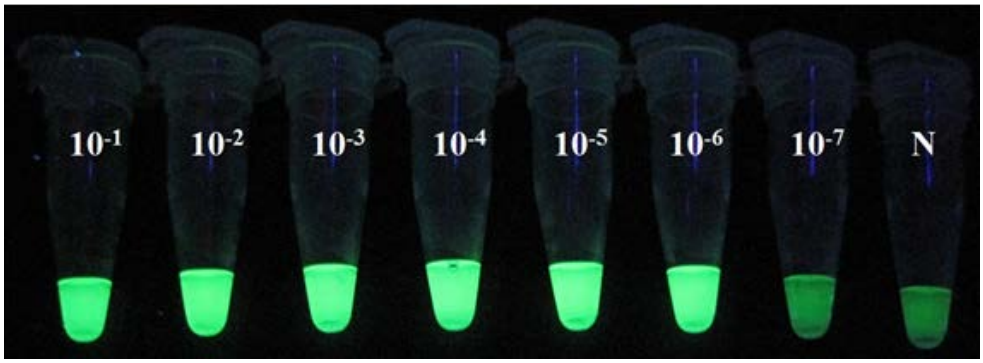

Figure 6 Sensitivity of the LAMP assay for the *amy1* gene of *A. nomius* using primer set Anom ID9. Calcein fluorescence under UV_{366nm} light. The original concentration of *A. nomius* genomic DNA was 3.8 µg/µl, so the amount of DNA in reaction was 10-folds diluted form 10^{-1} = 760 ng/reaction (rxn.) to 10^{-7} = 760 fg/rxn. N = negative control, water added instead of DNA.

3.3 Specificity of LAMP assays

Specificity of the LAMP assays with primer sets Anom ID9, Afla ID58, Apara ID153 and Aca ID56 (see table 5 for sequences) for the detection of *A. nomius*, *A. flavus*, *A. parasiticus* or *A. caelatus* was analyzed using genomic DNA extracted from pure cultures of 173 fungal strains as listed in table 6. LAMP reactions were run under isothermal conditions as given in table 5 for 60 min with addition of formamide to the master mix as indicated. Two hundred nanogram of purified DNA was added into LAMP master mixes for specificity testing. None of the highly purified genomic DNA of *Penicillium* spp., *Fusarium* spp., *Emericella* spp. and 34 species from other fungal genera resulted in a positive signal with none of the four primer sets. Testing DNA purified from *Aspergillus* species, only *A. nomius* gave a LAMP signal with primer set Anom ID 9, and all non-target *Aspergillus* spp. were tested negative. Testing of *Aspergillus* spp. DNA with primer set Apara ID153 resulted in a positive LAMP reaction with DNA of *A. parasiticus*, *A.*

sojae and *A. toxicarius*. Testing of *Aspergillus* spp. DNA with primer set Afla ID58 revealed that LAMP reaction was positive with *A. minisclerotigenes*, *A. oryzae*, *A. parasiticus*, *A. parvisclerotigenus*, *A. sojae* and *A. toxicarius*. Testing the specificity of primer set Aca ID56 for detection of *A. caelatus* resulted in positive LAMP reactions with DNA extracted from *A. caelatus*, *A. archidicola* and *A. bombycis*.

Table 6 Fungal isolates used during the current study and reaction of genomic DNA isolated from them in four different LAMP assays.

Genus	Species	Strain	LAMP primer set			
			Anom ID 9	Afla ID58	Apara ID153	Aca ID56
Alternaria	*alternata*	[9]TMW 4.0438	-	-	-	-
	mali	[3]CBS 106.24	-	-	-	-
	spec.	TMW 4.1428	-	-	-	-
Aspergillus	*aculeatus*	TMW 4.1776	-	-	-	-
	alliaceus	[4]DSM 813	-	-	-	-
	arachidicola	[5]IBT 27128	-	-	-	+
	auricomus	CBS 467.65	-	-	-	-
	awamori	CBS 101704	-	-	-	-
	bombycis	IBT 23536	-	-	-	+
	bridgeri	CBS 350.81	-	-	-	-
	caelatus	IBT 29700	-	-	-	P
	carbonarius	TMW 4.1512	-	-	-	-
	clavatus	CBS 513.65	-	-	-	-
	elegans	CBS 310.80	-	-	-	-
	ellipticus	CBS 707.79	-	-	-	-
	flavus	CBS 113.32	-	**P**	-	-
	foetidus	CBS 114.49	-	-	-	-
	fresenii	CBS 550.65	-	-	-	-
	fumigatus	CBS 113.55	-	-	-	-
	helicothrix	CBS 677.79	-	-	-	-
	heteromorphus	CBS 117.55	-	-	-	-
	insulicola	CBS 382.75	-	-	-	-
	japonicus	CBS 114.51	-	-	-	-
	minisclerotigenes	IBT 27177	-	+	-	-
	niger	CBS 101698	-	-	-	-
	nomius	CBS 260.86	**P**	-	-	-
	ochraceus	CBS 263.67	-	-	-	-
	ochraceoroseus	CBS 101887	-	-	-	-
	oryzae	IBT 28103	-	+	-	-
	parasiticus	CBS 126.62	-	+	**P**	-
	parvisclerotogenes	IBT 3850	-	+	-	-
	petrakii	CBS 105.57	-	-	-	-
	pseudotararii	IBT 21092	-	-	-	-
	rambellii	IBT 14580	-	-	-	-
	sclerotiorum	CBS 549.65	-	-	-	-
	sojae	IBT 21643	-	+	+	-
	tamarii	CBS 591.68	-	-	-	-
	terreus	CBS 377.64	-	-	-	-
	toxicarius	CBS 822.72	-	+	+	-

	tubingensis	[6]ITEM 4496	-	-	-	-
	usamii var.shiro-usamii	CBS 101700	-	-	-	-
	versicolor	CBS 245.65	-	-	-	-
Beltraniella	*portoricensis*	CBS 856.70	-	-	-	-
Bipolaris	*sorokiniana*	CBS 311.64	-	-	-	-
Cladobotryum	*dendroides*	[8]NRRL 2903	-	-	-	-
Colletotrichum	*acutatum*	CBS 295.67	-	-	-	-
	fragariae	CBS 142.31	-	-	-	-
	gloeosporioides	CBS 285.50	-	-	-	-
Cryptomela	*acutata*	CBS 157.33	-	-	-	-
Drechslera	*teres*	CBS 378.59	-	-	-	-
	tricici-repentis	CBS 265.80	-	-	-	-
	spec.	TMW 4.0428	-	-	-	-
Epicoccum	*nigrum*	TMW 4.1407	-	-	-	-
Fusarium	*acuminatum*	TMW 4.0298	-	-	-	-
	anguioides	CBS 172.32	-	-	-	-
	annulatum	CBS 258.54	-	-	-	-
	anthophilum	CBS 222.76	-	-	-	-
	aquaed.var.aquaed	CBS 837.85	-	-	-	-
	armeniacum	CBS 485.94	-	-	-	-
	arthrosporioides	CBS 173.32	-	-	-	-
	avenaceum	[1]BBA 70723	-	-	-	-
	bactridioides	CBS 177.35	-	-	-	-
	beomiforme	BBA 69406	-	-	-	-
	brasilicum	TMW 4.1391	-	-	-	-
	buharicum	CBS 796.70	-	-	-	-
	camptoceras	ITEM 1235	-	-	-	-
	caucasicum	CBS 179.35	-	-	-	-
	cavispermum	CBS 171.31	-	-	-	-
	cerealis	CBS 589.93	-	-	-	-
	chlamydosporum	CBS 145.25	-	-	-	-
	coeruleum	CBS 836.85	-	-	-	-
	compactum	CBS 466.92	-	-	-	-
	concolor	CBS 183.34	-	-	-	-
	crookwellense	CBS 623.85	-	-	-	-
	culmorum	DSM 62191	-	-	-	-
	decemcellulare	CBS 113.57	-	-	-	-
	detonianum	CBS 736.79	-	-	-	-
	dimerum	CBS 175.31	-	-	-	-
	dlamini	[7]MRC 3024	-	-	-	-
	equiseti	CBS 406.86	-	-	-	-
	eumartii	DSM 62809	-	-	-	-
	flocciferum	CBS 831.85	-	-	-	-
	fujikuroi	DSM 63217	-	-	-	-
	globosum	MRC 6646	-	-	-	-
	graminearum	DSM 4529	-	-	-	-
	graminum	DSM 62224	-	-	-	-
	heterosporum	DSM 62231	-	-	-	-
	inflexum	DSM 63203	-	-	-	-
	javanicum	DSM 62233	-	-	-	-
	kyushuense	BBA 70812	-	-	-	-
	langsethiae	TMW 4.0072	-	-	-	-
	larvarum	CBS 783.79	-	-	-	-
	longipes	CBS 739.79	-	-	-	-
	lunulosporum	CBS 636.76	-	-	-	-
	macroceras	CBS 146.25	-	-	-	-
	melanochlorum	CBS 202.65	-	-	-	-

	merismoides var. chlamydosporum	DSM 62256	-	-	-	-
	musarum	ITEM 1294	-	-	-	-
	napiforme	BBA 67629	-	-	-	-
	nelsonii	ITEM 1681	-	-	-	-
	neoceras	CBS 147.25	-	-	-	-
	nygamai	BBA 67375	-	-	-	-
	oxysporum	DSM 62292	-	-	-	-
	oxysporum	CBS 310.87	-	-	-	-
	poae	DSM 62376	-	-	-	-
	proliferatum	DSM 62261	-	-	-	-
	reticulatum	DSM 62395	-	-	-	-
	robustum	CBS 637.76	-	-	-	-
	sacchari v. subglutinans	CBS 215.76	-	-	-	-
	sambucinum	CBS 185.29	-	-	-	-
	scirpi	CBS 448.84	-	-	-	-
	semitectum var. majus	DSM 63310	-	-	-	-
	setosum	CBS 574.94	-	-	-	-
	solani	DSM 62416	-	-	-	-
	sporotrichioides	CBS 412.86	-	-	-	-
	subglutinans	TMW 4.0947	-	-	-	-
	sublunatum	CBS 189.34	-	-	-	-
	succisae	DSM 63162	-	-	-	-
	sulphureum	DSM 62433	-	-	-	-
	tabacinum	DSM 2125	-	-	-	-
	torulosum	BBA 64465	-	-	-	-
	trichothecioides	CBS 136.73	-	-	-	-
	tricinctum	CBS 410.86	-	-	-	-
	tumidum	CBS 486.76	-	-	-	-
	udum	DSM 62451	-	-	-	-
	verticillioides	CBS 218.76	-	-	-	-
	venenatum	CBS 458.93	-	-	-	-
	xylarioides	CBS 258.52	-	-	-	-
Geotrichum	*candidum*	TMW 4.0508	-	-	-	-
Gliocephalotrichum	*spec. nov.*	NRRL 2993	-	-	-	-
Gliocladium	*roseum*	TMW 4.0425	-	-	-	-
	viride	TMW 4.1916	-	-	-	-
Hypomyces	*rosellus*	CBS 521.81	-	-	-	-
Memnoniella	*echinata*	CBS 627.61	-	-	-	-
Microdochium	*majus*	TMW 4.0496	-	-	-	-
	nivale	TMW 4.0495	-	-	-	-
Monascus	*ruber*	TMW 4.1426	-	-	-	-
Mucor	*mucedo*	DSM 809	-	-	-	-
Penicillium	*verrucosum*	IBT 12935	-	-	-	-
	aurantiogriseum	CBS 225.90	-	-	-	-
	camemberti	DSM 1233	-	-	-	-
	chrysogenum	CBS 306.48	-	-	-	-
	chrysogenum	CBS 573.68	-	-	-	-
	commune	CBS 311.48	-	-	-	-
	commune	CBS 107.11	-	-	-	-
	corylophilum	CBS 321.48	-	-	-	-
	crustosum	CBS 499.73	-	-	-	-
	digitaum	DSM 62840	-	-	-	-
	expansum	DSM 62841	-	-	-	-
	glabrum	TMW 4.2027	-	-	-	-
	griseofulvum	TMW 4.1543	-	-	-	-
	italicum	DSM 62846	-	-	-	-

