Food Microbiology 90 (2020) 103469

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

A loop-mediated isothermal amplification (LAMP) assay for rapid detection of fumonisin producing *Aspergillus* species

Massimo Ferrara*, Antonio F. Logrieco, Antonio Moretti, Antonia Susca

Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Bari, Italy

ARTICLE INFO

Keywords:
Aspergillus niger
Aspergillus welwitschiae
Rapid molecular tool
Fumonisin
Maize

ABSTRACT

Fumonisins contamination of food commodities is a worldwide problem, especially for maize. The ability to produce fumonisinsis a trait of several species of Fusarium, mainly F. verticillioides and F. proliferatum on maize, and some Aspergillus species. *A. niger* and its sister species *A. welwitschiae*, can contribute to fumonisin B_2 (FB_2) accumulation in maize kernels, although to a lesser extent than fumonisin-producing *Fusarium* species.

Fumonisins risk monitoring represents an effective strategy in the integrated approach for mycotoxin risk management and reduction. The availability of a user-friendlymolecular assay for the detection oftoxigenic fungal species represents a valuable tool in understanding and managing upcoming mycotoxin contamination.

In this study, we developed a LAMP assay, based on the detection of *fum10*, for a rapid and specific molecular detection of FB_2-producing *A. niger* and *A. welwistchiae*, potentially useful to perform monitoring directly "on site" in maize chain. Results showed that very low amounts of conidia are suitable to detect the presence of the target gene, thus providing information about the presence of FB_2-producing *Aspergillus* species and the possible upcoming fumonisins contamination in maize. The assay was combined with a suitable protocol for "in field" crude DNA extraction and a colorimetric method for easy naked-eye evaluationof results, offering a reliable and user-friendly tool to support effective reduction strategies of mycotoxin contamination in crop management programs.

1. Introduction

Maize kernels are commonly infected by toxigenic fungi belonging to the genera *Aspergillus* and *Fusarium*, which can cause maize spoilage and mycotoxin contamination in the kernels. Among the different mycotoxins, fumonisins are the most common in maize-growing regions of the world and contamination of maize grains by fumonisinsis considered a serious problem due to its association with diseases of livestock and humans (Desjardins, 2006; Munkvold, 2003).

The fumonisins are responsible for equine leukoencephalomalacia, a serious and usually fatal disease in horses, and porcine pulmonary edema, an often fatal disease in swine (Desjardins, 2006). In humans, there is a link between high consumption of maize contaminated by fumonisins and the occurrence of oesophageal cancer in some areas of the world, and fumonisins also have been reported as potential risk factors for neural tube defects, craniofacial anomalies, and other birth defects arising from neural crest cells (Marasas et al.,2004).

The fumonisins, mainly fumonisin B_1, B_2 and B_3, can accumulate at harmful levels in maize kernels when environmental conditions favor the development of Fusarium kernel rot, caused by several species of *Fusarium* (e.g. *Fusarium verticillioides* and *F. proliferatum*). Moreover, natural occurrence of fumonisin B_2 has been reported in red wine from Italy (Logrieco et al., 2010). In a survey on maize carried out in Central Italy (Logrieco et al., 2014), 17 maize kernel samples showed *F. verticillioides* and/or *F. proliferatum* at a level ranging from 13% to 100% of kernels. However, 10 out of 17 samples were also contaminated by *Aspergillus* section *Nigri* with a range from 6% to 68% of kernels, showing a significant inverse logarithmic relationship between levels of *Fusarium* and *Aspergillus* contamination. The discovery of putative fumonisin biosynthesis gene clusters, and production of fumonisin B_2 (FB_2) and B_4 (FB_4), in some strains of *Aspergillus* sect. *Nigri* (Frisvad et al.,2011; Mogensen et al.,2010), has led to concern about the contribution of these fungi to fumonisin contamination in agricultural products (including maize grain) that they infect or colonize.

Among species of the black aspergilli, only *A. niger* and its sister species *A. welwitschiae* have been reported to produce fumonisin (FB) (Frisvad et al., 2007, 2011; Mogensen et al., 2010) and can contribute to FB_2 accumulation in maize kernels, although their levels of production and occurrence on maize are lower than those of fumonisin-producing *Fusarium* species.

* Corresponding author.
E-mail address: massimo.ferrara@ispa.cnr.it (M. Ferrara).

https://doi.org/10.1016/j.fm.2020.103469
Received 2 October 2019; Received in revised form 11 December 2019; Accepted 19 February 2020
Available online 28 February 2020

The genomes of three strains of *A. niger* have already been fully sequenced: ATCC 1015, NRRL 3 and CBS 513.88 (Baker, 2006; Pel et al., 2007; Andersen et al., 2011).

Those strains have subsequently been examined intensively using transcriptomics and metabolomics to explore and understand growth, differentiation, chemistry and physiology of the species (Nielsen et al., 2009; Andersen et al., 2008a, 2008b; Jorgensen et al., 2009; Guillemette et al., 2007; Sun et al., 2007). All three strains have a fumonisin gene cluster similar to the one responsible for the production of the known fumonisin mycotoxins by *Fusarium verticillioides* (Brown et al., 2005; Proctor et al., 2003). *A. niger* strains produce fumonisins B_2, B_4, and B_6 but not FB_1 (Frisvad et al., 2007; Månsson et al., 2010).

Species of *Aspergillus* do not possess orthologs of all genes in the *Fusarium* fumonisin cluster; in particular, they lack an ortholog of the *Fusarium* gene *fum2*, which is required for production of the fumonisin analogs fumonisins B_1 (FB_1) and B_3 (FB_3), but not the analog fumonisin B_2 (FB_2) (Susca et al., 2010).

Although FB_2 production has been reported in both *A. niger* and *A. welwitschiae*, not all strains of these species produce the mycotoxin (Susca et al.,2010, 2014a; Varga et al.,2010; Frisvad et al., 2011; Storari et al., 2012; Palumbo et al., 2013; Gherbawy et al., 2015; Massi et al., 2016). PCR, Southern blot analysis and genome sequence data indicate that FB non-producing strains of *A. welwitschiae* have a partially deleted FUM cluster (ITEM 7468, KJ934796; ITEM 11945, KJ934798). Analysis of a collection of FB non-producing isolates of *A. welwitschiae* indicated that all the isolates exhibited the same gene deletion pattern within the FUM cluster (Fig. 1). Deletion occurred for genes *fum21*, *fum14*, *fum13*, *fum8*, *fum3*, *fum7* and *fum10*. Conversely, FB non-producing isolates of *A. niger* had an intact fumonisin biosynthetic gene cluster (*A. niger* ITEM 10355, KJ934797), and are therefore considered potentially producing isolates. (Susca et al., 2014b).

