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Detection of post-harvest pathogens by loop-mediated isothermal amplification: a review

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Summary. Postharvest losses, which occur between harvest and consumption of agricultural commodities, are major causes of food waste. Minimizing food loss helps provide nutritious food for animals and humans, and alleviate adverse environmental effects on food production. These losses are often related to the presence of postharvest pathogens, including fungi and bacteria, which typically start by infecting crops in the field as well as during postharvest chain. Control of these pathogens relies on development of tools that ensure their early and accurate detection. Among these is loop-mediated isothermal amplification (LAMP), a molecular method for pathogen detection. LAMP characteristics of rapidity, specificity and simplicity have encouraged development of a number of LAMP assays for detection of postharvest pathogens. Each LAMP assay allows to detect a specific genetic region of the target microorganism, which can be directly related to mycotoxin production, fungicide resistance and phytotoxicity. The LAMP amplicons are rapidly visualized, either at a specific timepoint, or in real-time by taking measurements throughout reaction, thereby necessitating less sophisticated facilities than those needed for PCR assays. In addition, many studies have developed simple protocols for the direct detection of pathogens on fresh produce. This paper explains the LAMP reaction, and its importance for postharvest detection of fungi and bacteria. Previous studies that have developed LAMP assays are also discussed.

Keywords. Food losses, microorganism contamination, mycotoxins, LAMP.

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INTRODUCTION

Human population growth has created major concerns about food security. By 2050, global food production will have to increase by an estimated 70% to adequately feed humans and avoid an unprecedented food crisis (Mvumi and Stathers, 2015). Although intensifying food production seems an obvious solution, this is unpractical due to the challenges of cli-

mate change and the role of intensive agriculture in their escalation (Giovani *et al.,* 2022). A good way to improve this situation while protecting the environment would be to reduce the important amounts of wasted commodities (Parfitt *et al.*, 2010). Approximately one-third of all food produced for human consumption is lost or wasted along supply chains (FAO, 2011), thereby rendering postharvest food losses a leading cause of food insecurity. These losses occur between harvest and consumption, at rates of 20% to 50% in developing countries and 5% to 25% in developed ones, depending on product type, cultivar, environmental factors, and postharvest conditions (Kader, 2003; Yahia *et al.*, 2019). Presence of postharvest pathogens on produce, whether in the field or during product handling, are major factors in product commodity deterioration. For each fruit or vegetable species, pathogenic bacteria, fungi and yeasts can cause many postharvest diseases (Antunes and Cavaco, 2010). Several bacterial pathogens such as *Bacillus, Pseudomonas, Pectobacterium* and *Xanthomonas* can cause important losses in the field and postharvest. Nevertheless, fungi are considered the most important degrading agents that affect foods during storage, making the food products unfit for human consumption by decreasing their nutritive value. Many of these pathogens are also able to produce carcinogenic mycotoxins. The health hazards posed by these compounds for humans have led most countries to issue regulations of their consumption, which target the mycotoxins or, in some cases, the toxigenic agent (Wenderoth *et al.,* 2019). In addition, agricultural exports are subjected to maximum tolerated mycotoxin levels. In some cases, these have reshaped the trade patterns of economically important crops (Bui-Klimke *et al.*, 2014).

For a long time, synthetic fungicides were the primary means of controlling postharvest decays (Spadaro and Gullino, 2005). However, their use has decreased due to their potentially hazardous effects on human health and environments, as well as the development of fungicide-resistant strains of postharvest pathogens (Baibacova *et al.*, 2019). These factors have restricted the approval of many products and motivated researchers to find alternative ways to control postharvest pathogens. Consequently, new technologies, substances and practices have emerged for fresh produce storage which target these pathogens, to preserve agricultural products and extend their shelf-lives (Tripathi and Dubey, 2004).

Most postharvest pathogens start infection processes in the field and often remain latent in fresh produce before causing serious damage during storage (Suarez *et al.*, 2005; Wenneker and Thomma, 2020). Many farmers therefore apply treatments on their crops to avoid these contaminations. However, in some cases, these treatments may be unnecessary since they are applied without accurate verification of the presence of pathogens. This random decision-making contradicts the principles of precision agriculture, and can have severe effects on the environment. Furthermore, they are often costly. Successful treatment depends on early detection and accurate identification of spoilage agents. This relies on several methods, traditionally including morphological characterization after growth on agar media (Samson *et al.*, 2007). However, these methods are time-consuming and require laboratory facilities and mycological expertise (Luo *et al.*, 2012).

Molecular methods such as PCR and real-time PCR are more rapid, sensitive and specific than culturing techniques (Schaad *et al.*, 2002; Rodríguez *et al.*, 2012). They can be used to identify mycotoxigenic strains by targeting the genes linked to toxin production (Stakheev *et al.*, 2011). However, they are costly and require suitably trained personnel and well equipped laboratories. As an alternative technology, Loop-mediated isothermal AMPlification (LAMP) reaction was described as a specific, rapid, cost-effective, and easy-to-use method by Notomi *et al.* (2000). This method uses four to six primers from the target region of each organism which is amplified at a fixed temperature. The high levels of specificity and sensitivity obtained with LAMP, coupled with its robustness to inhibition substances and its user-friendliness, have encouraged researchers to improve this method by developing real-time way to visualize the amplification products such as real-time LAMP. This method is largely used for the detection of several pathogens in preharvest among viruses (Bhat *et al.*, 2022), fungi (Abderraouf *et al.*, 2022) and bacteria (Yaseen *et al.*, 2015; Valentini *et al.*, 2022). Previous studies that developed specific LAMP primer sets for the identification of postharvest pathogens also assessed the possibility to apply LAMP-based assays to rapidly detect pathogens directly from infected commodities (Niessen *et al.*, 2018). In these protocols, simplicity is often researched throughout all the steps of the analysis, from the nucleic acid extraction to the amplification and detection of results. This paper reviews these studies and offers insights on LAMP and its potential effects on the management of postharvest pathogens.