	nalgiovense	TMW 4.1371	-	-	-	-
	nordicum	[2]BFE 487	-	-	-	-
	olsonii	TMW 4.1362	-	-	-	-
	purpurescens	CBS 223.28	-	-	-	-
	purpurogenum	CBS 286.36	-	-	-	-
	roquefortii	CBS 221.30	-	-	-	-
	roseopurpureum	TMW 4.1770	-	-	-	-
	rugulosum	TMW 4.1902	-	-	-	-
	variabile	CBS 385.48	-	-	-	-
Stachybotrys	*chartarum*	TMW 4.0523	-	-	-	-
Trichoderma	*harzianum*	TMW 4.1502	-	-	-	-
	virens	CBS 344.47	-	-	-	-
	hamatum	TMW 4.1882	-	-	-	-
	longibrachiatum	TMW 4.1940	-	-	-	-
Trichoderma	*paucisporum*	TMW 4.2032	-	-	-	-
Trichothecium	*roseum*	CBS 567.50	-	-	-	-
Zygosaccharomyces	*bailii*	TMW 3.058	-	-	-	-
	bisporus	TMW 3.062	-	-	-	-
	rouxii	TMW 3.057	-	-	-	-

P = positive control
+ = green fluorescence under UV-365 nm light
- = no green fluorescence under UV-365 nm light
[1]BBA = Julius Kühn Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, DE
[2]BFE = Max Rubner Institut, Bundesforschungsanstalt für Ernährung, Karlsruhe, DE
[3]CBS = Centraalbureau vor Schimmelcultures, Utrecht, NL
[4]DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, DE
[5]IBT = Technical University of Denmark, Department of Biotechnology, Lyngby, DK
[6]ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, IT
[7]MRC = South African Medical Research Council, Tygerberg, SA
[8]NRRL = Northern Regional Research Laboratory, Peoria (Illinois), USA
[9]TMW = Technische Mikrobiologie Weihenstephan, Freising, DE

3.4 Identification of fungal pure cultures

The usefulness of the LAMP assays for the identification of pure cultures was tested with 17 fungal species incubated on SNA plates before analysis, including 15 fungal species closely related to the respective target organisms and two unrelated species. Cell lysate from cultures was added as template directly in the mater mix after simple mechanical pre-treatment with Fastprep for mycelium as described previously (see 2.2.2.2). Following the LAMP reactions, those species which already showed a positive signal in LAMP specificity testing with pure genomic DNA (compare table 6) also gave positive signals when crude mycelial extracts were used as template (see figure 7).

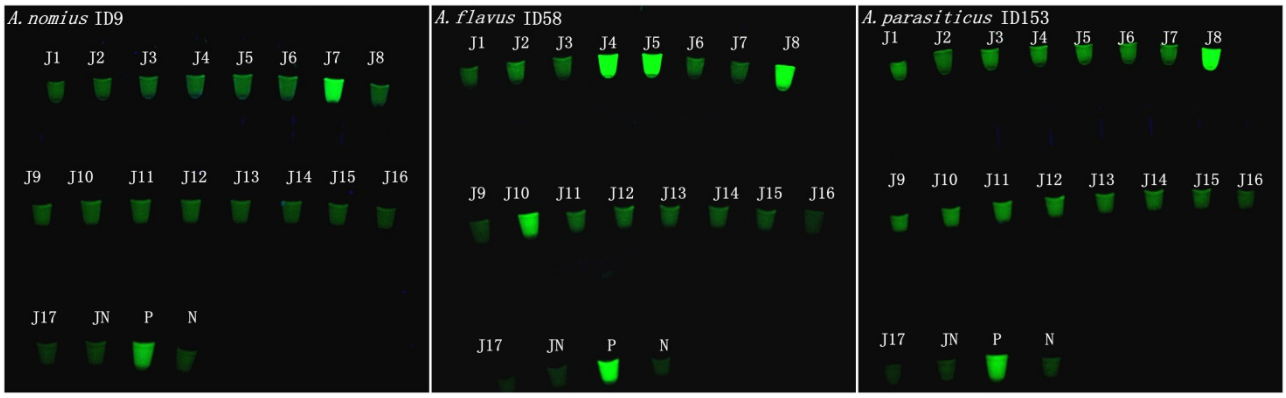

Figure 7 LAMP reactions with cell lysate of 17 fungal species from mycilia of SNA grown cultures using primer sets Anom ID 9, Afla ID58, and Apara ID153. J1 = *Aspergillus arachidicola* IBT27128; J2 = *A. bombycis* IBT 23536; J3 = *A. caelatus* IBT 29700; J4 = *A. minisclerotigenes* IBT 27177; J5 = *A. flavus* CBS 113.32; J6 = *A. niger* CBS 101698; J7 = *A. nomius* CBS 260.86; J8 = *A. parasiticus* CBS 126.62; J9 = *A. pseudotarmarii* IBT 21092; J10 = *A. parvisclerotoigenes* IBT 3850; J11 = *A. rambellii* IBT 14580; J12 = *A. tamarii* CBS 591.68; J13 = *Emmericella astellata* IBT21903; J14 = *E. olivicola* IBT26499; J15 = *E. venezuelensis* IBT20956; J16 = *Fusarium graminearum* DSM 4529; J17 = *Penicillium commune* CBS 311.48; JN = SNA substrate; P = positive control, highly purified genomic DNA of target species; N = negative control, water added instead of DNA.

3.5 LAMP assays conidia from fungal pure cultures

Detecting the conidia of *A. flavus* and *A. nomius*, the LAMP assays showed different sensitivity with primer sets Afla ID58 and Anom ID9. When intact conidia were added directly as template to LAMP reactions, the sensitivity of assays was 10^4 and 10^5 conidia per reaction for detection of *A. flavus* and *A. nomius*, respectively. When conidia were pre-treated by disruption and boiling, detection limits considerably decreased to 100 conidia per reaction and 10 conidia per reaction for *A. flavus* and *A.nomius*, respectively (figure 8). Similar results were obtained with the sensitivity of LAMP assay for detecting conidia of *A. parasiticus* and *A. caelatus*, which was increased 10 fold from 10^4 to 10^3 conidia detected per reaction and 1 000 fold from 10^4 to 10 conidia per reaction, respectively, after the same pre-treatment. Results demonstrate that higher sensitive detection can be achieved by conidium disruption prior to addition to LAMP reaction.

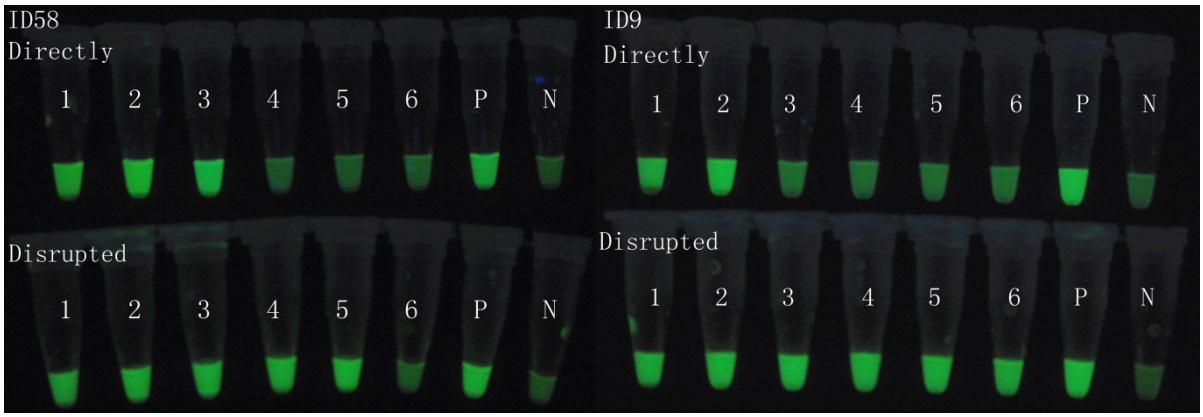

Figure 8 LAMP assays for *A. flavus* (left panel) and *A. nomius* (right panel) using 10-fold serial dilutions of conidia added directly (upper row) and after previous treatment (under row). 1-6: concentration of conidia of the respective species from 1 = 10^6 to 6 = 10 conidia per reaction; P: positive control (pure DNA of *A. flavus* and *A. nomius*, resp.); N: negative control (water added instead of DNA).

3.6 Identification of fungal isolates from Brazil nuts

Identification of 69 fungal pure cultures isolated from surface disinfected seeds and shells of Amazonian Brazil nuts (see table 7) was achieved with the LAMP assays for *A. flavus, A. nomius* and *A. caelatus*. *A. parasiticus* was not isolated from any of the samples. Positive and negative results obtained with the LAMP assays were compared with the results published by Gonçalves et al. (2012) and Calderari et al. (2013), who identified the same set of isolates using microscopic morphological examination. As compared to morphological identifications, results obtained with the LAMP assays had accuracy (percentage of the consistent results) of 85.3%, 92.6% and 100% for identification of *A. flavus*, *A. nomius*, and *A. caelatus*, respectively. In the LAMP assays for *A. flavus* using primer set Afla ID58, all of the strains assigned to *A. flavus* were confirmed by a positive LAMP reaction. Also 8 *A. caelatus* and 2 *A. nomius* strains reacted positive with the *A. flavus* specific primer set, however, none of the DNA from other fungal species gave a positive LAMP reaction. There seems to be a cross reaction of the *A. flavus* specific primer set with *A. caelatus* which was previously undetected during specificity testing with pure DNA as given in table 6. On the contrary, no cross reactions with *A. flavus* DNA occurred when primer set Aca ID56 was used in a LAMP assay. With these primers, all strains assigned to *A. caelatus* and one strain of *A. archidicola* were tested positive. As for the analysis of the *A. nomius* specific primer set Anom ID9, the assay identified 28 out of 32 isolates assigned to the species by morphological identification and failed to give a signal in 4 such isolates. There was one false positive reaction with *A. arachidicola*.

Table 7 Fungal isolates from Brazil nuts used during the current study and results of LAMP analysis.

