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REVIEW

Progress of loop-mediated isothermal amplification technique in molecular diagnosis of plant diseases

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Abstract Effective disease management of crops is crucial to sustain food security and safeguard potential losses in crop production that worth billions of dollars. The key to success in plant disease management is having the ability to detect the causal pathogen(s) early and accurately. Polymerase chain reaction (PCR) has been a gold standard in nucleic acid-based diagnostics. Apart from PCR, within the last decade, the development of a technique called "loop-mediated isothermal amplification" or LAMP has facilitated the development of hundreds of simple assays for plant disease diagnostics. There are now more than 200 LAMP publications per year, of which 20% identify plant disease pathogens. Among them, LAMP assays are available for pathogen detection of 50 plant viruses, 20 bacterial plant diseases, 7 fungal plant diseases and several phytoplasmas. Here, we provide a comprehensive analysis of all LAMP assays available for detecting plant diseases, including various detection chemistries used. We also discuss how to avoid pitfalls when developing LAMP assays. Finally, we offer perspectives of the applications of LAMP in plant disease management, addressing the questions as to which extent the assays are helpful and whether they should be used outside the laboratory. This review will be a "handbook" for researchers developing LAMP assays for plant disease diagnostics.

Keywords Crop disease management · LAMP · Molecular diagnosis · Nucleic acid-based diagnostics · Plant disease detection · RT-LAMP

Abbreviations

LAMP	Loop-mediated isothermal amplification
RT-LAMP	Reversed transcription LAMP
IC-LAMP	Immunocaptured LAMP
ELISA	Enzyme-linked immunosorbent assay

Crop productivity and yield gaps

Rapid population growth, urbanization and climate change are some of the factors threatening food security. Plant yield and crop productivity have never been more important to feeding the world. Besides germplasm, harvest yield depends on various factors, which prevents it from reaching potential yield. Potential yield is the productivity of a crop cultivar when grown in optimal conditions with regards to water, nutrients and biotic stress (e.g., pests and diseases) [1] (Fig. 1). The difference between actual harvested yields and potential yields is known as the yield gap. For example, in the Northeastern region of Thailand in 2001, the harvested yield was 38–65% of potential yield for paddy rice, 11–18% for upland rice, 25–67% for maize, 32–49% for soybean, and 11–33% for groundnut, respectively. In the Northern part of Vietnam, actual yield of maize was two-thirds of the potential yield, while groundnut and soybean had the harvested yield recorded

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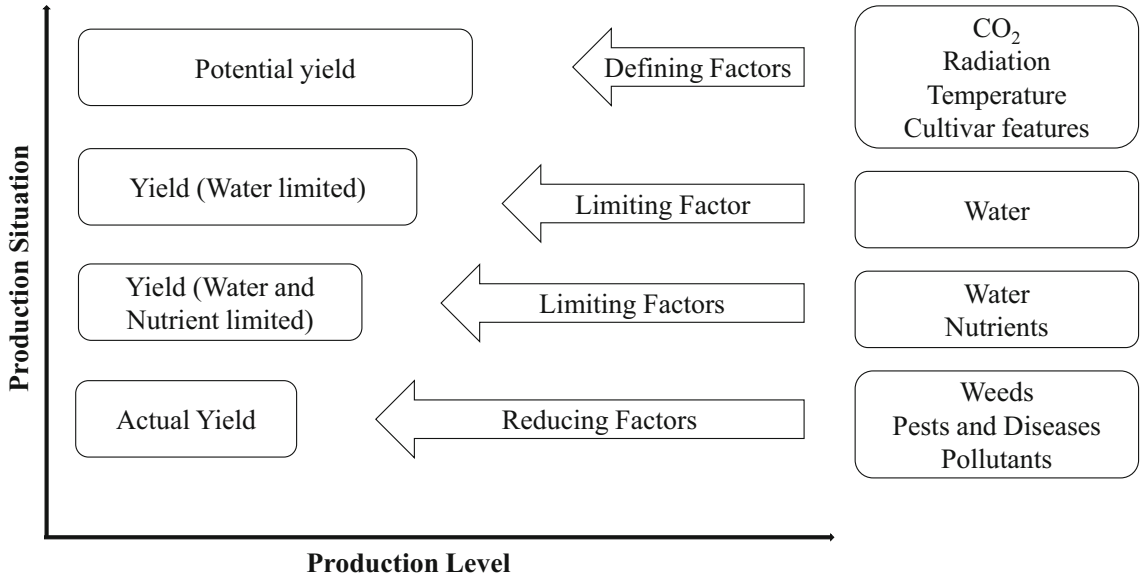

Fig. 1 Potential yield, actual yield and factors widening yield gaps ([1])

40–60% of potential yield. In India, the yields of soybean observed were 19–65% of potential yield [2].