POSTHARVEST PATHOGENS

Plant protection for economically important species is based on two essential pillars: the first is protection of plants throughout production stages to maximize yields by avoiding losses due to pest attacks; the second is pro-

tection of agricultural produce after their harvest to preserve food security and reduce waste due to postharvest pathogens. It is estimated that, in some cases, postharvest losses can be up to 50% of potential production (Kasso and Bekele, 2018; Kader, 2003; Yahia *et al.*, 2019). This can especially be the case when postharvest management lacks advanced technologies, such as continuous cold storage (Kitinoja *et al.*, 2019). Harvesting is the detachment of product from living plants, which renders products vulnerable to opportunistic spoilage agents that enter through wounds caused by agricultural practices, feeding animals, or the handling processes. These agents, such as *Ralstonia solanacearum* (Lemma *et al.*, 2014), *Erwinia carotovora* (Zhao *et al.*, 2013) and *Botrytis cinerea* (Suarez *et al.*, 2005), are often encountered in the field, and many can also cause damage at preharvest stages.

Several taxonomic groups of pathogens can cause postharvest infections. These include bacteria, yeasts and filamentous fungi. The most important filamentous fungi are species of *Alternaria, Aspergillus, Botrytis, Fusarium, Geotrichum, Gloeosporium, Monilinia, Penicillium, Mucor* and *Rhizopus* (Barkai-Golan, 2001). These are responsible for decay of agricultural commodities, they break barriers that would otherwise protect against other microorganisms such as bacteria and human pathogens, and many produce mycotoxins (Dukare *et al.*, 2019). Mycotoxins are secondary metabolites that can be highly toxic and carcinogenic, mutagenic and teratogenic to humans and animals (Omotayo *et al.*, 2019). They are mainly produced by species of *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Table 1). Due to the hazardous effects of these fungi, many countries have issued regulations to control the importation of mycotoxin-susceptible commodities (van Egmond *et al.*, 2007). In some cases, the costs imposed by these regulations have caused important economic losses to exporting countries. For example, in 1997, the EU banned pistachio nut imports from Iran due to high aflatoxin levels. This decision shifted the trade patterns when the United States of America became the main exporter of this crop to countries with strict aflatoxin tolerance regulations (Bui-Klimke *et al.*, 2014).

DETECTION METHODS FOR POSTHARVEST PATHOGENS

Successful management of postharvest pathogens is directly related to their early and accurate detection. Therefore, many detection methods have been developed and improved. These methods can be either microbiological, serological or molecular.

Table 1. The most important postharvest pathogens, the symptoms they cause, and the main mycotoxins they produce.

Pathogen group	Main species	Symptom	Main mycotoxins	Reference
Aspergillus section *flavi*	*A. flavus* *A. parasiticus*	Green mold	Aflatoxins	Varga *et al.*, 2011
Aspergillus section *nigri*	*A. carbonarius* *A. niger*	Black mold	Ochratoxin A Fumonisin B_2	Astoreca *et al.*, 2010 Palumbo *et al.*, 2011
Penicillium	*P. verrucosum* *P. expansum* *P. italicum*	Blue mold	Ochratoxin A Patulin	Perrone and Susca, 2017
Alternaria	*A. alternata*	Black spots	Alternariol Tenuazonic acid Altertoxins I, II, III	Ostry, 2008
Fusarium	*F. verticillioides* *F. moniliforme* *F. graminearum*	Dark to brown rot	Fumonisin	Duvick *et al.*, 2001
Colletotrichum	*C. acutatum* *C. gloeosporioides* *C. boninense*	Anthracnose	—	Shi *et al.*, 2020a
Geotrichum	*G. candidum*	Sour rot	—	Talibi *et al.*, 2012
Botrytis	*B. cinerea*	Gray mold	—	
Monilinia	*M. fructicola* *M. laxa* *M. fructigena*	Brown rot on stone fruit	—	Côté *et al.*, 2004
Rhizopus	*R. microsporus* *R. stolonifer*	Rhizopus rot, Black bread mold	Rhizonin	Partida-Martinez *et al.*, 2007

Microbiological methods

Microbiological methods are the traditional ways of identifying and differentiating postharvest pathogens. They are based on pathogen cultivation on agar media followed by the observation of microorganism macro and micro-morphological characteristics (Klich and Pitt, 1988). Microbial growth manifests differently depending on the medium and environmental conditions (Cotty, 1994). In addition, some media are selective or semi-selective, encouraging growth and/or sporulation of particular fungus species while preventing development of others. For example, Samson *et al.* (2007) described the boscalid MEA medium, which only allowed the sporulation of *Aspergillus carbonarius* amongst all other black aspergilli. Other media, such as coconut cream agar (CCA) (Dyer and McCammon, 1994) and *A. flavus* and *A. parasiticus* agar (AFPA) (Pitt *et al.*, 1983) are particularly suitable for the growth of toxigenic strains.