Species name	Strain (see Soares Gonçalves et al., 2012)	Clone	LAMP assay		
			Anom ID9	Afla ID58	Aca ID56
A. arachidicola	ITAL 189	TMW 4.2312	+	-	+
A. caelatus	ITAL 91	TMW 4.2353	-	-	+
A. caelatus	ITAL 97	TMW 4.2348	-	+	+
A. caelatus	ITAL 201	TMW 4.2303	-	+	+
A. caelatus	ITAL 212	TMW 4.2359	-	+	+
A. caelatus	ITAL 243	TMW 4.2287	-	-	+
A. caelatus	ITAL 467	TMW 4.2360	-	-	+
A. caelatus	ITAL 504	TMW 4.2286	-	+	+
A. caelatus	ITAL 562	TMW 4.2295	-	-	+
A. caelatus	ITAL 566	TMW 4.2349	-	+	+
A. caelatus	ITAL 584	TMW 4.2281	-	+	+
A. caelatus	ITAL 695	TMW 4.2308	-	-	+
A. caelatus	ITAL 787	TMW 4.2310	-	-	+
A. caelatus	ITAL 1574	TMW 4.2327	-	+	+
A. caelatus	ITAL 1576	TMW 4.2362	-	+	+
A. flavus	ITAL 58	TMW 4.2342	-	+	-
A. flavus	ITAL 59	TMW 4.2301	-	+	-
A. flavus	ITAL 71	TMW 4.2333	-	+	-
A. flavus	ITAL 73	TMW 4.2356	-	+	-
A. flavus	ITAL 92	TMW 4.2289	-	+	-
A. flavus	ITAL 99	TMW 4.2363	-	+	-
A. flavus	ITAL 112	TMW 4.2374	-	+	-
A. flavus	ITAL 136	TMW 4.2354	-	+	-
A. flavus	ITAL 168	TMW 4.2355	-	+	-
A. flavus	ITAL 179	TMW 4.2313	-	+	-
A. flavus	ITAL 295	TMW 4.2332	-	+	-
A. flavus	ITAL 758	TMW 4.2346	-	+	-
A. flavus	ITAL 1257	TMW 4.2364	-	+	-
A. flavus	ITAL 1306	TMW 4.2302	-	+	-
A. flavus	ITAL 1836	TMW 4.2361	-	+	-
A. flavus	ITAL 3919	TMW 4.2337	-	+	-
A. nomius	ITAL 255	TMW 4.2330	+	-	-
A. nomius	ITAL 256	TMW 4.2331	+	-	-
A. nomius	ITAL 438	TMW 4.2305	+	-	-
A. nomius	ITAL 486	TMW 4.2314	+	-	-
A. nomius	ITAL 532	TMW 4.2285	+	-	-
A. nomius	ITAL 587	TMW 4.2350	+	-	-
A. nomius	ITAL 608	TMW 4.2343	+	-	-
A. nomius	ITAL 618	TMW 4.2300	+	-	-

A. nomius	ITAL 638	TMW 4.2329	+	-	-
A. nomius	ITAL 648	TMW 4.2328	+	-	-
A. nomius	ITAL 650	TMW 4.2357	-	-	-
A. nomius	ITAL 657	TMW 4.2315	+	-	-
A. nomius	ITAL 696	TMW 4.2336	+	-	-
A. nomius	ITAL 699	TMW 4.2334	+	-	-
A. nomius	ITAL 709	TMW 4.2341	+	-	-
A. nomius	ITAL 723	TMW 4.2307	+	-	-
A. nomius	ITAL 730	TMW 4.2347	-	+	-
A. nomius	ITAL 741	TMW 4.2335	+	-	-
A. nomius	ITAL 743	TMW 4.2344	+	-	-
A. nomius	ITAL 764	TMW 4.2297	-	-	-
A. nomius	ITAL 849	TMW 4.2292	+	-	-
A. nomius	ITAL 1228	TMW 4.2298	+	-	-
A. nomius	ITAL 1230	TMW 4.2299	+	-	-
A. nomius	ITAL 1325	TMW 4.2283	-	-	-
A. nomius	ITAL 3052	TMW 4.2304	+	-	-
A. nomius	ITAL 3087	TMW 4.2311	+	-	-
A. nomius	ITAL 3333	TMW 4.2345	+	-	-
A. nomius	ITAL 4466	TMW 4.2294	+	-	-
A. nomius	ITAL 6071	TMW 4.2365	+	-	-
A. nomius	ITAL 6226	TMW 4.2284	-	+	-
A. nomius	ITAL 6245	TMW 4.2358	-	-	-
A. nomius	ITAL 6251	TMW 4.2309	+	-	-
A. pseudotamarii	ITAL 791	TMW 4.2339	-	-	-
A. pseudotamarii	ITAL 792	TMW 4.2338	-	-	-
A. tamarii	ITAL 119	TMW 4.2296	-	-	-
A. tamarii	ITAL 129	TMW 4.2352	-	-	-
A. tamarii	ITAL 226	TMW 4.2306	-	-	-

3.7 LAMP assays with artificially contaminated nuts and coffee beans

The application of the LAMP assays to detect *A. flavus*, *A. nomius* and *A. parasiticus* was evaluated by detecting the three target fungi in artificially contaminated sample materials. Before analyzed with LAMP assays, DNA preparation was performed by extracting DNA from samples of artificially contaminated commodities using either a very simple protocol or a DNA purification kit.

Whole peanut pods as well as peanuts separated into shells and kernels were innoculated by spraying with conidial suspensions of *A. flavus* and *A. parasiticus* in separate experiments. DNA extracted by a very simple protocol (see 2.2.2.3) was diluted 10 fold before it was used as

template in the *A. flavus* specific LAMP assay. As shown in figure 9A, only the DNA extracted from whole peanut pods showed a fluorescent signal in the *A. flavus* specific LAMP assay when samples were infected with the reference fungus. No fluorescent signal was obtained when 10 fold diluted extracts from either shells or nuts were tested. However, the 1 000 fold diluted crude DNA extracts of shell and nuts showed fluorescence signals in a subsequent LAMP assay (figure 9A1). Results suggested that inhibiting compounds may be present in the extracts of nuts and shells. Using a DNA isolation kit, the 10 fold diluted and purified DNA from peanut samples artificially inoculated with *A. parasiticus* conidia all showed positive results in the LAMP assay for detection of *A. parasiticu* (figure 9B).

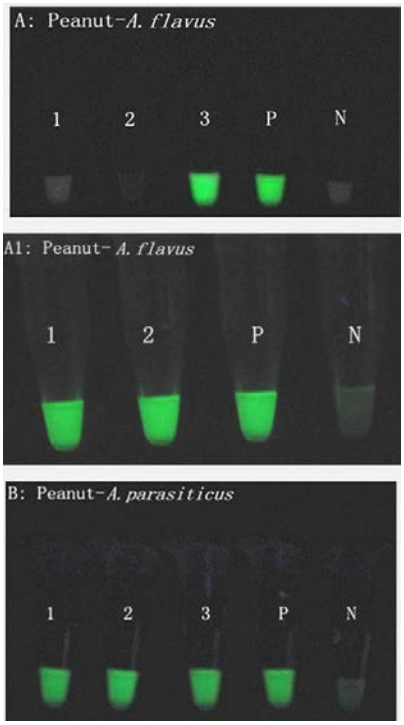

Figure 9 LAMP reaction with crude extracted DNA or Kit extracted DNA of artificially contaminated peanut samples. Calcein fluorescence under UV366nm light. A: LAMP assay for crude extracted DNA of artificially contaminated peanuts by *A. flavus*, 1.shell of peanut; 2. nut of peanut; 3. whole peanut pod; P. DNA of *A. flavus* as Positive control; N. Negative control, water added instead of DNA. A1: Further LAMP assay for crude extracted DNA of artificially contaminated peanuts by *A. flavus*, 1. shell of peanut; 2. nut of peanut; P. DNA of *A. flavus* as positive control; N. negative control, water added instead of DNA. B: LAMP assay for Kit extracted DNA of artificially contaminated peanuts by *A. parasiticus*, 1. shell of peanut; 2. nut of peanut; 3. whole peanut; P. DNA of *A. parasiticus* as positive control; N. negative control, water added instead of DNA.

Similarly, unroasted coffee beans as well as salted Brazil nuts from a China retail market and separated into nut and shell were infected by spraying with the *A. nomius* conidial suspension. Also whole shelled and chopped Brazil nuts from a German retail were inoculated with conidial

suspension of the same fungus. After incubation, no fungal growth was observed on the shells of salted Brazil nuts. In the analysis of unroasted coffee beans and salted Brazil nuts with the LAMP assay for *A. nomius*, DNA was extracted with a DNA isolation kit and diluted 10 fold before addition to LAMP reactions (see figure 10A). Nevertheless, the simple protocol was shown to be useful to prepare crude extracts of whole shelled Brazil nuts and Brazil nut fragments that also showed positive results in the LAMP assay after 10 fold dilution (figure 10B).

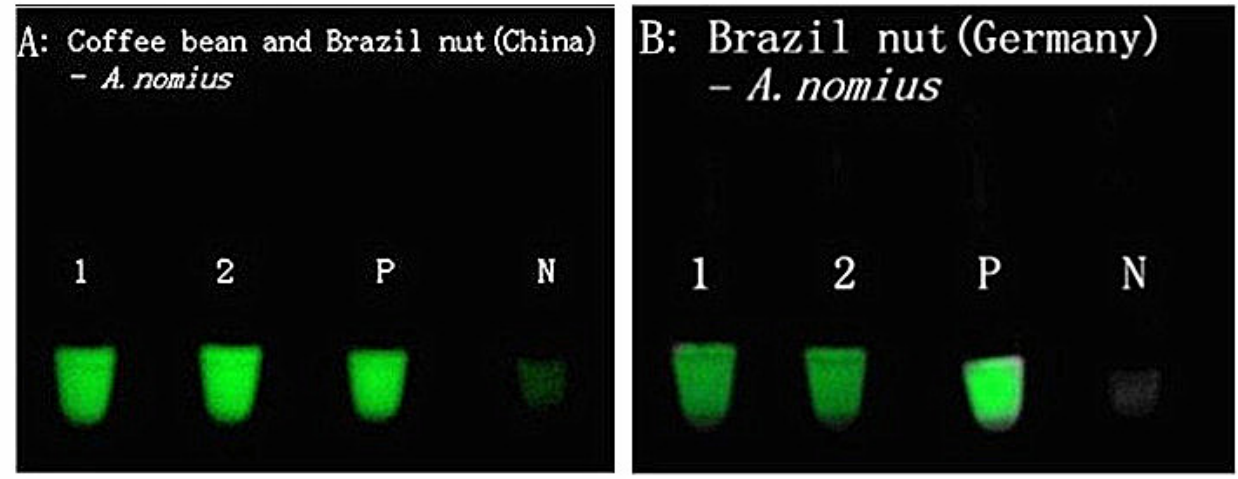

Figure 10 LAMP reactions with crude extracted DNA or Kit extracted DNA of unroasted coffee beans and Brazil nuts artificially contaminated with *A. nomius*. Calcein fluorescence under UV366nm light. A: LAMP assay for Kit extracted DNA of artificially contaminated unroasted coffee bean and salted Brazil nuts, 1. coffee bean; 2.salted Brazil nut (China); P. DNA of *A. nomius* as positive control; N. negative control, water added instead of DNA. B: LAMP assay for crude DNA extracts of *A. nomius* contaminated Brazil nuts (Germany), 1. whole nut; 2. pieces of nut; P. DNA of *A. nomius* as positive control; N. negative control, water added instead of DNA.