These findings suggested that the genetic basis for FB non-production could be exploited in order to set up a diagnostic tool for the early detection of fumonsin-producing *Aspergillus* species on crops.

Current diagnostic systems for toxigenic fungi are based mainly on microbial isolation and subsequent identification through morphological and PCR-based methods.

These approaches require a fully equipped laboratory and are expensive and time consuming. To overcome these limitations, the availability of diagnostic tools for rapid, simple, and cost-effective detection of toxigenic food-borne fungi is advantageous, especially if applicable directly “on-site” in food chain production or directly “in field”.

As an alternative to PCR-based methods, loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) has been described as an easy and rapid molecular diagnostic tool. LAMP reaction is based on application of four primers with six different binding sites in the target DNA which are required to bind properly in order to initiate DNA amplification by a thermophilic DNA polymerase with high strand displacement activity (Notomi et al., 2000). The further application of additional loop primers speeds up the amplification reaction reducing time of execution up to half time (Nagamine et al., 2002). In addition to high specificity and sensitivity, LAMP amplification has a relatively low sensitive to inhibitors, such as polyphenols or polysaccharides that can be very abundant especially in the food matrices (Kaneko et al., 2007). Therefore the LAMP technique is widely applied or the detection of pathogenic and mycotoxin-producing fungi, such as *Fusarium graminearum*, *Aspergillus carbonarius*, *A. niger* and three species of *Aspergillus* section Flavi, *Penicillium nordicum* (Niessen and Vogel, 2010; Luo et al., 2012; Storari et al., 2013; Ferrara et al., 2015).

Amplification products generated by LAMP can be visualized in different ways such as increase of turbidity, fluorescence or by complexometric dyes (Mori et al., 2001; Hill et al.,2008; Goto et al., 2009; Tomita et al., 2008). Moreover, aiming at developing a detection system that provides an easy readable result based on a visible color change that is not a prerogative of expert scientists, Tanner et al. (2015) proposed a colorimetric detection system based on the pH shift that occurs due to the release of protons during DNA synthesis. In a weak buffer system, pH is allowed to drop from pH 8.7 to mild acidity which leads to a color change of pH-indicators such as neutral red or phenol red. Since the method is quite new, only few assays involving potential mycotoxin producers have recently been based on these promising visual detection system (Niessen et al., 2017; Vogt et al., 2017; Frisch et al., 2019).

The objective of the current study was to develop a simple and rapid LAMP-based assay, applicable by mais chain operators straight on site, for the detection of fumonisin-producing *Aspergillus* species on maize samples.

2. Material and methods

2.1. Fungal cultures and DNA extraction

A collection of 24 fungal strains from Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production (ISPA-CNR, Bari, IT, http://server.ispa.cnr.it/ITEM/Collection/) were used for the set up of LAMP assay (Table 1). The *A. niger* (3) and *A. welwitschiae* (9) strains were selected according to genomic information about the presence/absence of the biosynthetic *fum10* gene and their ability to produce FB_2 (Susca et al., 2014a, 2016). Other strains (12) were selected for non-target species frequently isolated from maize or able to produce fumonisins and thus potentially having genetic similarities with the target gene. Working cultures were maintained on PDA medium (Conda Laboratories, Spain) at 25 °C for 5–7 days and stored as conidial suspension in 15% v/v glycerol at −20 °C. Total genomic DNA was extracted from mycelium grown in Wickerham medium (dextrose 4% w/v, peptone 0.5% w/v, yeast extract 0.3% w/v, malt extract 0.3% w/v, sterilized at 110 °C for 15 min) at 25 °C for 72–96 h. Mycelium was collected by filtration on sterile Whatman paper (No.4) and DNA was extracted from 100 mg of mycelium by using the Wizard Magnetic DNA Purification System for Food kit (Promega, USA), according to the manufacturer's instructions. Quality and concentration of DNA were determined by agarose gel electrophoresis and spectrophotometric analysis using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, USA).

2.2. Primer design and LAMP reaction

LAMP assay was specifically designed on *A. niger* fum10 gene (GenBank accession no. XM_001389070) coding for a peroxisomal-coenzyme A synthetase previously reported to be involved in fumonisins biosynthesis (Susca et al., 2016).

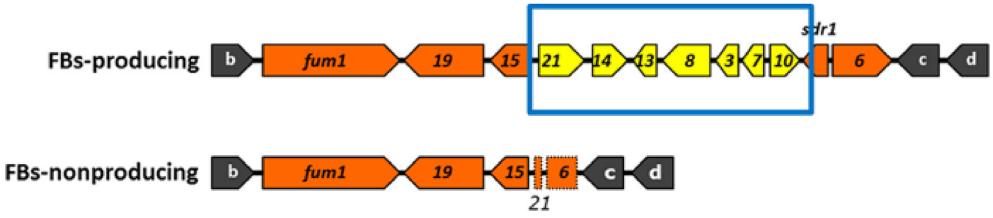

Fig. 1. Organization of genes in the FUM cluster region of FB_2-producing and non-producing strains of black *Aspergillus* species. Blue square indicates cluster region with possible gene deletion. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Fungal strains used for evaluation species specificity (o crossreactivity) of the developed LAMP.