Although these methods have played important roles in improving microbiological analyses, they are inadequate for current challenges, even though they are still needed if the pathogen is new and other kind of methods are still not available. They are time-consuming and require high levels of laboratory expertise and mycological knowledge in order to provide accurate diagnoses. Furthermore, these methods cannot be applied for every species and strain, and their results are strictly dependent on appropriate incubation conditions (Balajee *et al.*, 2007a; b). These methods also do not ensure high sensitivity due to low survival of fungal propagules under stressful conditions of selective and semi-selective media (Beuchat, 1993).

Serological methods

Serological diagnostic methods, such as Enzyme-Linked Immunosorbent Assay (ELISA), are based on detection of antibodies against pathogens and constitute a group of sensitive, rapid, specific and cost-effective tests (Clarck *et al.*, 1986). ELISA targets specific proteins based on the interaction between antigens specific to each pathogen, and their specific antibodies (Crowther, 1995). ELISA methods have been widely used to detect plant pathogens (Le and Vu, 2017), and were tested for the detection of pathogens in food products (Tsai and Cousin, 1990). Some researchers were interested in using ELISA for postharvest analyses, such as in the quantification of *B. cinerea* (Fernàndez-Baldo *et al.*, 2011) and for the detection of mycotoxins (Pei *et al.*, 2009). As field test, the lateral flow assay is applied for the rapid, equipment-free detection of different pathogens, e.g., *Phytophthora* spp. (Lane *et al.*, 2007). Nevertheless, sensitivity of these methods remains low compared to molecular methods, which are the most trusted tools for pathogen identification.

Molecular methods

Molecular methods are based on detection and amplification of target sequences from reference genes in pathogen nucleic acids. Therefore, they are used for species and strain differentiation and in phylogenic studies (Luo *et al.*, 2012). Among these methods, PCR is most commonly used. It amplifies target regions using polymerase and two specific primers throughout a series of repeated thermal cycles. PCR-based methods (PCR, real-time PCR, qPCR, multiplex qPCR) are powerful tools that provide high levels of specificity and sensitivity for detection of postharvest pathogens (Suarez *et al.*, 2005; Samson *et al.*, 2007). These methods have also been applied for amplification of genes relevant to mycotoxin biosynthesis (Shapira *et al.*, 1996) which, in some cases, can also be involved in pathogenicity (Sanzani *et al.*, 2012). However, PCR-based methods are costly due to the necessity for expensive reagents and high technology equipment such as thermocyclers. They also require advanced laboratory training and long DNA or RNA clean-up steps before amplification procedures. Therefore, these methods do not detect contaminants *in situ*. As an alternative molecular technology, LAMP was described by Notomi *et al.* (2000) as a specific, rapid, cost-effective, and easy-to-use method.

LAMP

LAMP is a molecular detection technique that amplifies DNA or RNA fragments using a strand displacing DNA polymerase (usually the Bst DNA polymerase from *Bacillus stearothermophilus*). This allows production of high amounts of DNA in a short time (Luo *et al.*, 2014). LAMP works under isothermal conditions (operating at a constant temperature), and can be highly specific since it uses four to six primers able to hybridize from six to eight regions of the target sequence. Usually, this technique requires no post-reaction processing because results can be quickly observed using indicators. Consequently, it can speed up the diagnostic process in comparison to a PCR-based method. LAMP is also highly tolerant to sample inhibitors, allowing it to be used directly on crude DNA extracted from infected or infested commodities (King *et al.*, 2019).

LAMP primer design

The most popular softwares to design LAMP primers are: Primer Explorer, a free, online tool with five versions released to date (the latest version is available at https://primerexplorer.jp/e/), OptiGene Limited (Horsham, UK) using Genie platforms, and "LAMP Designer" by PREMIER Biosoft (USA) (Le and Vu, 2017). While taking into consideration the four key factors in LAMP primer design (melting temperature, stability at the end of each primer, GC content, and secondary structure), these tools facilitate the design of the following primers (Figure 1): Forward inner primer (FIP), which consists of an F2 region complementary to the F2c region at the 3' end of the target sequence; whereas at the 5' end, it consists of an F1c region identical to the F1 region of the target sequence. Forward outer primer (F3), which is complementary to the F3c region of the target sequence. Backward inner primer (BIP), which consists of a B2 region complementary to the B2c region at 3' end of the target sequence; whereas at 5' end, it consists of the B1c region identical to the B1 region of the target sequence. Backward outer primer (B3), which is complementary to the B3c region of the template sequence. Forward loop primer (LF) which is complementary to the region between F1 and F2. Backward loop primer (LB) which is complementary to the region between B1 and B2.

The loop primers reduce the reaction time and increase the rate of amplification by binding the loops that are incorrectly oriented to bind to internal primers (Nagamine *et al.*, 2002).