3.8 LAMP assays with naturally infected Brazil nuts

During the current study, the LAMP assays for *A. flavus* and *A. nomius* were also applied to analyze the fungi in samples of naturally infected Brazil nuts. The results of both LAMP assays are listed in table 8 together with results of two previous studies which analyzed the presence of *A. flavus* and *A. nomius* using microbiological methods (Gonçalves et al., 2012) and the presence and concentrations of total aflatoxins (Calderari et al., 2013) in the same samples.

Table 8 Detection of the presence of *A. nomius* and *A. flavus* in naturally infected Brazil nuts by microbiological plating and LAMP assays together with data on presence or absence of total aflatoxin in corresponding samples.

Sample ID	Origin and State	Result plating (no. of infected nuts in lots of 50)		Result LAMP assay		Aflatoxin present in sample
		A. nomius	*A. flavus*	*A. nomius* Anom ID9	*A.flavus* Afla ID58	
2	Rainforest, Parà	0	12	+	-	-
8		5	21	+	-	+
10	Supermarket, Campinas	1	1	+	-	+
12		0	1	+	-	+
15	Market, Parà	0	0	+	+	-
17		0	0	+	+	+
21	Supermarket, Sao Paulo	0	0	-	+	+
26	Market, Amazon	8	1	+	-	-
32	Market, Parà	1	24	-	-	-
37	Processing, Parà	0	10	+	+	-
41		0	0	+	+	-
51		2	12	-	+	+
53		0	31	-	+	+
80	Market, Amazon	5	1	+	+	+
169	Processing, Parà	0	1	+	+	+
170		0	0	-	+	+
172		5	1	+	-	+
177		0	1	-	+	+
178		3	18	-	+	+
179		8	17	-	+	+
180		1	1	-	+	+
181		0	1	+	+	+
182		0	0	-	+	+
195		0	2	+	-	+
196	Supermarket, Sao Paulo	0	1	+	+	+
197		0	7	-	+	+
198		0	2	+	+	+
199		3	2	+	+	+
202		0	0	-	+	+
222	Market, Amazon	0	0	-	-	-
233		10	18	+	+	-
234		5	11	+	+	-

Results given in table 8 for the microbiological analysis showed that *A. flavus* was present in 24 out of 32 samples (75%) with frequencies (number of contaminated particles per total number of particles analyzed per sample) varying between 2% and 62%. *A. nomius*, which always co-occurred together with *A. flavus*, was present in 13 of the samples (40%) with frequencies ranging from 2% to 20%. Eight of the samples (25%) were found to be uninfected with either of the two species. Preliminary to LAMP assays for the two species, the DNA template was prepared from the debris fraction after disruption and boiling of surface washings (see 2.2.2.3)

obtained by shaking 10 Brazil nut seeds per sample with water containing 0.1% Tween 20. Results obtained from the LAMP assays suggested that 30 of the samples (94%) were contaminated with either of the two fungi whereas two samples (6%) showed no reaction in either LAMP assay. In particular, 23 out of 32 samples (72%) showed a positive result in the LAMP assay for *A. flavus* while 19 of the samples (59%) were positive in the *A. nomius* specific LAMP assay. Moreover, 12 samples (37%) were positive in both assays. When compared with the results of mycological analysis, 11 of the samples (34%) were positive in the LAMP assay for *A. nomius* whereas no presence of that fungus was detected in these samples. In turn, five other samples were negative in the *A. nomius* specific LAMP assays although *A. nomius* had been detected in microbiological analysis, even with high frequency (16%) in one sample (no. 179). According to results obtaines with the Afla ID58 LAMP assay for *A. flavus*, 7 samples (22%) were determined to be contaminated by the fungus whereas no detection had been recorded for *A. flavus* according to the microbiological analysis. On the other hand, no LAMP signal occurred in the LAMP assay in 7 other samples (22%) even though they had been tested to be contaminated by the fungus. By comparing the results of LAMP assays to those of mycological analysis as the reference method, positive and negative predictive values of the respective LAMP assays were calculated. The positive predictive value was defined as the number of samples positive (true positive) in both mycological analysis and respective LAMP assay for the respective species divided by the number of samples positive in the respective LAMP assay. The negative predictive value was defined in analogy (Fletcher et al., 1988). The Afla ID58 LAMP assay had positive and negative predictive values of 69.6% and 11.1%, respectively, while that for the Anom ID9 LAMP assay was 42.1% and 61.5%, respectively.

In the HPLC-based aflatoxin analysis, 23 samples (72%) had measurable concentrations of total aflatoxins (sum of aflatoxins B_1, B_2, G_1, G_2) whereas 9 (28%) contained no detectable aflatoxin. Comparing the results of mycotoxin analysis, microbiological analysis, and both LAMP assays, it was found that there was only one out of 32 samples (no. 222) in which results agreed completely among all the analyses with no mycotoxins, fungal contamination and LAMP positive signal detected. In addition, the predictive values of both LAMP assays were analyzed in regard to the presence of aflatoxin contamination of samples. The *A. flavus* LAMP assay had positive and negative predictive values for the presence of total aflatoxin contamination of samples being above the limit of detection (LOD= 0.05 µg/kg) of 78% and 44%, respectively, while that of the

A. nomius LAMP assay was found to be 63% and 15%, respectively. If combining the results of both LAMP assays, the positive predictive value for total aflatoxin contamination in the samples increased to 77%, however, the negative predictive value could not be improved substantially.

3.9 Quantification of *A. flavus, A. parasiticus,* and *A. nomius* conidia using real-time LAMP assays

Specificity of the three sets of LAMP primers has previously been established using calcein based indirect in-tube detection of LAMP signals (see 3.3). However, due to slight adjustments of the reaction conditions which were necessary to run LAMP reactions in a real-time turbidimeter, specificity of the assays was re-assessed using DNA isolated from the same array of tester strains as previously used. Specificity of all three assays was fully verified as previously established (see table 6). The time to threshold (T_t) defined as dturb./dt = 0.05 was used to quantify the signal intensity of the LAMP reaction. Turbidity was continuously monitored and OD600nm was plotted against time. In order to analyze quantitative correlations between conidial numbers and T_t for the three species, DNA was prepared (see 2.2.2.3) from solutions with defined conidial numbers for each species and used as template in a real-time LAMP with the respective primers set. Calibration curves were generated for *A. flavus*, *A. parasiticus* and *A. nomius* real-time LAMP by plotting log number of conidia per reaction against T_t. Each conidial dilution was analyzed repeatedly in three individual experiments. As shown in plot A of figure 11, the calibration curve for *A. flavus* conidial suspensions in the real-time LAMP assay spanned a range from 10 to 10^5 conidia/reaction corresponding to average T_t values between 21.0 and 40.1 min. When testing conidial suspension of *A. parasiticus* (plot B in figure 11) and *A. nomius* (plot C in figure 11), conidial concentrations in a range from 10^2 to 10^5 conidia/reaction were tested corresponding to T_t values from 52.7 min to 68.3 min and from 20.9 min to 28.8 min for *A. parasitcus* and *A. nomius*, respectively. In all assays, coefficients of correlation (R^2) between conidial numbers and T_t values were positive being 0.9258, 0.9352, and 0.9333 for *A. flavus*, *A. parasiticus*, and *A. nomius*, respectively.

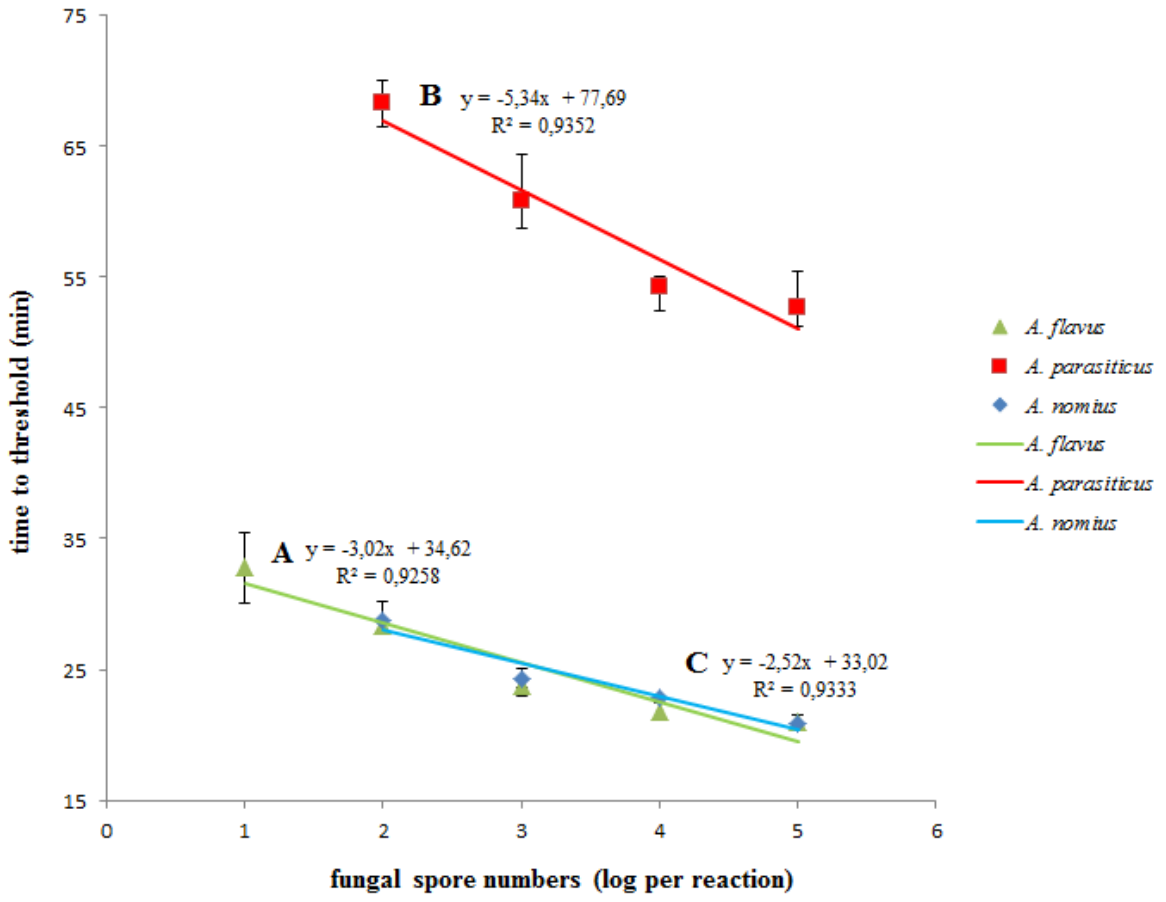

Figure 11 Linear correlation between conidial numbers (log per reaction) and T_t (min) in real-time LAMP assays for *A. flavus*, *A. paraciticus*, and *A. nomius*. A. Correlation for *A. flavus* specific real-time LAMP with conidial numbers ranging from 10 to 10^5 conidia/reaction; B. Correlation for *A. paraciticus* real-time LAMP with conidial numbers ranging from 10^2 to 10^5 conidia/reaction; C. Correlation for *A. nomius* real-time LAMP with conidial numbers ranging from 10^2 to 10^5 conidia/reaction.