Genus	Species	Strain	FUM10	FB_2	LAMP reaction
Aspergillus	*niger*	ITEM 9568	+	+	+
	niger	ITEM 4501	+	+	+
	niger	ITEM 10927	+	+	+
	welwitschiae	ITEM 4552	–	–	–
	welwitschiae	ITEM 6142	–	–	–
	welwitschiae	ITEM 7097	+	+	+
	welwitschiae	ITEM 10353	–	–	–
	welwitschiae	ITEM 10935	+	+	+
	welwitschiae	ITEM 14303	+	+	+
	welwitschiae	ITEM 11945	+	+	+
	welwitschiae	ITEM 11778	–	–	–
	welwitschiae	ITEM 11943	–	–	–
	tubingensis	ITEM 7040	–	–	–
	flavus	ITEM 8115	–	–	–
Fusarium	*verticillioides*	ITEM 231	+	+	–
	proliferatum	ITEM 5351	+	+	–
	fujikuroi	ITEM 7584	+	+	–
	subglutinans	ITEM 4400	–	+	–
	poae	ITEM 9138	–	–	–
	sporotrichioides	ITEM 7637	–	–	–
	equiseti	ITEM 11296	–	–	–
	graminearum	ITEM 8600	–	–	–
Penicillium	*chrysogenum*	ITEM 4518	–	–	–
Alternaria	*alternata*	ITEM 752	–	–	–

Specific primers were designed using the PrimerExplorer V. 4 software tool (http://primerexplorer.jp/e/.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). Sequence analysis resulted in several sets of primers for *fum10* gene. The specificity of primers was tested in silico using the nucleotide BLAST search tool against the NCBI nt sequence database (Altschul et al., 1997).

Primers designed were outer primers Fum10-F3 and Fum10-B3, inner primers Fum10-FIP and Fum10-BIP and loop primers Fum10-LoopF and Fum10-LoopB (Table 2).

In order to select the optimal temperature, LAMP reaction was tested in a temperature gradient. Reactions were set up as follows: 2.5 μl 10x thermopol buffer (New England Biolabs, Hertfordshire, UK), 8 mM $MgSO_4$ (Sigma-Aldrich, Munich, Germany), 0.8 M betaine (Sigma-Aldrich, Munich, Germany), 1.6 mM dNTPs (Euroclone), 1.6 μMeach of FIP and BIP (HPLC-purified, Invitrogen), 0.2 μMeach of F3 and B3, 20 ng of ITEM 9568 genomic DNA, 8 U of the Bst 2.0 WarmStart DNA polymerase (New England Biolabs, MA, USA) and sterile deionized water up to 25 μl.The reaction mix was incubated, using an Eppendorf Mastercycler pro 384 (Eppendorf AG, Germany), in a temperature gradient between 63 and 68 °C for 60 min and final heating at 85 °C for 2 min to terminate the reaction.

Optimal temperature and reaction mixture were selected when the highest amount of amplicons for target DNA was generated.

To confirm the specific region targeted by LAMP assay, a PCR amplification of genomic DNA of type strains *A. niger* ITEM 9568 and *A. welwitschiae* ITEM 11945 was performed by using the F3 and B3 outer LAMP primers. The PCR reaction was carried out in 25 μl reaction mixture containing Platinum Hot Start SuperFi PCR 2x Master Mix (Invitrogen, Carlsbad, CA, USA), 500 nM of each primers and 20 ng of template DNA from ITEM 9568 or ITEM 11945. PCR amplification conditions were: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 30sec, followed by a final extension at 72 °C for 5 min. LAMP and PCR amplification products were separated by 2% (w/v) agarose gel electrophoresis and stained with GelRed (Biotium, USA). PCR products were sequenced with an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA) to confirm the nucleotide sequence of targeted gene.

Table 2
List of LAMP primers for *fum10* gene. Degenerate base is defined as R (G/A).

PRIMER NAME	5' > 3' OLIGONUCLEOTIDE SEQUENCE
FUM10-F3	**CGGTAGCGATCTTTCTCCG**
FUM10-B3	**CGTCTGGATGRTTTCCTGC**
FUM10-FIP	**ATAGCCGGTGCTGTTTCTGTCA-GCCTTCATGAGCTTGGGAC**
FUM10-BIP	**TTGACAGTTCGATGCGCCGG-TCTACCCTCGCTTGGGAG**
FUM10-LOOPF	**CCCGTAGACCTCAATTACTTGC**
FUM10-LOOPB	**TGGCACACTACCCTATCGCATC**

2.3. Specificity and sensitivity of LAMP assay

The specificity of the selected primer set was verified using DNA isolated from fungal strains listed in Table 1. LAMP assay was conducted as reported above at the optimal temperature of 67 °C.

LAMP products were analyzed using 2% (w/v) agarose gel electrophoresis and stained with GelRed (Biotium, USA).

Moreover, the sensitivity of LAMP assay was tested on crude DNA isolated from 10-fold serial dilutions of *A. niger* ITEM 9568 conidial suspensions. The conidial suspensions were obtained by scraping the surface of a PDA plate inoculated with ITEM 9568 after 7 days of incubation at 25 °C. Conidia were suspended in sterile distilled water and the concentration was determined by using a "Thoma" chamber. Crude DNA was extracted from 100 μL aliquots of conidial suspensions, ranging from 10^2 to 10^6 conidia/total volume, with ION-Liquid Force Extraction buffer (Generon Spa, MO, Italy) according to the manufacturer's instructions, and 5 μL of crude DNA extracts, were directly used for LAMP assay. A colorimetric assay for naked-eye evaluation of positive and negative reactions has been implemented. The colorimetric assay was based on phenol red (CAS n. 34,487-61-1). Accordingly, the LAMP reaction was set up as described above except for Bst 2.0 reaction buffer. The phenol red modified Bst 2.0 reaction buffer (pH ~8.6–8.8) was prepared as fallow: 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 50 mM KCl, 0.1% v/v Tween 20, 0.8 M Betaine, 1.4 mM dNTP solution mix, phenol Red 0.1 mM. Crude DNA extraction and amplification reactions were performed in triplicates using a H2O3-PROIII programmable portable thermoblock (Coyote Bioscience, CA, USA). Positive and negative reactions were recorded according to color transition from red (negative) to yellow (positive). Limit of detection was defined as the lowest conidia amount that induced recognizable color change of phenol red from red to yellow.