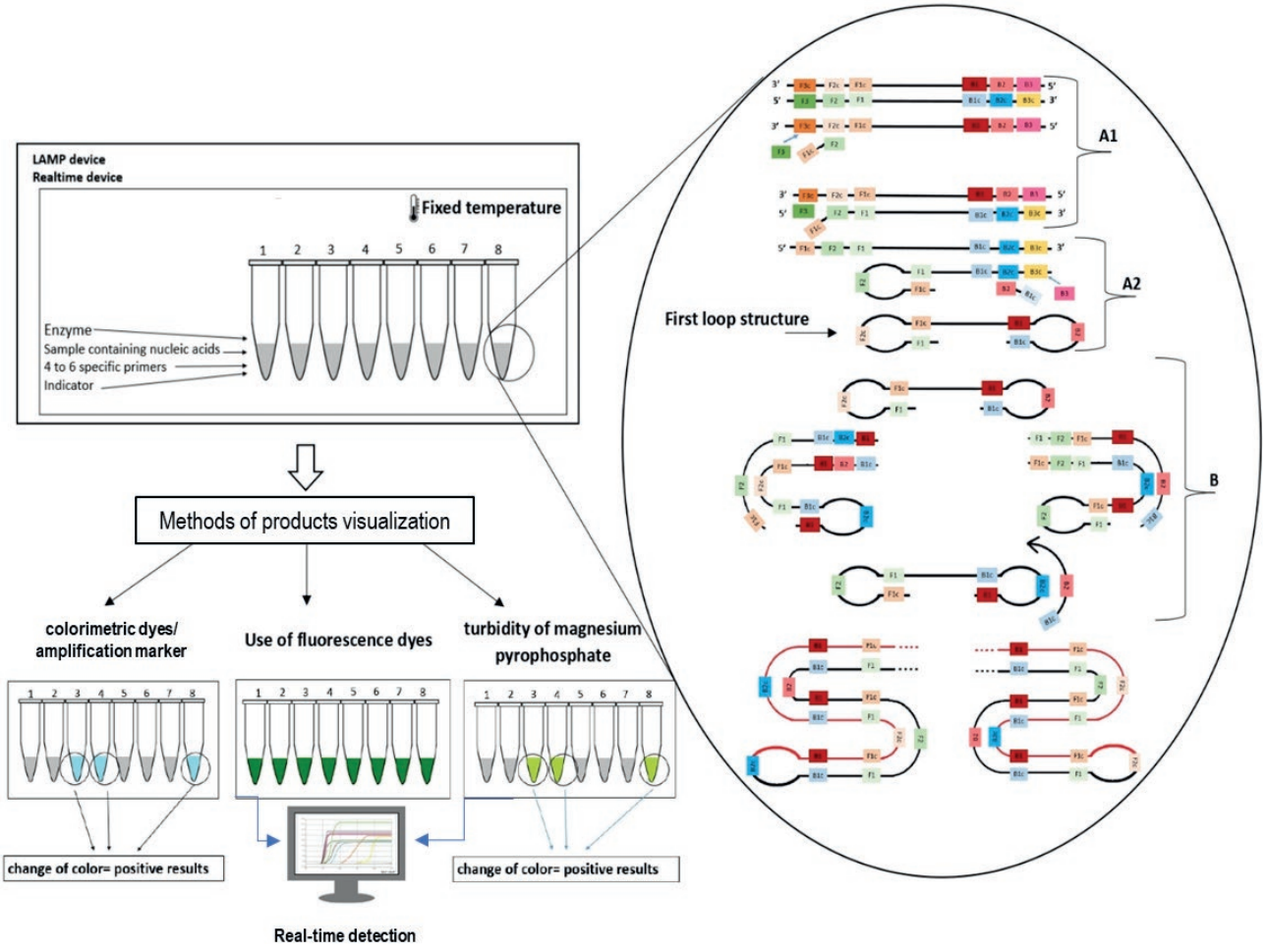

Figure 1. LAMP reaction and amplicon detection. Loop structure production: (A1) annealing and elongation of primer F2 (on primer FIP), followed by annealing and elongation of primer F3 which allows the first strand displacement and first loop formation through the annealing of F1(on primer FIP); and (A2) the annealing of primer B2 (on primer BIP), followed by annealing and elongation of primer B3 with the displacement of the polymerized strand and the formation of the second loop through B1 (on primer BIP). Target amplification (B): repetition of the annealing and elongation cycles on the produced loop and primer sites.

LAMP reaction

Two main steps can be differentiated in the LAMP reaction: loop structure production (Figure 1, A1 and A2) and amplification (Figure 1, B).

Loop structure production begins when FIP anneals the target sequence and separates the amplified sequence from the template by extending the primer, thereby forming the first product. This product is then displaced by synthesis when F3 anneals to an upstream target region (F3c), and the end of it forms a self-hybridizing loop structure due to the presence of the reverse complementary sequence F1c (Figure 1, A1). The same cycle repeats on the other end of the target sequence by the backward primers (BIP and B3) to form the first loop dumbbell structure (Figure 1, A2).

During elongation and amplification, the nucleic acid structure resulting from the previous step serves as a template for carrying on the amplification. It contains several sites from which the synthesis can initiate including the 3' end of the loop and the annealing sites of FIP and BIP. This allows the distinction of two elongation cycles: self-elongation from the loop and binding elongation of the inner region (Notomi *et al*., 2015) (Figure 1, B). The amplification step allows synthesis of complex structures with multiple loop sites that allow for exponential amplification of the sequence chosen as target.

Detection of LAMP products

Detection of LAMP amplicons can be accomplished by agarose gel electrophoresis. However, the most widely used methods are those that can ensure rapid observation of results without requiring further experimental steps (Figure 1). These methods can be either end-point tests (measured at specific timepoints) or real-time tests (measurement of amplification progress throughout the reactions). These methods can be classified into two groups (Moore *et al*., 2021). Sequence-independent methods rely on the detection of concentration changes of substrates or products produced throughout the reaction related to the amplification of the target sequences, and include changes in turbidity pH reactive dyes, intercalating fluorescent dyes, or bioluminescence. Conversely, sequence-dependent methods generate a signal directly dependent on the specific sequence targeted and allow the multiple target detection in a single tube; they include Quenching of Unincorporated Amplicon Signal Reporters (QUASR), Detection of Amplification by Releasing of Quenching (DARQ), CRISPR-Cas cleavage systems, one step strand displacement, and molecular beacons.