3.10 Influence of DNA from food raw materials on real-time LAMP assays

Usually, DNA extracted from food samples contains excess amounts of background DNA derived from the sample matrix. Therefore, the effect of background DNA on the reaction of real-time LAMP assays was investigated by analyzing the mixtures of fungal reference DNA with DNA isolated from the different matrices. The results in figure 12 show threshold times for the three LAMP reactions obtained with different ratios between reference and backgroung DNA. Amplification of reference DNA of *A. flavus* and *A. nomius* was independent from the concentration of Brazil nut background DNA added even when the ratio of reference DNA to background DNA was 1:1 × 10^4. Testing *A. flavus* and *A. parasiticus* specific real-time LAMP

assays for their response to background DNA revealed that nearly no amplification occurred when peanut and maize background DNA was added to *A. flavus* and *A. parasiticus* reference DNA 1:1× 10^4 ratios, respectively. However, addition of 5-fold and 10-fold dilutions of peanut DNA and maize DNA showed only negligible inhibition of the real-time LAMP assays for *A. flavus* and *A. parasiticus*, respectively. No LAMP signal was obtained with none of the pure background DNA from either source in none of the three real-time LAMP assays.

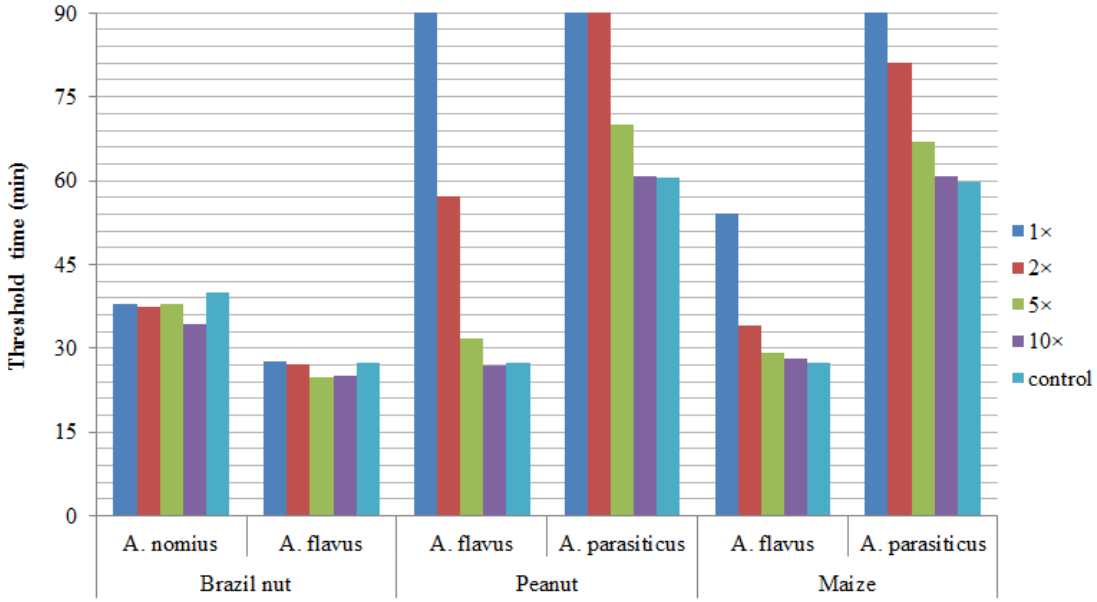

Figure 12 Influence of background DNA from different sample matrices on the performance of real-time LAMP assays for *A. flavus*, *A. parasiticus*, and *A. nomius*. Time to threshold (T_t (min)) of real-time LAMP performed with constant concentrations of the respective target DNA and addition of 1 to 10-fold diluted DNA extracted from uninfected samples of three different commodities are compared to untreated control reactions. Signals with T_t values exceeding 90 min were defined as negative. The concentrations of *A. flavus*, *A. parasiticus*, and *A. nomius* DNA were 1.6 ×10^{-4} µg/µl, 1 ×10^{-4} µg/µl and 2.6 ×10^{-4} µg/µl, respectively. The concentration of DNA of untreated Brazil nut, peanut, and maize were 1.08 µg/µl, 5.9 µg/µl, and 1.57 µg/µl, respectively.

3.11 Estimation of fungal infection in model food samples

To assess the sensitivity of the three turbidimetric real-time LAMP assays developed here for the detection of the respective target organisms in contaminated commodities, Brazil nuts, peanuts, and maize were artificially inoculated with conidial solutions of *A. flavus*, *A. parasiticus*, and *A. nomius*, respectively. Conidia of three tester strains were 10-fold serially diluted in sterile tap water and spray inculated to the ground sample materials. Conidial concentrations ranged from 1 × 10^6 to 10 conidia per g sample. DNA was extracted by disruption with ultrasonication and modificated CTAB precipitation (see 2.2.2.4) immediately after mixing conidia with ground

sample material in order to prevent germination of conidia. The results of real-time LAMP done with the total DNA isolated from inoculated samples confirmed the detection limits found for pure conidial solutions in all tested commodities. In Brazil nut samples, LAMP assays for *A. flavus* and *A. nomius* both showed a detection limit of 10 conidia/g. For the detection of *A. flavus* in peanuts and maize, the real-time LAMP assay had detection limits of 10^2 conidia/g and 10^4 conidia/g, respectively. In the same commodities, the real-time LAMP assay for *A. parasiticus* had detection limits of 10^5 conidia/g and 10^4 conidia/g in peanuts and in maize, respectively (see table 9).

Table 9 Limits of detection (conidia/g) of the real-time turbidimeteric LAMP assays for conidia of *A. nomius*, *A. flavus* and *A. parasiticus* in different commodities after artificial contamination with conidial suspensions.

Artificially contaminated food products	Inoculated mould strain	Detection limit (conidia/g)
Brazil nut	*Aspergillus nomius* CBS 260.86	10
	A. flavus CBS 113.32	10
Peanut	*A. flavus* CBS 113.32	10^2
	A. parasiticus CBS 126.62	10^5
Maize	*A. flavus* CBS 113.32	10^4
	A. parasiticus CBS 126.62	10^4

4 Discussion

Nowadays, 80 000-100 000 fungal species have been described (Hawksworth and Miadlikowska, 1997; Rossman, 1994) and some are known to produce toxic secondary metabolites which lead to adverse effects in animals and humans. Aflatoxins, which are mainly produced by *Aspergillus* spp., are highly toxic and carcinogenic compounds which are of high concern for the safety of food. The taxonomy of species in the genus *Aspergillus* is highly complex and ever evolving and there is no single method (morphological, physiological or molecular) that works flawlessly in identifying and recognizing all the species described so far. Furthermore, there may be disadvantages of classical identification methods as they are highly time consuming and require considerable mycological knowledge and experience to be accurately performed. Molecular biological techniques are less time-consuming but are even complicated because of cumbersome preparation of high quality DNA and purification of PCR product for sequencing. Moreover, reliable identification of species depends on the availability of sufficient data to compare the analyzed sequence with. Oftenly it is necessary to analyze phylogenetic trees containing the specimen analyzed and several closely related taxa in order to fully asses its taxonomic position. These LAMP assay developed during the current study may be a useful novel alternative method to rapidly identify and detect three of the most common aflatoxin producing *Aspergillus* species, namely *A. flavus*, *A. nomius* and *A. parasiticus*, in food matrices which typically become contaminated with aflatoxin.

4.1 Determination of target species and genes

During the current study, four specific LAMP assays were developed and optimized for the detection and identification of typical aflatoxigenic moulds, *A. flavus*, *A. nomius*, and *A. parasiticus*, as well as a common but non-aflatoxingenic mould *A. caelatus*, all of which belong to *Aspergillus* section *Flavi*, a group of several closely related fungal species. Based on the importance for food safety, these three species were selected as target for the respective assays during the current study. The frequently occurring *A. flavus* was selected as the most common producer of B type aflatoxins, while the less common *A. parasiticus* is the most frequently reported producer of both B and G type aflatoxins in naturally contaminated materials (Frisvad et al., 2005). When Freire et al., (2000) analyzed Brazil nuts for aflatoxins and fungal contamination they found contamination with high levels of *A. flavus* but did not only detect B type aflatoxins

but also G type aflatoxins in equal concentrations. Since the authors did not detect *A. parasiticus* in contaminated samples they were able to shown that the presence of G type aflatoxins was linked to the presence of *A. nomius* in high numbers, a fungus, which regularly produces both B and G type aflatoxins in pure culture, Brazil nuts, and other tropical tree nuts (Molyneux et al., 2007; Olsen et al., 2008). For this reason, *A. nomius* was selected as another target for LAMP based detection and identification during the current work. In addition, *A. caelatus* was chosen as the fourth species to be detected. Calderari et al. (2013) indicated that *A. caelatus*, which showed the same conidium color on CYA as *A. flavus*, is a common non-aflatoxin producing species with very high frequency of occurrence in Brazil nut in addition to *A. nomius* and *A. flavus*.

To set up LAMP-based identification and detection assays, different genes were chosen as amplification target of primer design for analysis of *Aspergillus* spp.. For the current study, primers applied in LAMP assays for the detection of *A. nomius* and *A. parasiticus* were generated from the sequences of the genes both coding for alpha amylase (*amy*1) in the respective species. Fakhoury and Woloshuk (1999) showed that *amy*1 is indirectly connected to aflatoxin biosynthesis in *Aspergillus* spp.. They also demonstrated that production of α-amylase encoded by the gene in *A. flavus* helps the fungus to generate sugar concentrations sufficient to induce aflatoxin biosynthesis in maize. Therefore, it can be assumed that the *amy*1 gene plays a similar role in other aflatoxigenic species and may thus be similarly useful as a target for the detection of aflatoxigenic fungi as any of those belonging to the cluster of aflatoxin biosynthesis gene. Attemps made during the current study to use the *amy*1 homologous sequence for the design of LAMP primers for *A. flavus* failed since primers showed cross reactions with a wide array of *Aspergillus* species, among which were species such as *Aspergillus niger* that are not known to be typical producers of aflatoxin. As a consequence, the primer set presented here for the LAMP assay for *A. flavus* was rather designed based on the sequence of the gene coding for ATP citrate lyase subunit 1 (*acl*1) which was also the target in the LAMP assay for *A. caelatus*. Niessen et al. (2012) applied this housekeeping gene successfully in a LAMP assay for discrimination of genetically losely related fungal taxa, i.e. *F. tricinctum* and *F. avenaceum*.