2.4. Implementation of LAMP assay on food crop material

A specific protocol for the implementation of LAMP assay on food crop material was set up. In details, 200 g of maize kernels were soaked (25% w/v) with sterile distilled water and incubated overnight at room temperature. Next, soaked maize kernels were sterilized at 121 °C for 15 min and incubated at room temperature for additional 2 days. Sterilized maize kernels were divided in 10 g aliquots and inoculated separately with 1 mL of a conidial suspension of *A. niger* ITEM 9568 in order to obtain a final contamination rate ranging from 10^4 to 10 conidia/g of kernels. Inoculated maize kernels aliquots were dried for 12 h at room temperature under a sterile hood. Then, each inoculated aliquots was washed with 10 mL of 0.1% (v/v) Tween 20 sterile solution. Washing solutions were filtered on 45 mm sterile 0.22 μm nitrocellulose filters (Merck Spa, Darmstadt, Germany). Filters were than washed with 1 mL of ION-Liquid Force Extraction buffer, incubated at 98 °C for 10 min and extraction mixture was than diluted 1:10 (v/v) with sterile distilled water. After crude DNA extraction, 5 μL of crude extracts were used as template for the FUM10 specific LAMP assay. As positive controls, 5 μL of crude DNA from 10^2 conidial suspension of ITEM 9568 extracted with ION-Liquid Force Extraction buffer (as previously described) and 20 ng of genomic DNA of ITEM 9568 were included. Reactions were performed in triplicates.

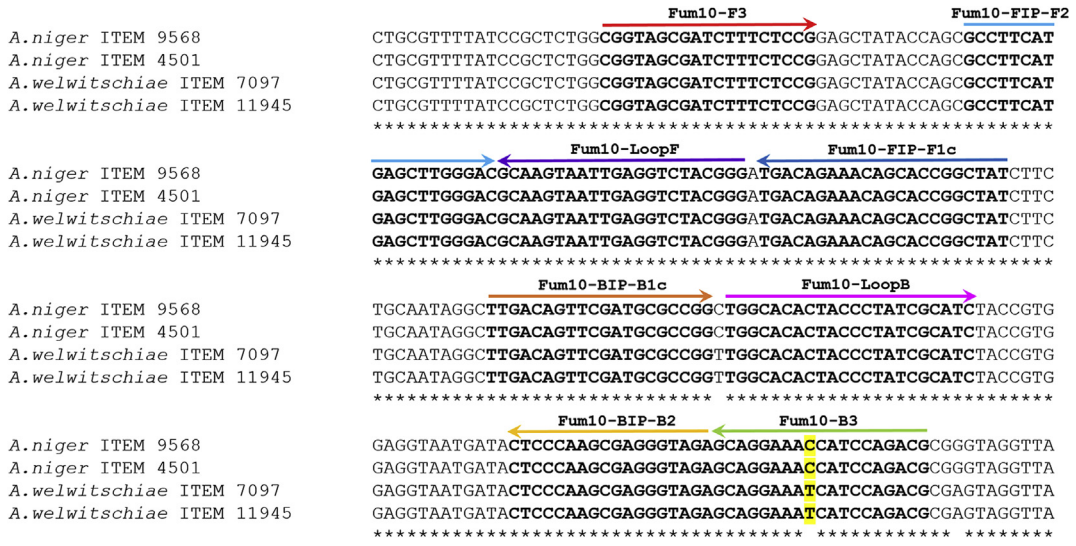

Fig. 2. Multiple alignment of a partial *fum10* gene sequence (240 nt) in fumonis in producing *A. niger* and *A. welwitschiae*. Nucleotides corresponding to annealing sites of LAMP primers (F3, F2, F1c, B3, B2, B1c) and loop primers (LoopF and LoopB) are marked in bold. Position marked in yellow indicates sequence variation between the 2species. Arrowheads indicate orientation of the primers (→ = forward primer, ← = reverse primer). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

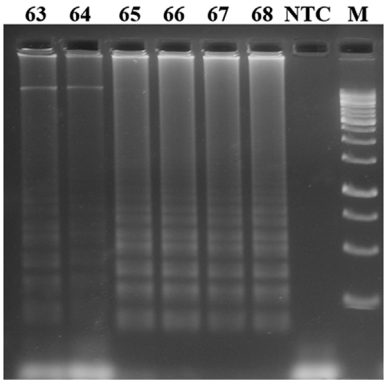

Fig. 3. Optimization of LAMP reaction conditions. Amplification pattern is displayed for each temperature tested. NTC = no template control; M = 1 Kb DNA ladder.

3. Results

3.1. LAMP primer set

Primer set Fum10-ID27was selected among primer sets generated for *fum10* gene. The other predicted primer sets were discarded due to inadequate primer stability at the 3' end and the possible risk of secondary structures formation as self dimers or cross dimers. Positions of the selected primers within the target *fum10* nucleotide sequence of *A. niger* are shown in Fig. 2.

Specificity of the primers was tested in silico using the nucleotide BLAST search tool on the NCBI sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). No significant hits different from *fum10* gene were detected, confirming high primer specificity for the target sequence. Optimization of LAMP reaction conditions resulted in the selection of 67 °C as the optimal temperature. Positive amplification reaction resulted in the production of the typical ladder like pattern of DNA fragments ranging approximately from 200 to several thousand base pairs (Fig. 3).

3.2. Specificity and sensitivity of LAMP assays

Specificity of the primer set for the detection of fumonisin-producing *Aspergillus* species was analyzed using DNA isolated from pure cultures of 12 fungal strains listed in Table 1, after 60 min reaction time at 67 °C. No amplification was observed from non-producing *Aspergillus* isolates of the two analyzed species (Fig. 4). Moreover, specificity of the molecular assay was verified on 12 non target species belonging to *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* genera (Table 1, Fig. 5). The sensitivity of LAMP assays for FBs-producing strains was tested against 10-fold serial dilutions of *A. niger* ITEM 9568 conidial suspensions ranging from 10^5 to 10 conidia/reaction. The colorimetric LAMP assay was performed according to the optimized protocol for phenol red.