LAMP assays for postharvest molds

The review of Niessen (2018) identified 23 research publications describing development of LAMP assays to detect mycotoxigenic fungal pathogens on food matrices. Among these, two assays were panfungal, detecting presence of any fungal contamination in samples (Zhang *et al.,* 2017). Since then, similar studies have shown increased interest in LAMP as effective for distinguishing mycotoxigenic pathogens. However, other fungi, such as *Botrytis* spp. and *Monilinia* spp., which are unknown for mycotoxin production, can also severely damage harvested commodities. Therefore, these fungi have been the subject of several LAMP assays. In addition, some important postharvest bacterial pathogens have been subjects for development of rapid LAMP detection assays. In total, the present review lists 42 articles for fungi, and many of the studies provide simple and rapid protocols for detection of postharvest fungal contaminants directly from food. Since these pathogens are often present in preharvest as latent infections (Sanzani *et al*., 2012), the assays conducted on plant parts or seedlings have also been taken into consideration in the present review (Table 2).

Aspergillus

Among postharvest fungal pathogens, *Aspergillus* spp. are the most studied for development of rapid detection LAMP assays. Luo *et al.* (2012) were the first to aim to detect aflatoxigenic *Aspergillus* spp. directly from food samples, including Brazil nuts, peanuts and coffee beans. Their assay targeted the *acl1*-gene of *A. flavus* and *amy1*-genes of *A. nomius* and *A. parasiticus,* and positive results were detected by bright green fluorescence under UV 366 nm light. The detection limits were 2.4, 7.6 and 20 pg of pure DNA per reaction, respectively, for *A. flavus, A. nomius* and *A. parasiticus*. Specificity of the assays was also high with the *A. nomius* primer set not detecting any non-target isolate, and the other two primer sets detecting only some *Aspergillus* spp., which are very closely related to the targets.

The same primers were further tested by Luo *et al.* (2014) as parts of species-specific turbidimeter-based real-time LAMP assays, where turbidity was measured at 600 nm at intervals of 6s. These assays attempted to define contamination levels in samples of shelled Brazil nuts, maize, and peanuts. The detection limit was 10 conidia g^{-1} for *A. flavus* and *A. nomius* in Brazil nuts. The assay detection limits for *A. flavus* were 10^2 conidia g^{-1} for peanuts, and 10^4 conidia g^{-1} for maize, and for *A. parasiticus* were 10^5 conidia g^{-1} for peanuts and 10^4 conidia g^{-1} for maize.

Table 2. LAMP assays developed for the detection of postharvest fungal pathogens.

Pathogen	Target gene	Sensitivity	Food matrix	Reference
Aspergillus flavus *A. nomius* *A. parasiticus*	Alpha amylase (*amy1*) *amy1* ATP citrate lyase subunit 1	2.4 pg of pure DNA/ reaction 7.6 pg of pure DNA/ reaction 20 pg of pure DNA/ reaction	Brazil nuts, peanuts, green coffee beans	Luo *et al.*, 2012
A. flavus *A. nomius* *A. parasiticus*	a*my1* *amy1* ATP citrate lyase subunit 1	10 spores 100 spores 100 spores (sensitivity according to matrix and pathogen)	Brazil nuts, peanuts, maize	Luo *et al.*, 2014
A. flavus, *A. flavus* (toxygenic strains)	ITS1–5.8S–ITS2 rDNA region aflatoxin-encoding gene *aflP*	10 fg 1 pg of pure DNA	Peanuts, maize	Luo *et al.*, 2014
Aflatoxigenic Aspergilli	*nor1*	9.03 pg of DNA 211 conidia	Rice, nuts, raisins, dried figs	Niessen *et al.*, 2018
A. flavus, *A. parasiticus*	Aflatoxin efflux pump gene *aflT*	100-999 pg of DNA	Hazelnuts	Ortega *et al.*, 2020
A. carbonarius *A. niger* (ochratoxigenic)	polyketide synthase genes *pks*	Between 0.01 and 0.1 ng	Grapes	Storari *et al.*, 2013 Storari and Broggini, 2017
Ochratoxigenic strains of *Aspergillus* spp.	*pks*	Not mentioned	Peanuts	Al-Sheikh, 2015
A. niger *A. welwistchiae*	*fum10* (Fumonisin production)	10 conidia g^{-1} of maize	Maize	Ferrara *et al.*, 2020
A. caelatus *A. flavus* *A. nominus*	*acl1*	10^1 for *A. nomius*, 10^2 for *A. flavus*	Brazil nuts	Luo *et al.*, 2012
Botrytis cinerea	*bcos5*	10^{-3} ng μL^{-1}	Tomato and strawberry petals	Duan *et al.*, 2014a
B. cinerea	β-tubulin gene (*tub2*) mutation that causes resistance to benzimidazole	2×10^5 copies per μL of the plasmid	_____	Fan *et al.*, 2019
B. cinerea	Intergenic spacer (IGS) of nuclear ribosomal DNA	65 pg *B. cinerea* DNA	Detached rose petals, pelargonium leaves	Tomlinson *et al.*, 2010
B. cinerea	β-tubulin gene (*tub2*) mutation that causes resistance to benzimidazole	2×10^3 copies per μL of the plasmid	_____	Duan *et al.*, 2018
Penicillium expansum	*pex2_044840*	25 pg genomic DNA of *P. expansum*	Apples, grapes, apple juice, apple puree, grape juice	Frisch *et al.*, 2021
Patulin producing *Penicillium* spp.	isoepoxydon dehydrogenase *idh*	2.5 pg of purified genomic DNA	Grapes, apples	Frisch and Niessen, 2019
P. oxalicum	*pde_07106*	100 pg genomic DNA	Grapes	Vogt *et al.*, 2017
Monilinia laxa; M. fructicola	Intron in the cytochrome b, 166 associated with the *qoi* fungicides resistance	100-999 fg of DNA (*M. fructicola)*, 100-999 fg of DNA (*M. laxa)*	Nectarines	Ortega *et al.*, 2019
Fusarium graminearum	F167Y mutation of carbendazim-resistance of the b2-tubulin gene	Not mentioned	Perithecia produced on rice, infected wheat spikelets	Duan *et al.*, 2014b
Fusarium fujikuroi	*nrps31*	1 to 10 fg of DNA extracted from pure culture	Rice seeds and seedlings	Zhang *et al.*, 2019
Fumonisin-producing *Fusarium* spp.	*fum1* polyketide synthase involved in the biosynthesis of fumonisins	5 pg of genomic DNA 10^3 spores per reaction	Maize	Wigmann *et al.*, 2020