4.2 Specificity of LAMP assays

The primer sets designed according to the *amy*1 gene homologs were demonstrated to be highly specific in the LAMP assays of *A. nomius* and *A. parasiticus*, respectively. As shown in Table 6,

the results indicated that the LAMP assay for *A. nomius* had very high specificity for this fungus since no other species was tested positive. Similarly, the LAMP assay developed for the detection of *A. parasiticus* was also specificity for its target. However, according to the results given in Table 6, some cross reactions of the primers occurred with other taxa, namely *A. toxicarius* and *A. sojae*. The cross reactions observed with this set of primers can be explained by the fact that both taxa are synonyms for *A. parasiticus* (Kurtzman et al., 1986; Pildain et al., 2008). *A. sojae* is a name used for domesticated strains of *A. parasiticus* which are traditionally used for the production of fermented soy products (Pildain et al., 2008). It was demonstrated that *A. sojae* can scarcely be differentiated from its parent species *A. parasiticus* using neither morphological nor genetic parameter. The only differentiating parameter is the inability of *A. sojae* to produce aflatoxins (Rigó et al., 2002; Chang et al., 2007). Therefore, these taxa should be retained separately because of the legal confusion that conspecificity might generate in the food industry as it is morphologically indistinguishable from *A. parasiticus* (Geiser et al., 1998).

As regards the LAMP assay with the primer set based on the *acl*1 gene for detection of *A. flavus*, the results shown in table 6 do not imply a similarly high specificity like that of the other two *Aspergillus* specific LAMP assays. Aside from reactions with *A. flavus* strains, cross reactions of the primer set occurred with genomic DNA of *A. parvisclerotigenus, A. minisclerotigenes, A. oryzae, A. parasiticus, A. toxicarius* (syn. *A. parasiticus*) and *A. sojae* (syn. *A. parasiticus*). Some of the cross reactions can again be explained by synonymy as described for *A. parasiticus* /*A. sojae*. Similar to *A. sojae,* the name *A. oryzae* has been used for domesticated *A. flavus* strains also used in the production of traditional soy products. Both sister species showed high degrees of nuclear DNA complementarity and can be used as synonyms as revealed by Kurtzman et al. (1986). Comparision of the whole genomes of *A. oryzae* and *A. flavus* recently also showed striking similarities between the two taxa (Varga et al., 2011). To be more specific, 129 genes were found unique to *A. oryzae* whereas only 43 genes were unique to *A. flavus* in an array based genome comparison (Georgianna and Payne, 2009). Moreover, as a result of its production of a similar spectrum of toxic metabolites, also *A. parvisclerotigenus* was considered to be a variety of *A. flavus* at its first description (Saito and Tsuruta, 1993). However, later the fungus was raised to species level as Frisvad et al. (2005) neotypified it with an African isolate because the original type material from Thailand was no longer available (Frisvad, pers.comm.). Also, *A. parvisclerotigenus* was the only species that could not be differentiated from *A. flavus* in the

differentiation of 10 most closely related taxa with a combination of molecular identification methods (Godet and Munaut, 2010). Varga et al. (2011) showed however that the neotype of *A. parvisclerotigenus* formed a taxonomic entity separate from *A. flavus* when analysis of the phylogenetic relations was performed within species belonging to section *Flavi* of *Aspergillus* based on *β*-tubulin and calmodulin sequences. The species were not differentiated however when sequences from the internal transcribed spacer region of the rRNA (ITS) were compared. Similarly problems have been found in the taxonomic discussion of *A. minisclerotigenes* which was formerly included in subgroup II of *A. flavus* (Geiser et al., 2000). Discussion is still ongoing about the taxonomic status of *A. flavus* and its closest relatives. In the *A. flavus* specific LAMP assay, many of the false positive reactions do only exist in the light of current taxonomic discussion. All cross reactions observed might therefore as well be included into a wider definition of *A. flavus* under more practical and toxicological point of view. Using a wider taxonomical definition of *A. flavus* as a species here would be an advantages since all (cross) reacting taxa except *A. oryzae* and *A. sojae* have been described as producers of aflatoxin. The latter two species are both domesticated forms of *A. flavus* and *A. parasiticus* which can be assumed to be absent from the niches where *A. flavus* naturally occurs and will therefore not interfere with assay results obtained from naturelly contaminated food raw materials. The only cross-reaction of major concern was the one with *A. parasiticus*. From a practical point of view, however, this cross-reaction can be seen as an advantage here because *A. flavus* and *A. parasiticus* are both common producers of aflatoxin. Therefore, the cross reactivity of the *A. flavus* LAMP assay may be turned into an advantage for practical reasons since the two major aflatoxin producers can be detected in one analysis with the primer set. This is of special interest in the analysis of food samples rather than for the analysis of pure cultures where results should be unambiguous.

Similarly, the results of the LAMP assay with the primer set based on the *acl*1 gene for detection of *A. caelatus* shown in table 6 do not imply a similarly high specificity like that of *A. nomius* and *A. parasiticus* specific LAMP assays. Cross reactions of the primer set were detected with genomic DNA of two aflatoxingenic species, *A. bombycis* and *A. archidicola*. Both species can be found in Brazil nuts but much less common than *A. caelatus* (Gonçalves et al., 2012), so that the *A. caelatus* specific LAMP assay may still be helpful to the analysis of the biodiversity of *Aspergillus* section *Flavi* in Brazil nuts.

In conclusion, the LAMP assays developed in this study may be potentially useful to identify *A. nomius*, and *A. parasiticus* when DNA purified from pure cultures is used as template. DNA from species in other genera or even from more remotely related *Aspergillus* spp. was never amplified with none of the primer sets. The *A. nomius* specific LAMP assay can be used for the absolute identification of pure cultures. Whereas in the cases when cultures are tested to be *A. flavus* or *A. parasiticus*, samples have to be analyzed with both LAMP assays and results have to be compared. Isolates can be identified as *A. flavus* (including its synonyms) when the positive signal is showed only in the *A. flavus* specific test (primer set ID58). Those isolates for which a positive reaction occurs in separate LAMP assays with both primer sets (Afla ID58 and Apara ID153) can be identified as *A. parasiticus* (including its synonyms). Moreover, *A. flavus* and *A. parasiticus* are easily distinguished microscopically beyond doubt.

4.3 Influence of samples to the LAMP reaction

On dry food, moulds may occur in a variety of different structures (Pitt and Hocking, 2009). They basically exist as vegetative cells (aireal and substrate hyphae) which die off quite rapidly after drying. Moreover they can appear in masses as asexual spores (conidia) or as sexual spores (ascospores) (Ingold, 1971). Many fungi form chlamydopores which are rigid thick-walled structures within the mycelium that function as dormant survival stadia. All forms of mycelia contain a similar amount of DNA in a species, depending on the degree of ploidy and the number of nuclei per cell in the respective form (Kokoa, 2006). However, different structures can have a very different degree of resisitance to cellular disruption which is crucial point in DNA preparation (Shapira et al., 1996). Moreover, preparation of genomic DNA from a food matrix can only be performed properly and without co-preparation of inhibiting compounds if several steps of DNA isolation and purification are performed in succession. Therefore, DNA preparation often is the most time consuming step in molecular biological analysis and is often the reason for missing sensitivity of an assay. LAMP can circumvent many of the problems described because of its high sensitivity per se and its low susceptibility to DNA impurities. It has repeatedly been shown to work well despite the influences of pH changes and suboptimal incubation temperatures and even proved to be widely insensitive against inhibitors typically present in natural sample materials (Francois et al., 2011). Njiru et al., (2008) confirmed that LAMP assay for *Trypanozoon* spp., the causal agent of human African trypanosomiasis, still can amplify the target DNA even

with the addition of whole blood. During the current study, only small amounts of supernatant from mycelia after simple pre-treatment as the template in LAMP assays resulted in positive identification of target *Aspergillus* species from agar grown pure cultures. However, larger volumes of crude sample materials caused inhibition of the LAMP reactions. Niessen and Vogel (2010) demonstrated the situation for other fungi that blending spores directly into the LAMP master mix before incubation can be more convenient in the LAMP assay for detection and identification of *F. graminearum* directly from agar cultures.

Studying the influence of matrix or background DNA on the effectiveness of real-time PCR, Mayer et al. (2003) found that during analysis of samples containing low concentrations of target fungal DNA, high concentrations of unspecific matrix DNA may act as an inhibitor of DNA amplification, apparently by competitive binding to primers resulting in a decrease of primer concentrations accessible to specific amplification. Specifically, an inhibition on amplification efficiency of *Taq* DNA polymerase was described since high amounts of DNA from fresh figs were present during PCR-based detection of aflatoxigenic moulds (Färber et al., 1997). Therefore, an analysis about the inhibitive effects of background DNA was carried out during the current study by parallel amplification of pure DNA of *A. flavus*, *A. nomius* or *A. parasiticus* mixed with different dilutions of DNA extracted from non inoculated commodities. No influence of DNA and other components co-extracted from Brazil nut on the sensitivity of the *A. nomius* and *A. flavus* specific assays was observed, while DNA from peanut and maize both showed gradual inhibition in the LAMP reaction. However, the inhibiton of DNA from peanut or maize was minimized after 5 fold dilution of background DNA prior to addition to the real-time LAMP reaction. It is calculated that an inhibition exists when the amount of peanut DNA was 3×10^4 fold in excess of target DNA and the DNA from maize was 8×10^4 fold in excess.

4.4 Rapid DNA preparation from conidia

When pure culture mycelia were used as the sample for rapid DNA preparation during the current study, there was a prerequisite that mycelia had to be harvested before sporulation occurred from cultures grown on a synthetic agar medium (SNA) in the dark (Luo et al., 2012). However, usefulness of this method was challenged by the fact that an extra cultivation step added more time consumption to the analysis. Moreover, the method die not allow flexible use of different growth media for identification of cultures. In order to add more rapidness and flexibility, a

protocol for rapid template DNA preparation from conidia of *Aspergillus* spp. without the need for previous spore free cultivation was developed and applied to the cultures grown on typical lab media directly after cultivation under usual light and temperature conditions. Our results show that it is possible to analyze pure cultures by using conidia directly from MEA grown cultures as template added to the LAMP master mix before reaction. The sensitivity of detection was high enough to identify pure cultures since number of conidia is no limitation in *Aspergillus* pure cultures. However, results demonstrated that the sensitivity of the assay was considerably higher after disruption and boiling of conidia before additon to the master mix. However, the sensitivity of conidium detection could be increased greatly with only a little more input in time and simple pre-treatment that disruption of conidia with a mixture of two different sizes of zirconia/silica beads and subsequent boiling. The bead-beating process was identified as the most effective way to disrupt conidia to release fungal DNA (Zhou et al., 2000). Moreover, Williams et al. (2001) used microscopic examination and found that airborne fungal spores were still visibly intact even after a 10 min heating step at 95℃ while disruption of spores provided enough DNA for performing maximum sensitivity in a PCR-based detection assay for fungal spores. In addition, during the current study the sensitivity of the *A. nomius* specific LAMP assay increased much more than that of *A. flavus* with conidium disruption treatment. Feibelman et al. (1998) verified that conidia of *A. nomius* have a coarser surface ornamentation when compared with conidia of *A. flavus*. From our results we deduced that the difference in surface ornamentation may contribute to strengthen the friction between beads and conidia of *A. nomius*, which may result in a higher efficiency of disruption for the conidia of that particular species.