Soil nutrients, cultivation practices, environmental conditions, pests and diseases are major factors widening yield gaps, responsible for a 30–50% reduction in crop production globally [3]. To narrow down the yield gap caused by abiotic stress, researchers are developing a microbial approach to mitigate the effects of high salinity, including the strains that improve fertilizer-use efficiency [4–6]. In the meantime, other groups studied the genes that could be used to improve stress tolerance of crops [7, 8]. On the biotic stress aspects, more than 15,000 species of fungi, 80 species of bacteria and 900 species of plant pathogenic viruses have been found to cause plant diseases; and more than 150 new diseases are identified every year [9–11]. The disease outbreaks namely "emerging infectious diseases" (EIDs) caused by new pathogens can lead to huge economic losses as well as ecological and environmental consequences [12]. For example, Cassava Mosaic Virus Disease caused by African Cassava Mosaic Geminivirus was found at an averaged incidence of 50–60% of investigated areas in 18 African countries. Pathogens are also responsible for 11–24% of declined production in the world's most important food crops, such as rice, wheat, corn, peanuts, potatoes, tomatoes, with economic losses estimated to be over US$ 10 billion for each crop (Fig. 2) [13].

To protect yield and improve crop productivity, growers usually combine different methods to establish effective disease management, of which early diagnosis of the pathogens is one of the key components. Therefore, development of methods for early diagnosis plays an important role in crop disease management strategies. In principle, plant diseases can be identified based on one or more of the followings: (1) typical symptoms on infected plants, (2) specific protein targets and (3) specific nucleic acid targets.

Methods for plant disease detection

Disease identification by symptomatic phenotypes

Pathogens—after infecting their host plants—will alter gene expression and metabolite status leading to changes in the phenotypes of host plants. There are methods that rely on the observation of unique or typical symptoms of the infected plants for diagnosis. The greatest advantage of these methods is that it does not require any equipment. However, there are several limitations. Typically, to exhibit disease symptoms, it takes time for the pathogen to infect, multiply and spread throughout the plant. Therefore, detecting diseases by their symptoms is often too late for effective management. In addition, each disease may have been caused by more than one pathogen leading to different symptom variations. In other cases, many diseases have similar symptoms that make them difficult to distinguish [14]. Some diseases even exhibit their symptoms underground, such as on roots or tubers, making it impossible to observe phenotype changes using nondestructive methods. For instance, the root rot disease of cassava caused by *Phytophthora* spp. causes changes in root color from white to creamy white as well as producing a bad smell at the root [15].

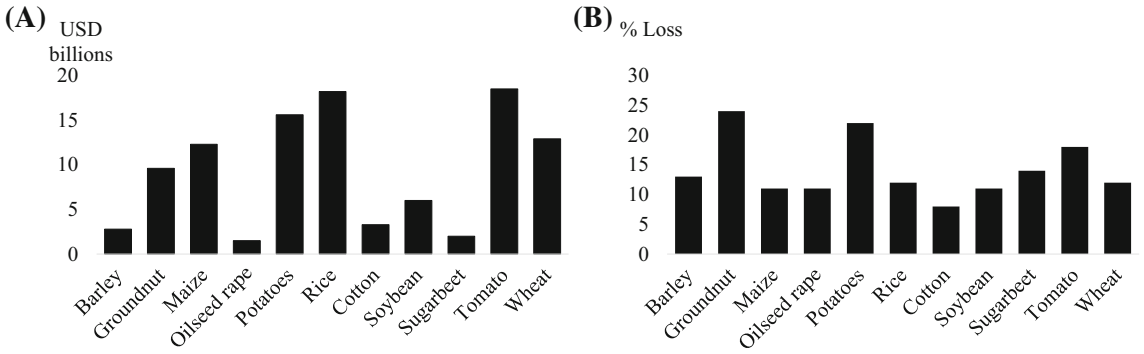

Fig. 2 Estimated global crop losses due to pathogens based on [13] in crop protection compendium. **A** illustrated economics loss (in US$ billion) and **B** represented as the % loss of crop production

Disease diagnostics by detecting target proteins

The molecular approach for plant disease diagnostics is to detect the presence of specific target nucleic acids or their protein products. Enzyme-linked immunosorbent assay (ELISA), also named as EIA (Enzyme Immunoassay), is the most popular and cost-effective test to detect target proteins. The method relies on the interaction between pathogens' antigens and their specific antibody [16]. The standard versions of ELISA, including direct, indirect and sandwich, have been powerful tools for the detection of plant pathogens since the 1980s. These methods do not require sophisticated equipment or specially trained personnel but still offer very good scalability for high throughput [17–19]. Nonetheless, several factors such as quality of antibodies, samples, the way test components are prepared and stored, the incubation time and temperature can impact the reliability of an ELISA test [14]. Since ELISA is also an enzyme reaction, high standards of the buffer elements, molarities and pH of the reagents and the purity of those chemicals are required.