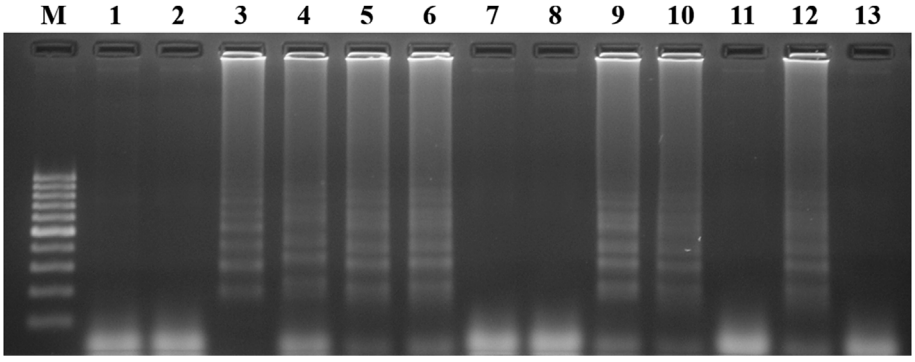

Fig. 4. Specificity of LAMP assay for Fum10-ID27 primer set: M = Marker 100 bp (ThermoFisher Scientific); Lane 1 = ITEM 11943; 2 = ITEM 6142; 3 = ITEM 10927; 4 = ITEM 11945; 5 = ITEM 9568; 6 = ITEM 10935; 7 = ITEM 10353; 8 = ITEM 11778; 9 = ITEM 4501; 10 = ITEM 7097; 11 = ITEM 4552; 12 = ITEM 14303; 13 = no template control.

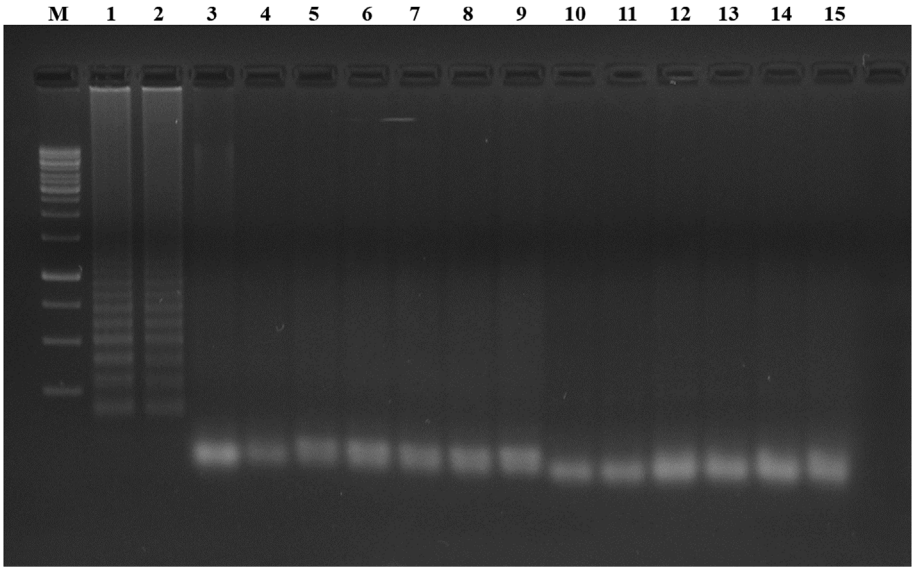

Fig. 5. LAMP assay for Fum10-ID27 specificity on non target species: M = Marker 1 kb (ThermoFisher Scientific): 1 = *Aspergillus niger* ITEM 9568; 2 = *Aspergillus welwitschiae* ITEM 11945; 3 = *Fusarium fujikuroi* ITEM 7584; 4 = *Fusarium subglutinans* ITEM 4400; 5 = *Fusarium sporotrichioides* ITEM 7637; 6 = *Fusarium verticillioides* ITEM 231; 7 = *Fusarium proliferatum* ITEM 5351; 8 = *Fusarium equiseti* ITEM 11296; 9 = *Fusarium poae* ITEM 9138; 10 = *Fusarium graminearum* ITEM 8600; 11 = *Aspergillus flavus* ITEM 8115; 12 = *Aspergillus tubingensis* ITEM 7040; 13 = *Penicillium chrysogenum* ITEM 4518; 14 = *Alternaria alternata* ITEM 752; 15 = no template control.

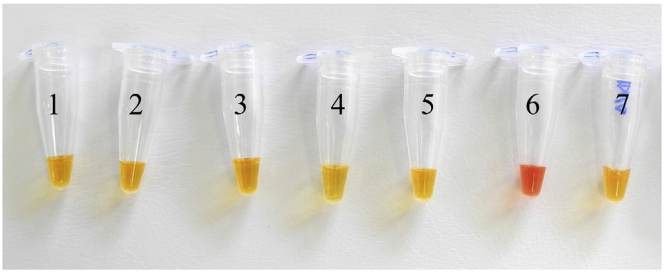

Fig. 6. Sensitivity of colorimetric LAMP assay on different conidia concentration of *A. niger* ITEM 9568. (1) 10^5conidia; (2) 10^4conidia; (3)10^3conidia; (4) 10^2conidia; (5) 10 conidia; (6) no template reaction control; (7) genomic DNA of ITEM 9568.

The limit of detection for LAMP assay was 10 conidia/reaction (Fig. 6). The color intensity of positive reactions was not correlated to DNA concentration and color variation was clear also at the lowest conidia concentrations. No intermediate colors between red and yellow were observed. Negative control samples without template showed no color variation under the selected reaction conditions.

3.3. Implementation on maize of LAMP assay for detection of fumonisin-producing Aspergillus species

A rapid and user-friendly protocol suitable for "in field" execution of LAMP assay on food crop material has been implemented (Fig. 7).