4.5 Identification of isolates from Brazil nuts

It was demonstrated in the current study that analysis of pure cultures and samples could be a useful example for the application of rapid identification and detection methods with LAMP assays to support a wide range of microbiological studies. Calderari et al. (2013) and Gonçalves et al. (2012) resently published the results of the identification for a total of 2 447 fungal isolates from 173 and 115 samples of seeds and shells of Brazil nuts, respectively, and analyzed the biodiversity of *Aspergillus* section *Flavi* in Brazil nuts collected from the Amazonian rain forest till the consumer´s household. With high frequency of occurrence in Brazil nut, also *A. caelatus* was found to be a very common species. LAMP assay for identification of the species may be

helpful to the analysis of the biodiversity of *Aspergillus* section *Flavi* in Brazil nuts due to its high specificity that only had cross reaction with *A. archidicola* and *A. bombycis* both of which are infrequent species in Brazil nuts. In this study, 68 fungal isolates from Brazil nuts were screened for identification by LAMP assays for identification of *A. nomius* and *A. flavus*. Results showed the rate of accuracy of both assays was 78%. With both LAMP assays, 10 isolates were misidentified as *A. flavus* and one as *A. nomius*. No cross-reactions of the two LAMP assays occurred previously with pure DNA of the respective other species, which might indicate that the original identification of the strains during the current study was not correct. Moreover, eight *A. caelatus* strains tested positive in both the *A. flavus* and *A. caelatus* specific LAMP assays while other six strains gave positive results only in the *A. caelatus* specific LAMP assay but were negative in other two LAMP assays. With the repeated re-isolation of the analyzed cultures, contamination of the tested cultures could be ruled as a possible explanation. The results of a BLAST analysis of calmodulin gene sequences deposited in GenBank showed that the *cmd* gene of *A. caelatus* shares a very high degree of homology with that of *A. flavus* (Luo et al., 2014). Peterson et al. (2000) stated that *A. caelatus* strains could mistakenly be identified as *A. tamarii* since non aflatoxin-producing strains of *A. tamarii* were found to be taxonomically more closely related to *A. caelatus* rather than to *A. tamarii*. Furthermore, Wang et al. (2001) described a strain defined as *A. flavus* which was closely related to *A. tamarii* based on the mitochondrial cytochrome *b* gene, with similarly brownish colonies, and similarities of the conidium surface profile by SEM. Recently, Varga et al. (2011) described *A. pseudocaelatus* producing aflatoxins B and G as a new species closely related to the non-aflatoxin producing *A. caelatus*. From the facts described, we deduced that the strains of *A. caelatus* which were tested positive in both LAMP assays for *A. flavus* and *A. caelatus* may have been misidentified in morphological analysis.

4.6 Application of LAMP assays

As shown by Niessen and Vogel (2010), LAMP was a useful tool for the detection of *F. graminearumin* in contaminated grains. Similarly, the LAMP assays developed during the current study were shown to be useful, rapid, and simple tools in identification of *A. nomius*, *A. flavus*, and *A. parasiticus*. Compared with the LAMP assay for *F. graminearumin*, however, sample preparation needed a little more effort during the current study. In addition to identifying pure

fungal cultures, another goal of the current research was detection of the target fungi in food raw materials. Brazil nuts, ground nuts (Kumara et al., 2008; Williams et al., 2004), other tree nuts (Molyneux et al., 2007), maize and cereals as well as other oil seeds are common food sources easily contaminated with aflatoxin (Klich, 2007). Even green coffee beans were identified as a possible source of aflatoxin contamination by Nakajima et al. (1997). Due to consumer relevant health concerns related to aflatoxin, aflatoxin contaminated food and food raw materials have repeatedly been rejected by law enforcement authorities, which results in high economic losses both for producers and traders (Robens and Cardwell, 2005). The usefulness of the current method was demonstrated by detecting three major aflatoxin producing *Aspergillus* species in artificially infected representative foods, i.e. peanuts, unroasted coffee beans, and Brazil nuts.

The results indicated that LAMP assays can be an interesting alternative for detection of target *Aspergillus* species directly from contaminated materials as the time for complete analyses was only hours while microbiological detection usually takes at least 5-7 d to be completed. It was shown in figure 9A that direct analysis of crude washings of whole artificially contaminated peanuts showed a positive signal in the *A. flavus* specific LAMP assay only after 10 fold dilution prior to its use as template while 10 fold diluted crude washings of either shell or nut of artificially contaminated peanuts showed no LAMP reaction. However, higher dilution (1 000 fold) of the crude washings of both shell and nut resulted in positive signals, which means an even better detectability of the fungus can be achieved after higher dilution (figure 9A1). It turned out that detection of *A. parasiticus* from either parts of the contaminated peanuts generally failed with crude extracted DNA. Therefore, use of a DNA extraction kit was necessary for the testing of that fungus from food raw materials. The results indicated that compounds, which showed substantial inhibition of the LAMP reactions applied here, certainly exist in the crude sample washings. For circumvention of false negative results, sufficient dilution of crude extracts will be needed before they can be used as target of LAMP assays. A commercially available extraction kit should be used to purify DNA from the crude sample washings. Nevertheless, it gave reliable results with all three assays. Kit extracted DNA will offer the benefit of much higher sensitivity and improved detectability with all LAMP assays developed here. However, higher time consumption for sample preparation and higher costs per analysis are the disadvantages of using a commercial extraction kit. In order to obtain reproducible results,

optimization of sample preparation protocols is needed before LAMP assays for routine analysis of naturally contaminated sample materials.

The results obtained here show an interesting potential of all three LAMP assays for detecting *A. nomius*, *A. flavus* and *A. parasiticus* from artificially contaminated commodities. It indicated that LAMP assays can be a rapid, simple and useful alternative to PCR-based methods presently used to detect aflatoxigenic fungi in food and food raw materials (Shapira et al., 1996; Geisen, 1996; Mideros et al., 2009).

4.7 Comparison of microbiological analysis, aflatoxin analysis and LAMP assays

The capability of LAMP assays for detection of *A. nomius* and *A. flavus* in naturally infected Brazil nut samples was tested with application of disruption and boiling of surface washings as a pre-treatment for DNA release. During the current study, 32 Brazil nut samples collected from different regions of Brazil and from different steps in the production process were analyzed for their fungal contamination by microbiological analysis, for the concentration of total aflatoxins by HPLC and the presence of target fungi with LAMP assays in parallel studies. Based on the assumption that the results of microbiological analysis showed the real contamination state of Brazil nuts as reference, the results of the LAMP assays indicated that the positive predictive value was about 65% for either of both species when compared with that of microbiological analysis. There was high deviation between results of LAMP assays and microbiological analysis in some of the samples. This circumstance may be explained by the fact that LAMP analysis was performed based on surface contamination while the microbiological analysis used plated slices of whole nuts including both surface and internal infections, which may also explain the fact that the number of infected samples by one of the two species was higher than microbiological analysis. Another important reason may be that the number of nuts used for one LAMP analysis may have been too low to discover minor levels of contamination, although *A. flavus* occurred with high frequencies and cfu values on the surface of Brazil nuts (Pacheco et al., 2010). However, during the current study it was found that washing solutions for DNA release could not be obtained from more than 10 nuts because of the high oil content of Brazil nuts (Ryan et al., 2006) which may decrease the sensitivity of LAMP assays.

In LAMP analysis, five of the Brazil nut samples were infected by one or both *Aspergillus* spp. whereas no aflatoxin was detected in the samples. It is an established fact that, according to the source of isolation, only a portion of *A. flavus* strains (25% (Rodrigues et al., 2009) to 69% (Vaamonde et al., 2003)) is capable of producing aflatoxin. In addition, the toxin production is influenced by environmental factors such like temperature, humidity and substrate composition in *A. flavus* (Northolt et al., 1977), Therefore, positive LAMP results with no aflatoxin found in corresponding samples may be due to the presence of non-aflatoxin-producing strains or adverse physiological conditions during growth.

4.8 Quantification of fungal conidia by real-time LAMP assays

Following optimization of conditions for real-time LAMP assays run on a turbidimeter, the level of the specificity was tested in the same way as that of the previously described end-point LAMP assays, using purified DNA from a total of 39 *Aspergillus* spp. including three target fungi as well as 135 strains belonging to various other genera.

With regard to sensitivity of real-time LAMP assays, the developed method showed high sensitivity to detect conidia previously harvested from pure cultures of *A. flavus*, *A. nomius* and *A. parasiticus*, with limits of detection of 10, 10^2 and 10^2 conidia/reaction, respectively. Moreover, all assays applied were sensitive enough to detect target DNA directly from the treated conidial solutions without any further manipulation for DNA purification or concentration. Compared with results of the corresponding conventional LAMP assays, the real-time LAMP assays showed 10-fold higher sensitivity for detection of *A. flavus* while sensitivity was 10-fold lower for the *A. nomius* assay. The coefficients of correlation between conidial numbers in the real-time LAMP per reaction and the cprresponding T_t values were found to be within a small range of R^2 = 0.92-0.94, which means that parameters are highly correlated. The results clearly showed the quantitative capability of the real-time LAMP assays when applied in detection of their respective target species in pure cultures or in conidial solutions

4.9 Prediction of the presence of aflatoxins in food raw materials

The study of Shapira et al. (1996) revealed that concentrations of aflatoxins can be correlated with the level of cfu of aflatoxigenic species detected on naturally contaminated samples.

Confirmation of fungal contamination levels in food matrices is therefore essential since previous studies have also demonstrated the association between levels of aflatoxigenic fungi and aflatoxin concentrations at a level exceeding legal limits (Lund and Frisvad, 2003). During the current study, the real-time LAMP assays were developed for the determination of contamination levels, at which consumers might encounter an increased risk of excessive amounts of aflatoxin in commodities. Olsen et al. (1998) concluded from their microbiological study of typical aflatoxin producers in a variety of commodities that there was a considerable risk of exceeding the European legislative limit for aflatoxin (4 µg/kg) when the level of *A. flavus*/*parasiticus* contamination exeeds 100 cfu/g of commodity. Moreover, the probability of excess total aflatoxin levels exceeding the European legislative limit of 4 µg/kg increased rapidly from approx. 30% to above 80% when the mould levels increased from 100 to 1000 cfu/g, respectively (Johnsson et al., 2008). In order to prevent aflatoxin from entering the food chain, systems for early detection of aflatoxin-producing species are a pressing need with capacity for detection of target fungi at the critical levels discussed in previous studies. To this end, we tested the real-time LAMP assays for detection of *A. flavus*, *A. nomius* and *A. parasiticus* in artificially infected Brazil nuts, peanuts and maize, respectively. Usefulness of the primer sets also for detection of natural contaminations has been verified for the *A. flavus* and *A. nomius* specific primers which were applied to the analysis of naturally contaminated Brazil nut samples. The results showed that the sensitivity of real-time LAMP assays was lower in the analysis of peanuts and maize which was mainly due to the dilution of DNA before being used as template in real-time LAMP reaction. Comparion of the detection limits found for conidia of the three *Aspergillus* spp. to the critical cfu levels discussed previously, the real-time LAMP assays for detection of *A. flavus* and *A. nomius* in Brazil nuts fully met the criteria suggested by Olsen et al. (1998), which suggested that a negative result with both assays in Brazil nut samples implies a safe product with very low potential to be contaminated with aflatoxin. The same holds true for detection of *A. flavus* in peanuts. However, limits of detection for *A. parasiticus* conidia in peanuts as well as for all species in maize samples did not meet the mentioned criteria meaning that consumer risk cannot be ruled out even in negative samples in the real-time LAMP assays. In order to considerably lower the detection limits of assays applied to the analysis of maize, a more sophisticated protocol for DNA preparation should be elaborated in order to meet criteria of safety.