(Continued)

Table 2. (Continued).

Pathogen	Target gene	Sensitivity	Food matrix	Reference
F. graminearum	galactose oxidase gene *gaoA* of *F. austroamericanum*		Cereal	Niessen, 2013
Fusarium spp. *F. graminearum*	*hyd5*	0.74 pg of DNA	Barley	Denschlag *et al.*, 2012
Fusarium spp	hyd5	27 gene copies	Barley	Denschlag *et al.*, 2013
Fusarium spp.	*tri6*, *tri5* combination of the two sets	1.7 pg of DNA	_____	Denschlag *et al.*, 2014
A. carbonarius *A. niger* *A. awamori*	polyketide synthase genes	100 and 10 pg of DNA	Grapes	Storari *et al.*, 2013
Alternaria alternata	*actts2*	15 pg of DNA		Moghimi *et al.*, 2016
Alternaria spp.	cytochrome b (*cytb*)	15 pg	Pears	Yang *et al.*, 2019
Monilinia fructicola	*mfcyp51*	10 fg of purified target DNA	Peaches	Chen *et al.*, 2019
Phomopsis longicolla	1-α (tef1-α)	100 pg μL^{-1}	Soybeans	Dai *et al.*, 2016
Venturia carpophila	rDNA-ITS	56.6 Fg μL^{-1}	Peaches	Zhou *et al.*, 2021
A. fumigatus *P. expansum* *P. marneffei* *Histoplasma capsulatum*	rRNA-28S	20 copies of plasmid DNA for *A. fumigatus*	Mycelium culture	Tone *et al.*, 2017
F. graminearum	gaoA	2 pg of DNA	Wheat grains	Niessen and Vogel., 2010 Abd-elsalam *et al.*, 2011 Almoammar *et al.*, 2013
F. asiaticum	cyp51C	100 pg of DNA	Wheat grain	Xu *et al.*, 2017
F. culmorum	cyp51C	100 pg of DNA	Soybeans	Zeng *et al.*, 2017
F. equiseti	cypP51C	10 pg μL^{-1} 4 conidia per g of soil	Soybean roots	Lu *et al.*, 2015
Aflatoxin producers section *Flavi*	nor1	9 pg gDNA per rxn 211 conidia per rn after disruption	Rice, maize, raisins, figs, hazelnuts, almonds, paprika, ginger	Niessen *et al.*, 2018
Claviceps purpurea	cpn60	50 genome copies per rxn	Cereal grains	Comte *et al.*, 2017

Ferrara *et al.* (2020) aimed for rapid detection by targeting the *fum10* gene of *A. niger* and *A. welwitshiae,* which can produce the mycotoxin fumonisin (FB2) in maize kernels. The amplification was carried out in a portable thermal block, and the results were detected using phenol red according to colour change from red (negative) to yellow (positive). These assays were highly specific when tested using the nucleotide BLAST search tool on the NCBI sequence database. The detection limit was as low as 10 conidia per reaction. Ferrara *et al.* (2020) also developed a user-friendly "in field" analysis protocol based on extraction of crude DNA from contaminated maize kernels using a programmable, portable device with long-life battery. Since *Aspergillus* spp. have wide host ranges, matrix-specific assays must be developed, especially for species/host combinations targeted by regulations. Currently, the present authors are developing specific real-time LAMP assays for detection of *A. carbonarius, A. flavus* and aflatoxigenic aspergilli, on pistachios and almonds which are some of the most susceptible commodities to mycotoxigenic contamination. These assays are based on detection of the pathogens directly from samples, without laborious DNA purification steps, aiming to offer simple product tests for growers.