The developed protocol was effective to achieve positive LAMP results in all the tested inoculation rates (Fig. 8). The assay was suitable

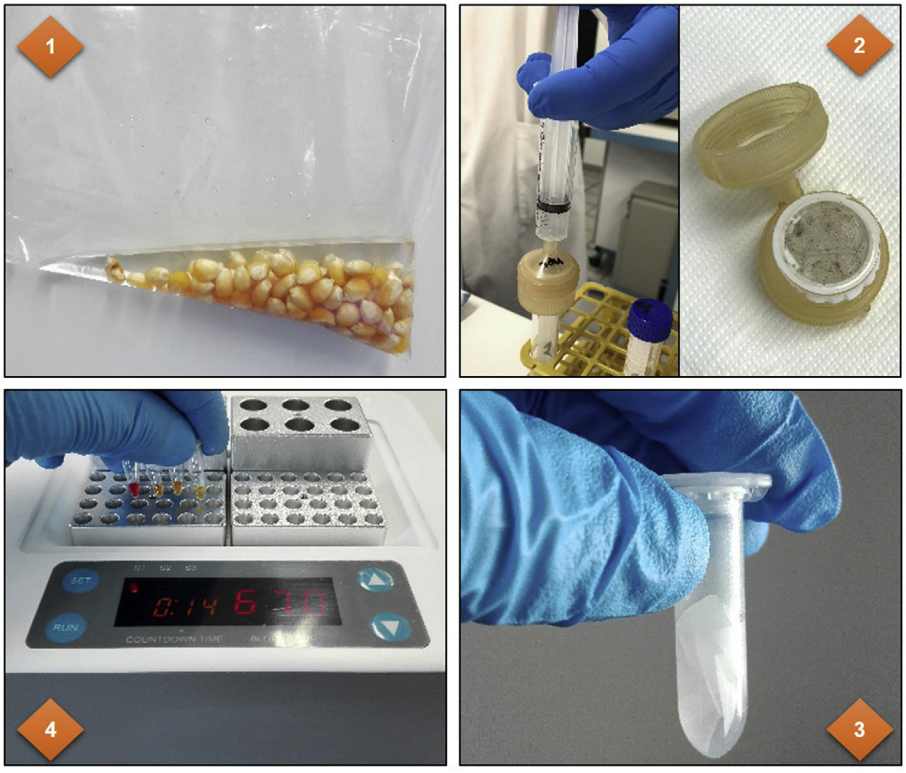

Fig. 7. Schematic representation of the developed protocol for LAMP assay on food crop material. Maize kernels are washed in sterile bags (panel 1). Washing solutions are filtered on sterile filters (panel 2). Crude DNA is extracted from collected material on filters (panel 3). Colorometric LAMP assay is conducted on a portable thermoblock (panel 4).

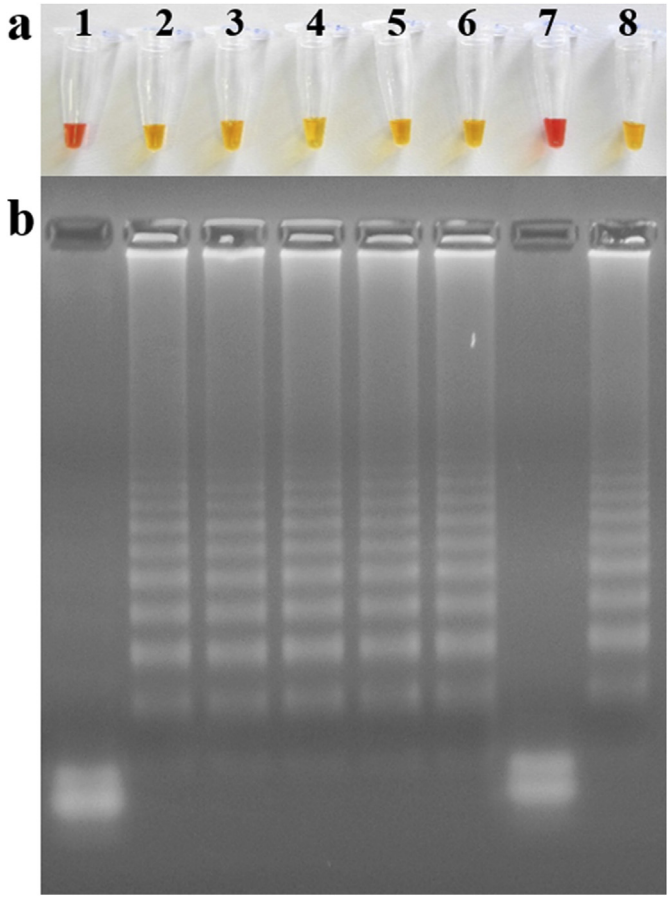

Fig. 8. LAMP assay on food crop material. In tube colorimetric assay (panel a) and the respective reaction on 2% agarose gel (panel b). (1) extraction buffer (2–5) maize kernels inoculated with 10^4 (2), 10^3 (3), 10^2 (4), 10 (5) conidia/g (6) crude DNA from 10^2 conidia of ITEM 9568 (7) no template reaction control (8) genomic DNA of ITEM 9568.

for the detection of FBs producers species up to a contamination kernels rate of 10 conidia/g. The new colorimetric assay was effective to detect positive reactions. Positive target amplification determined color transition from red to yellow, while negative reactions remained red. No intermediate color transition was observed.

4. Discussion

Species of *Aspergillus* section *Nigri* are commonly associated with maize kernels, and some strains can produce fumonisin mycotoxins. However, there is little information about the extent to which these fungi contribute to fumonisin contamination in grain, the damage they cause to maize ears, or their effects on maize seed germination and seedling health. FBs contribute in natural maize sample is not measurable, because *Aspergillus A. niger* and *A. welwistchiae* co-occurr with *Fusarium* FBs-producing spp. However, the evidence of their contribute has been deduced analyzing the ratio of FB_2/FB_1 contamination in the maize samples, where high values can be explained by *Aspergillus* section *Nigri* contribution to FB_2 accumulation. Hence, a continuous survey of fumonisin-producing *Aspergillus* species directly in the field, during the different maturation stages of maize crop, could support safety assurance in maize production and in the food chain.

In this study, for the first time, a user-friendly LAMP assay has been set up for the detection of FB_2-producing *Aspergillus niger* and *A. welwitschiae*. The rapid molecular assay is based on the detection of *fum10* gene, a structural gene of fumonisin cluster in *Aspergillus* toxigenic species, affected by deletion in strains non-producing FB_2. To date, no tests are available for the detection of FB_2-producing strains of the two species in a specific, rapid and robust way, which would avoid the need of sophisticated laboratory equipment, giving the opportunity to potentially execute the molecular essay directly on-site in maize chain production.

The developed LAMP assay can be used to rapidly detect the presence of fumonisins-producing strains of *Aspergillus* species on maize kernels. Since *Aspergillus* species are saprophytic fungi known to be secondary pathogens in maize crop, the prediction of an up-coming fumonisin contamination could be achieved by the molecular detection on maize surface of fumonisin-producing *Aspergillus* species. Its sensitivity and low susceptibility to DNA impurities offer the possibility to be applied directly on conidia or with crude DNA extracts. Therefore, no fungal neither maize DNA isolation are needed to perform this LAMP assay.