In addition, since mycotoxins are produced by the vegetative mycelia of the fungus rather than by its conidia, similar disproportionality exists between cfu and mycotoxin production in many species. The fact may explain why high correlation was found between mycotoxin concentrations and results of real-time PCR or real-time LAMP in *Fusarium* contaminated samples. *Fusarium* species have a lower sporulation rate per vegetative hyphal biomass which will result in a more balanced relation between hyphal and conidia biomass. When compared with quantitative real-time PCR which showed a detection limit at spore concentrations equal or higher than 10^6 conidia/g in flour samples (Sardiñas et al., 2011), the real-time LAMP assays developed in the current study had a 10 to 10^5 fold higher sensitivity even without pre-incubation of samples prior to DNA extraction and could be completed within 6 h including DNA preparation.

SUMMARY

Aflatoxins are the most thoroughly studied mycotoxins produced by several members of the genus *Aspergillus* in section *Flavi*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* are aflatoxin producers frequently isolated from contaminated food sources. In order to identify and detect the three species and another non-aflatoxin producing species *Aspergillus caelatus* which is frequently isolated from Brazil nuts, we developed and evaluated a novel method, loop-mediated isothermal amplification (LAMP) assay, in separate analyses in which the target genes are *acl*1-genes of *A. flavus* and *A. caelatus* and *amy*1-genes of *A. nomius* and *A. parasiticus*. The detection limits for pure DNA of *A. flavus*, *A. nomius*, *A. parasiticus* and *A. caelatus* were 2.4, 7.6, 20 and 458 pg/reaction, respectively. The specificity testing of the specific LAMP primer sets developed for the three target species were performed with the pure DNA extracted from mycelia of 174 representative strains including 39 *Aspergillus* species, 23 *Penicillium* species, 75 *Fusarium* species and 37 other fungal species. The LAMP assay was combined with a fast-prep DNA extraction method for the analysis of pure fungal culture, which showed the same signal as the specificity testing with pure DNA. In the further study, a rapid DNA release method for DNA extraction from conidia was developed and combined with LAMP assay. Compared with the detection limits for conidia added directly to the reaction, the sensitivity was increased from 10^5 to 10^1 and from 10^4 to 10^2 conidia per reaction for *A. nomius* and *A. flavus*, respectively, provided that a conidium disruption step was included in sample preparation. Basing on this, pure cultures of 68 isolates of both species obtained from Brazil nuts were identified and showed that the LAMP assays had an accuracy of 83.8% when morphological identification was used as a reference.

In addition, the LAMP assay was applied for the analysis of three reference *Aspergillus* species from artificially contaminated Brazil nuts, peanuts and green coffee beans. With using either a very simple protocol or a DNA purification kit, the contaminating fungi could be detected by the respective LAMP assays. Furthermore, to verify the practicability of LAMP assays, 32 naturally infected Brazil nut samples from different regions of Brazil and from different steps in the production process of the commodity were analyzed. The positive predictive value was defined as the number of samples positive in both mycological analysis and LAMP assay for the respective species (true positive) divided by the number of samples positive in the respective LAMP assay. The negative predictive value was defined in analogy. When LAMP results were

compared with mycological analysis of the samples, the negative predictive value of LAMP assays was 42.1% and 12.5% while the positive predictive value was 61.5% and 66.7% for *A. nomius* and *A. flavus*, respectively. Whilst the results were compared with the presence of aflatoxin in corresponding samples, the negative predictive value was 22.2% and 44.4% and the positive predictive value was 52.2% and 78.3% for aflatoxins produced by *A. nomius* and *A. flavus*, respectively.

A further part of the work deals with the development and application of three specific turbidimeter based real-time LAMP assays for detection and quantification of all three species in pure culture conidium solutions, as well as for the definition of contamination levels for the three species in Brazil nuts, peanuts and maize. The results show that DNA amounts equivalent to 10 conidia of *A. flavus* and 100 conidia both of *A. parasiticus* and *A. nomius* can be detected by the respective assays. Calibration curves relating conidium numbers to time to threshold (T_t) values were generated for each of the species. In the analysis of contaminated samples materials, the *A. nomius* specific real-time LAMP assay detected a minimum of 10 conidia per gram in Brazil nuts without pre-incubation of samples, whilst real-time LAMP assays specific for *A. flavus* and *A. parasiticus* had detection limits of 10^2 conidia/g and 10^5 conidia/g, respectively in peanut samples as well as 10^4 conidia/g and 10^4 conidia/g, respectively in samples of maize.

All in all, this thesis gives a detailed study of development and evaluation of LAMP assays for detection, identification, and quantification of *A. flavus*, *A. nomius* and *A. parasiticus*. It is suggested that the developed LAMP assay is a promising tool in the prediction of a potential aflatoxin risk at an early stage and in all critical control points of the food and feed production chain may therefore be suitable for low tech environments where resources may be limited since they are specific, sensitive and easy to use.

ZUSAMMENFASSUNG

Aflatoxine sind die am besten untersuchten Mykotoxine. Sie werden von verschiedenen Angehörigen der Pilzgattung *Aspergillus* aus der Sektion *Flavi* gebildet, *Aspergillus flavus, Aspergillus parasiticus* und *Aspergillus nomius* wurden häufig als Aflatoxin-Produzenten von befallenen Nahrungsmitteln isoliert.

Basierend auf dem Verfahren der loop-mediated isothermal amplification (LAMP) wurden im Rahmen der vorliegenden Dissertation Nachweise entwickelt, verifiziert und angewandt, mit deren Hilfe die wichtigsten Spezies in getrennten Analysen detektiert und identifiziert werden können. Die für den Nachweis verwendeten Zielsequenzen lagen jeweils im *acl*1 Gen von *A. flavus* und *A. caelatus* sowie jeweils im *amy*1 Gen von *A. nomius* und *A. parasiticus*. Die Nachweisgrenzen der erstellten Assays für gereinigte genomische DNA von *A. flavus, A. nomius, A. parasiticus* und *A. caelatus* waren 2,4, 7,6, 20 und 458 pg/Reaktion. Die Überprüfung der Spezifität der verschiedenen LAMP Primersets erfolgte durch Zugabe reiner genomischer DNA aus insgesamt 174 verschiedenen Pilzstämmen. Darunter waren 39 *Aspergillus* Arten, 23 *Penicillium* Arten, 75 *Fusarium* Arten und Vertreter von 37 anderen Pilzarten. Zur Analyse von pilzlichen Reinkulturen wurden die LAMP Assays mit einem Verfahren zur schnellen Präparation von genomischer DNA aus Pilzmyzelien kombiniert. Im weiteren Verlauf der Studie wurde dazu noch ein Verfahren zur schnellen DNA-Gewinnung aus pilzlichen Konidien entwickelt und ebenfalls mit den LAMP Assays kombiniert. Im Vergleich mit den Nachweisgrenzen für Sporen, die direkt zu der LAMP Reaktion zugegeben wurden, war die Nachweisempfindlichkeit nach der Schnellextraktion bei *A. nomius* 10.000 fach und bei *A. flavus* 100 fach erhöht. Der Grund für die Erhöhung der Nachweisempfindlichkeit war die zusätzliche Zerstörung der Sporen in der Probenvorbereitung. Basierend auf dieser Schnellextraktion wurden Reinkulturen von 68 Isolaten getestet, die nach morphologischer Analyse als *A. flavus, A. nomius* und *A. caelatus* bestimmt worden waren. Alle Isolate waren aus brasilianischen Paranüssen gewonnen worden. Es konnte gezeigt werden, dass die LAMP Assays gegenüber der morphologischen Identifizierung als Referenz eine Genauigkeit von 83.8% hatten.

Darüber hinaus wurden die entwickelten LAMP-Assays für die Analyse von *A. flavus, A. nomius* und *A. parasiticus* nach künstlicher Inokulation von Paranüssen, Erdnüssen und grünen Kaffeebohnen verwendet. Unter Verwendung eines einfachen DNA-Isolierungs Protokolls

konnten die Pilzkontaminanten mit den entwickelten LAMP Assays in diesen Proben nachgewiesen werden.

Vergleicht man die Ergebnisse der LAMP Assays mit der mykologischen Analyse der untersuchten Proben, so wurden für *A. nomius* und *A. flavus* negative Vorhersagewerte der jeweiligen LAMP Assays von jeweils 42,1% und 12,5 % festgestellt. Die positiven Vorhersagewerte für die beiden Arten lagen bei jeweils 61,5% und 66,7%. Vergleicht man die LAMP Ergebnisse mit der Anwesenheit oder Abwesenheit von Aflatoxinen in den entsprechenden Proben, so betrug der negative Vorhersagewert jeweils 22,2% und 44,4%. Die positiven Vorhersagewerte lagen bei 52,2% und 78,3% für die *A. nomius* und *A. flavus* spezifischen Assays.

Weiterhin beschäftigte sich die vorliegende Arbeit mit der Entwicklung und Anwendung dreier real-time LAMP-Assays auf der Basis der während der Reaktion auftretenden Trübung (Turbidimetrie). Ein Trübungsmessgerät wurde für die Erstellung von Kalibrationskruven für die Quantifizierung reiner Sporenlösungen von *A. flavus, A. nomius* und *A. parasiticus* eingestzt. Mit den so erstellten real-time LAMP assays erfolgte die Bestimmung des Kontaminationsgrades von Paranüssen, Erdnüssen und Mais mit den drei genannten Pilzarten. Die Ergebnisse zeigen, dass die DNA-Menge äquivalent zu 10 Sporen von *A. flavus* und 100 Sporen von *A. nomius* und *A. parasiticus* von den jeweiligen Assays detektiert werden können. Bei der Analyse von kontaminierten Materialproben ohne Vorinkubation detektierte der *A. parasiticus* spezifische real-time LAMP assay ein Minimum von 10 Sporen pro Gramm in Paranüssen während die Assays für *A. flavus* und *A. parasiticus* eine Nachweisgrenze von jeweils 10^2 Sporen/g und 10^5 Sporen/g in Erdnussproben sowie 10^4 Sporen/g und 10^4 Sporen/g in Maisproben detektierten.

Zusammenfassend stellt diese Doktorarbeit eine detaillierte Studie über die Entwicklung und Evaluierung von LAMP-basierten Systemen für Detektion, Identifizierung und Quantifizierung der wichtigen Aflatoxinproduzenten *A. flavus, A. nomius* und *A. parasiticus* sowie von *A. caelatus* dar. Es ist zu erwarten, dass die entwickelten LAMP Assays ein vielversprechendes Instrument in der Vorhersage möglicher Risiken durch Aflatoxine zu einem frühen Zeitpunkt darstellt. Die Systeme könnenn somit im Rahmen von HACCP Konzepten in der Nahrungs- und Futtermittelproduktionskette angewendet werden, da sie spezifisch, empfindlich und leicht zu handhaben sind. Durch ihren einfachen Aufbau und ihre geringe Störanfälligkeit eignen sie sich

insbesondere für den Einsatz in sog. *Low-Tech-Environments*, also in Situationen, in denen technische Ressourcen begrenzt sind.

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