Penicillium

The study of Sun *et al.* (2010) was the first to focus on detecting *Penicillium* species by LAMP, targeting the human pathogen *P. marneffei* in archived human tissues. Later, LAMP assays were developed to detect *Penicillium* spp. in food samples (Tone *et al.*, 2017; Frisch and Niessen, 2019). Frisch and Niessen (2019) focused on rapid detection of *P. expansum,* which causes blue

mold decay, an important postharvest fruit disease. This assay targeted the *pex2_044840* gene, and was able to detect *P. expansum* DNA at high specificity and sensitivity of 25 pg per reaction. Results were visualized using neutral red as indicator. The assay was further tested on artificially contaminated food samples including apples, grapes, apple juices, apple puree and grape juice. The protocol required different DNA preparations depending on the type of sample; for grapes and apples, detectable amounts of DNA were obtained after simple steps of washing and mechanical treatment. For juices and purees, extraction of pure DNA was necessary for LAMP amplification. Despite the importance of *Penicillium* spp. as dominant food pathogens which produce hazardous mycotoxins, only a few LAMP assays targeting these fungi have been developed, compared to other important toxigenic postharvest pathogens such as *Aspergillus* and *Fusarium*. Development of rapid species-specific real-time LAMP assays would facilitate detection and treatment of these common mold fungi.

Botrytis

LAMP assays were developed both for toxigenic postharvest fungi and those such as *Botrytis cinerea*, whose damaging effects are unrelated to mycotoxin production. This pathogen causes grey mold, an important pre- and postharvest disease on many high value crops such as grapes, strawberries and kiwifruit (Droby *et al.*, 2007; Williamson *et al.*, 2007). This pathogen is often present as latent infection, and causes damage on fresh produce after periods of quiescence. For this reason, the fungus must be detected in early stages on plant material, rather than later, when the damage has occurred. The first rapid LAMP protocol for *B. cinerea* detection was published by Tomlinson *et al.* (2010), detecting the pathogen on plant material. Their study compared this protocol with the two previously used detection methods, TaqMan real time PCR and lateral flow devices. The LAMP assay targeted the intergenic spacer (IGS) of the *B. cinerea* nuclear ribosomal DNA (rDNA) sequence, which was the same sequence targeted by the Taq-Man real time PCR assay of Suarez *et al.* (2005). LAMP was carried out on DNA extracted from inoculated rose and pelargonium, and the results were visualized by electrophoresis on 1.4% agarose gels, followed by staining with ethidium bromide. The assay was further optimized and tested in real time, with sensitivity of 6.5 pg of DNA. Comparison of LAMP and TaqMan PCR showed that both methods were very specific by only detecting *B. pelargonii* among other closely related species. When tested directly on inoculated rose petals, only real-time PCR gave results 5 h after inoculation. Both methods detected the pathogen 29 h after inoculation. *Botrytis cinerea* is an important pathogen that often commences infection in the field and spreads rapidly at postharvest stages. However, few LAMP studies have been carried out with this fungus, but two have targeted the b-tubulin gene (*tub2*) mutation that causes resistance to benzimidazole (Duan *et al.,* 2014). While those studies are important for the detection of fungicide-resistant strains, further research is required to develop protocols for field detection and identification of this pathogen.

LAMP assays developed for the detection of postharvest bacteria

Postharvest bacteria often begin infections in the field and continue to cause damage on products after harvest. They mostly cause rotting which makes pathogen differentiation difficult from symptoms. Management of these pathogens is complicated and often requires extreme measures such crop eradication. Regular monitoring for presence of these organisms in susceptible crops is mandatory. In addition, these pathogens are strictly regulated by countries which classify them in quarantine lists. Development of new, rapid and sensitive tools for detection of postharvest bacteria will ease monitoring processes and border surveillance. Several studies have aimed to develop simple LAMP protocols for the detection of postharvest bacterial pathogens (Table 3).

Kubota *et al.* (2008) were the first to attempt to develop a LAMP assay on a postharvest bacterial pathogen, for *Ralstonia solanacearum*. This pathogen is important because of its wide host range (at least 200 plants, including economically important potato, tomato and peanuts). *R. solanacearum* is also of quarantine importance in several countries, so susceptible imported crops are often tested for this pathogen at borders, making it important to develop rapid and simple detection. Kubota *et al.* (2008) targeted the *fliC* sequence of the *R. solanacearum* genome to design specific LAMP primers. They also developed an assay for direct detection from edible ginger plants. The amplification results were electrophoresed at 85V for 90 min through 2% agarose gel (1× Tris-acetate-EDTA), followed by staining with ethidium bromide. Detection was by observation of white turbidity in reaction mixtures using magnesium pyrophosphate (Mori *et al.,* 2001). This assay was highly specific when tested on other soil-borne bacteria, but sensitivity varied according to the *R. solanacearum* strain. Efficacy of the same primers was also tested by Kubota *et al.* (2008) on potato tubers in a real-time LAMP assay, that

Table 3. LAMP assays for postharvest bacterial pathogens.