The specificity of the LAMP assay was demonstrated in rapidly detecting (about 1 h) selectively between FBs-producing *Aspergillus* strains among non-producing strains, that is *A. niger* and *A. welwitschiae*.

Additionally, based on our knowledge, the occurrence of *A. niger* isolates not able to produce FBs is very rare, as well as possible fumonisins production inducibility should be further studiedfor those *A. niger* isolates with intact FUM cluster but not able to produce FBs in standard laboratory conditions. Possible further speculations could be focused on other genetic or regulatory elements linked to their incapability to synthetize FBs. Above all, we should consider that, similarly to other DNA-based detection techniques, LAMP is only informative about the presence of the fungal target and does not give any information about the real presence of FB_2 in the sample.

In order to set up a molecular assay suitable for “in field” analysis, a protocol for crude DNA extraction from contaminated maize kernels has been set up and optimized for LAMP assay. The DNA extraction and reaction amplification were performed in a programmable portable device, provided with a long life battery for the execution of extraction and amplification steps directly in the field.

The application of an *in-tube* colorimetric detection method avoids additional handling of tubes after reaction and reduces potential cross-contaminations between samples. Moreover, the use of phenol red as colorimetric indicator offers the possibility of a simple and clear positive/negative reading of results by non-qualified staff without the need of any UV illumination equipment or sophisticated image analyzer. These aspects contribute to improve the potential transferability of the assay directly “in the field”, offering the opportunity to monitor the safety of food products during crucial steps of maize production.

Results showed that very low amounts of conidia are suitable to detect the presence of the target gene, thus providing an early information about a possible upcoming fumonisin contamination in maize and/or the presence of fumonisin-producing *Aspergillus* species. Although the described LAMP assay has been developed and optimized on maize, it could be potentially applied also for the detection of FB_2-producing *Aspergillus* species on grapes. In our opinion, the developed LAMP assay represents, in fumonisins risk monitoring, a reliable and promising molecular tool supporting crop management for application of appropriate and effective reduction strategies of contamination.

5. Conclusions

A user-friendly LAMP assay has been set up for the detection of FB_2-producing *Aspergillus niger* and *A. welwistchiae.* Results revealed that the *fum10* gene could be a reliable target to detect the presence of fumonisin-producing *Aspergillus* species on maize kernels within about 60 min. The optimization of a suitable protocol for crude DNA extraction from artificially contaminated maize kernels together with the use of a portable device make the assay potentially suitable for “in field”. Moreover, visual detection of results under daylight conditions enables fast and easy discrimination of positive/negative results. These aspects make the developed LAMP assay a reliable and promising molecular

tool for monitoring toxigenic risk during the maturation steps of maize crop and could be implemented as diagnostic tool in crop management strategies.

Author contributions

M. F. and A. S. set up and performed the experiments; M. F., A. F. L., A. M. and A. S. wrote the paper.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This work was supported in part by H2020-project 678781-MycoKey- "Integrated and innovative key actions for mycotoxin management in the food and feed chain".

References

Altschul, et al., 1997. Gapped BLAST and PSI-BLAST: a New Generation of Protein Database Search Programs.

Andersen, M.R., Vongsangnak, W., Panagiotou, G., Salazar, M.P., Lehmann, L., Nielsen, J., 2008a. A trispecies Aspergillus microarray: comparative transcriptomics of three *Aspergillus* species. Proc. Nat. Acad. Sci. USA 105, 4387–4392.

Andersen, M.R., Nielsen, M.L., Nielsen, J., 2008b. Metabolic model integration of the bibliome, genome, metabolome and reactome of *Aspergillus niger*. Mol. Syst. Biol. 4, 178.

Andersen, M.R., Salazaar, M.P., Schaap, P.J., van de Vondervoort, P.J.I., Culley, P., et al., 2011. Comparative genomics of citric-acid-producing Aspergillus niger ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res. 21, 885–897.

Baker, S.E., 2006. Aspergillus Niger genomics: past, present and into the future. Med. Mycol. 44, S17–S21.

Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A.E., Zheng, L., et al., 2005. Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. Fungal Genet. Biol. 42, 848–861.

Desjardins, A.E., 2006. Fusarium, Mycotoxins, Chemistry. Genet. Biol. APS, St Paul, USA.

Ferrara, M., Perrone, G., Gallo, A., Epifani, F., Visconti, A., Susca, A., 2015. Development of loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Penicillium nordicum* in dry-cured meat products. Int. J. Food Microbiol. 202, 42–47.

Frisch, L.M., Niessen, L., 2019. Development and optimization of a group-specific loop-mediated isothermal amplification (LAMP) assay for the detection of patulin-producing *Penicillium* species. Int. J. Food Microbiol. 298, 20–30.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin B2 production by *Aspergillus niger*. J. Agric. Food Chem. 55, 9727–9732.

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson, R.A., et al., 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoSONE 6, e23496. https://doi.org/10.1371/journal.pone.0023496.

Gherbawy, Y., Elhariry, H., Kocsube, S., Bahobial, A., Deeb, B.E., Altalhi, A., et al., 2015. Molecular characterization of black *Aspergillus* species fromonion and their potential for ochratoxin A and fumonisins B2 production. Foodb. Pathog. Dis. 12, 414–423 10.1089/fpd. 2014.1870.

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxynaphthol blue. Biotechniques 46, 167–172.

Guillemette, T., van Peij, N.N.M.E., Goosen, T., Lanthaler, K., Robson, G.D., et al., 2007. Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. BMC Genom. 8, 158.

Hill, J., Beriwal, S., Chandra, I., Paul, V.K., Kapil, A., Singh, T., Wadowsky, R.M., Singh, V., Goyal, A., Jahnukainen, T., Johnson, J.R., Tarr, F.I., Vats, A., 2008. Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli*. J. Clin. Microbiol. 46, 2800–2804.