Pathogen	Gene	Sensitivity	Food matrices	Reference
Pectobacterium parmentieri	*petF1* gen	10 CFU mL^{-1} 100 fg of DNA	Potato plants and tubers	Domingo *et al*., 2021
P. atrosepticum	*sorA* *cfa6*	2.5×10^2 CFU mL^{-1}	Edible ginger plants	Li *et al*., 2011
P. carotovorum	(*p4h*) (α subunit)	1 ng μL^{-1} – 5 fg μL^{-1}	Potato	Yasuhara-Bell *et al*., 2016
P. atrosepticum	*gyr*B	3 CFU/reaction from pure cultures 22 CFU/reaction from samples	Potato tubers	Hu *et al*., 2016
Dykeya spp.	*mglC*	5 pg/reaction	Pineapple, Potato	Yasuhara-Bell *et al*., 2017
P. carotovorum	*pmrA*	10^4 CFU mL^{-1}	Celery	Shi *et al*., 2020b
Xanthomonas euvesicatoria	*recG*	100 fg of pure DNA 1,000 fg of DNA in samples spiked with tomato DNA	Tomato plants	Larrea-Sarmiento *et al*., 2018
X. arboricola pv. pruni	ABC transporter ATP-binding protein	1.8 ng μL^{-1} of genomic DNA	Peach orchards	Li *et al*., 2019
Ralstonia solanacearum	*fliC*	10^4 to 10^6 CFU mL^{-1}	Edible ginger plants	Kubota *et al*., 2008
R. solanacearum	*egl*	10^4 cells mL^{-1} (25 cells per LAMP reaction) for strains of phylotypes I and III 10^5–10^6 cells mL^{-1} for strains of phylotypes II	Tomato plants, potato plants and tubers	Lenarčič *et al*., 2014
R. solanacearum	*orf428*	100 fg mL^{-1} of DNA 10^3 CFU mL^{-1} of bacterial fluid	Sweet potato	Li *et al.,* 2021

gave a detection limit of 1.25×10^5 CFU g^{-1}. This is low sensitivity, compared to results previously reported from PCR, which amplified the gene at 2×10^2 CFU g^{-1} (Horita *et al*., 2004). Improvements of LAMP techniques have allowed development of more sensitive LAMP protocols, with lower detection limits. For example, Li *et al.* (2021) designed a LAMP primer set targeting the *orf428* gene, with a detection limit of 100 fg mL^{-1} of DNA and 10^3 CFU mL^{-1} of bacterial fluid.

In addition to *Ralstonia* spp., *Pectobacterium* spp. have also been subjects for development of rapid LAMP assays. Li *et al.* (2011) developed a LAMP assay for the detection of *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atrosepticum*), which causes potato blackleg associated with pre- and postharvest losses in potato crops. The assay targeted the gene cluster encoding a pathogenicity-related phytotoxin, specifically PKS *cfa6* and *Polyangium cellulosum* soraphen polyketide synthase A (*sorA*) genes. The assay had a specificity and a low detection limit of 2.5×10^2 CFU mL^{-1}. However, the assay described by Hu *et al.* (2016) for the same pathogen, which targeted the *gyr*B gene, had a detection limit of 3 CFU per reaction from pure cultures, and 22 CFU per reaction from samples of contaminated potato tubers. Improvement of LAMP has resulted in assays with greater specificity and simpler detection protocols. Domingo *et al.* (2021) aimed to specifically detect among *Pectobacterium* spp. and *Dickeya* spp. that can cause the soft rot of potato. This highly specific real-time LAMP assay allowed detection of the target species, and no other very closely related species. This is due to the signature region within the *petF1* gene that was not found in other *Pectobacterium* spp.. In addition to real-time measurement, results were also observed from orange to bright green colour change after adding SYBR green before the reaction. Domingo *et al.* (2021) also developed a simple and effective protocol for the detection of *P. parmentieri* from potato plants and tubers. Several LAMP assays have been developed for food-borne bacteria such as *Salmonella* spp. and *Escherichia coli*, in association with foods such as meat, milk and juice. Some studies tested LAMP on fresh agricultural produce, such as that targeting the *invA* gene of *Salmonella* spp. (Zhang *et al*., 2011). This assay had a detection limit of 2 CFU per 25 g and was tested on coriander, lettuce, parsley, spinach, tomato, jalapeno

and pepper. Yokoyama *et al.* (2010) developed a LAMP assay for *E. coli* associated with radish sprouts, broccoli sprouts, ready-to-eat salads, ground pork and beef, which targeted the *aggR* gene of this bacterium, and was able to detect 6.3 CFU per reaction.

CONCLUSIONS

Postharvest waste threatens food security, and is mainly caused by decay-inducing pathogens. Protection of harvested commodities relies heavily on the early detection of these pathogens, which often commence host infections in the field (Logrieco, 2022). LAMP is a rapid, sensitive and specific method for detecting and accurately identifying these pathogens, even in the field. Indeed, LAMP is easily performed as requires no special expertise, is less expensive than other molecular identification tools. To amplify a target sequence of target nucleic acid, LAMP uses four to six primers designed specifically according to the relevant DNA. Many assays have been developed to detect postharvest fungi using this method as reported in Table 3. These assays have targeted several mold species, particularly *Aspergillus* and *Fusarium* spp. For postharvest bacteria, however, assays have been designed for only a few agricultural product hosts, and tests for this general group of products should be more widely developed. This can be achieved utilizing the current revolution in molecular biology, specifically in gene sequencing, which provides material to design specific and functional LAMP primer sets. The continuous improvement of real-time LAMP using simple extraction methods, with crude extract instead of highly purified DNA (Kogovšek *et al.,* 2017; Yaseen *et al.,* 2015), in combination with lyophilized primers contribute to the suitability of this technique for *in situ* detection of postharvest pathogens.

Since most of these pathogens and their mycotoxins are regulated by many countries, LAMP represents an easy way of testing commodities at production sites, to facilitate future treatment decisions at borders and to alert importing countries about the presence of potentially harmful and damaging pathogens.

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