Jorgensen, T.R., Goosen, T., van den Hondel, C.A.M.J.J., Ram, A.F.J., Iversen, J.J.L., 2009. Transcriptomic comparison of *Aspergillus niger* growing on two different sugars reveals coordinated regulation of the secretory pathway. BMC Genom. 10, 44.

Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys. Methods 70, 499–501.

Logrieco, A.F., Ferracane, R., Visconti, A., Ritieni, A., 2010. Natural occurrenceof fumonisin B_2 in red wine from Italy. Food Addit. Contam. 27, 1136–1141. https://doi.org/10.1080/19440041003716547.

Logrieco, A.F., Haidukowski, M., Susca, A., Mulè, G., Munkvold, G.P., Moretti, A., 2014. Aspergillus section Nigri as contributor of fumonisin B_2 contamination in maize. Food Addit. Contam. 31, 149–155. https://doi.org/10.1080/19440049.2013.862349.

Luo, J., Vogel, R.F., Niessen, L., 2012. Development and application of a loop-mediated isothermal amplification assay for rapid identification of aflatoxigenic molds and their detection in food samples. Int. J. Food Microbiol. 159, 214–224.

Månsson, M., Klejnstrup, M.L., Phipps, R.K., Nielsen, K.F., Frisvad, J.C., et al., 2010. Isolation and characterization of fumonisin B_6, a new fumonisin from Aspergillus Niger. J. Agric. Food Chem. 58, 949–953.

Marasas, W.F., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C., Allegood, J., Martínez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E., Merrill Jr., A.H., 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J. Nutr. 134, 711–716.

Massi, F.P., Sartori, D., de Souza Ferranti, L., Iamanaka, B.T., Taniwaki, M.H., Vieira, M.L., et al., 2016. Prospecting for the incidence of genes involved in ochratoxin and fumonisins biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*. Int. J. Food Microbiol. 221, 19–28. https://doi.org/10.1016/j.ijfoodmicro.2016.01.010.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010. Production of Fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J. Agric. Food Chem. 58, 954–958 10. 1021/jf903116q.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.

Munkvold, G.P., 2003. Cultural and genetic approaches to managing mycotoxins in maize. Ann. Rev. Phytopathol. 41, 99–116.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16, 223–229.

Nielsen, K.F., Mogensen, J.M., Johansen, M., Larsen, T.O., Frisvad, J.C., 2009. Review of secondary metabolites and mycotoxins from the Aspergillus Niger group. Anal. Bioanal. Chem. 395, 1225–1242.

Niessen, L., Bechtner, J., Fodil, S., Taniwaki, M.H., Vogel, R.F., 2017. LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section Flavi in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection. Int. J. Food Microbiol. 266, 241–250.

Niessen, L., Vogel, R.F., 2010. Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. Int. J. Food Microbiol. 140, 183–191.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, e63.

Palumbo, J.D., O'Keeffe, T.L., Gorski, L., 2013. Multiplex PCR analysis of fumonisins biosynthetic genes in fumonisin-nonproducing *Aspergillus niger* and *A. awamori*strains. Mycologia 277–284. https://doi.org/10.3852/11-418.

Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofman, G., et al., 2007. Genome sequencing and analysis of the versatile cell factory Aspergillus Niger CBS 513. 88. Nat. Biotechnol. 25, 221–231.

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E., 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet. Biol. 38, 237–249.

Storari, M., Dennert, F.G., Bigler, L., Gessler, C., Broggini, G.A.L., 2012. Isolation of mycotoxins producing black aspergilli in herbal teas available on the Swiss market. Food Contr. 26, 157–161. https://doi.org/10.1016/j.Foodcont.2012.01.026.

Storari, M., Rohr, R., Pertot, I., Gessler, C., Broggini, G., 2013. Identification of ochratoxin A producing *Aspergillus carbonarius* and *A. niger* clade isolated from grapes using the loop-mediated isothermal amplification (LAMP) reaction. J. Appl. Microbiol. 114, 1193–1200.

Sun, J.B., Lu, X., Rinas, U., Zeng, A.P., 2007. Metabolic peculiarities of *Aspergillus niger* disclosed by comparative metabolic genomics. Genome Biol. 8, R192.

Susca, A., Proctor, R.H., Mulè, G., Stea, G., Ritieni, A., Logrieco, A., et al., 2010. Correlation of mycotoxin fumonisin B_2 production and presence of the fumonisin biosynthetic gene fum8 in *Aspergillus niger* from Grape. J. Agric. Food Chem. 58, 9266–9272 10. 1021/jf101591x.

Susca, A., Moretti, A., Stea, G., Villani, A., Haidukowski, M., Logrieco, A., et al., 2014b. Comparison of species composition and fumonisins production in *Aspergillus* section Nigri populations in maize kernels from USA and Italy. Int. J. Food Microbiol. 188, 75–82 10. 1016/j.ijfoodmicro.2014.06.031.

Susca, A., Proctor, R.H., Butchko, R.A., Haidukowski, M., Stea, G., Logrieco, A.F., et al., 2014a. Variation in the fumonisin biosynthetic gene cluster in fumonisin-producing and non producing black aspergilli. Fungal Genet. Biol. 73, 39–52. https://doi.org/10.1016/j.fgb.2014.09.009.

Susca, A., Proctor, R.H., Morelli, M., Haidukowski, M., Gallo, A., et al., 2016. Variation in fumonisin and ochratoxin production associated with differences in biosynthetic gene content in *Aspergillus niger* and *A. welwitschiae* isolates from multiple crop and geographic origins. Front. Microbiol. 7, 1–15.

Tanner, N.A., Zhang, Y., Evans Jr., T.C., 2015. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. Biotechniques 58, 59–68.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., et al., 2010. Fumonisin contamination and fumonisins producing black Aspergilli in dried vine fruits of different origin. Int. J. Food Microbiol. 143, 143–149. https://doi.org/10.1016/j.ijfoodmicro.2010.08.008.

Tomita, N., Mori, Y., Kanda, H., Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3, 877.

Vogt, E.I., Kupfer, V.M., Bechnter, J.D., Frisch, L.M., Vogel, R.F., Niessen, L., 2017. Detection of *Penicillium oxalicum* in grapes with a loop-mediated isothermal amplifcation (LAMP) assay. J. Microbiol. Biotechnol. Food Sci. 7, 265–270.