Disease diagnostics by detection of pathogen-specific nucleic acids

Apart from proteins, pathogen-specific nucleic acid sequences are also a target for plant disease diagnostics. In molecular diagnostics of plant diseases, PCR sets a gold standard and still plays an increasingly important role [20]. PCR is superior in its sensitivity, specificity and ability to identify RNA targets when coupled with a reversed transcription enzyme [21]. PCR provides not only qualitative results, but when coupled with a real-time detection module, it can also provide quantitative data, therefore, giving an estimate of pathogen titer. Real-time PCR is a fast and effective method to diagnose plant diseases through on-the-fly data calculation with minimal contamination risk and greater flexibility to detect multiple pathogens [22]. Moreover, nested PCR with two or more pairs of primers is able to amplify a low abundance target sequence, providing significantly increased reliability and sensitivity compared to conventional PCR [23, 24]. PCR can be combined with restriction digestion of the amplified targets (RFLP-PCR) to offer additional specificity of the test [25].

The advantages of the nucleic acid-based methods are their sensitivity, specificity and reliability [26]. PCR-based techniques, especially real-time PCR, can detect and quantify pathogens even before the infected host exhibits symptoms. Nested PCR provides sensitivity 100–1000 times higher than that of conventional PCR [27, 28].

PCR is recognized as a highly effective method for detecting plant diseases, but it does have its inherent limitations: high investment cost, greater time investment and requirement for trained personnel. In addition, the presence of inhibitors of polymerase reactions such as polyphenol, polysaccharides, xylene, etc., may reduce or degrade the polymerase activity, even leading to false negative results [29]. Another major drawback of PCR is the high cost of materials for quality control, test preparation, nucleic acids cleanup and result interpretation [30]. These extensive requirements have made PCR-based techniques suitable to perform only in well-equipped facilities. Because PCR-based assays are highly sensitive, they are prone to contamination, which may lead to false positives, especially with nested PCR [27].

The need for a handy tool in plant disease diagnosis and the invention of LAMP

Plant diseases and their management are becoming more complicated because of climate change. It is necessary to have new tool(s) capable of detecting and monitoring the pathogens early. The new tools must meet following criteria: (1) high sensitivity, (2) high specificity, (3) low per-test cost, (4) simple, (5) rapid and (6) less instrument-

intensive [31]. PCR-based detection methods may have the sensitivity and the specificity, but are lacking in other metrics like speed, and instrument affordability [32]. A recently developed technique based on isothermal amplification of nucleic acids satisfies these requirements, the loop-mediated isothermal amplification (LAMP) technique. This method was first reported by Notomi and his colleagues and was widely applied in various biological fields because of the ease at which it can be performed [33]. In this review, we focus on the application of LAMP for detection of plant diseases.

LAMP (loop-mediated isothermal amplification) is a highly effective and specific amplification technique to diagnose plant disease pathogens. It applies the strand displacement activity of Bst DNA polymerase (a polymerase enzyme from *Bacillus stearothermophilus* [34]) to amplify the target DNA through two or three pairs of specific primers in an isothermal condition. LAMP is a highly exponential amplification method that produces the target DNA at amounts of 10^9–10^{10}-folds within 45–60 min at 60–65 °C [33].

LAMP principle (loop primers and isothermal amplification by Bst DNA polymerase)

LAMP is a one-step amplification assay that amplifies the target DNA or RNA sequence under isothermal conditions. LAMP requires two or three pairs of primer to detect six distinct regions in the target sequence, in which four primers are compelling. Those four primers are named as F3 (Forward outer), B3 (Backward outer), FIP (Forward inner) and BIP (Backward inner). FIP and BIP are hybrid primers that contain two regions (F1c and F2 for FIP, B1c and B2 for BIP, respectively, in which F1c and F2c are complementary to the F1 and B1 regions in the target sequence). Two more primers, namely LF (Loop forward) and LB (Loop backward), can be applied to accelerate the reaction [35].

The LAMP process—explained as in Fig. 3—includes three steps: initial steps (1–2), loop structure production (3–5) and the cycling amplification (6–11). In the initial steps, the inner primers (FIP) target the specific complement sequence (B3c) and start the DNA synthesis. At the opposite end of the target sequence, the DNA amplification of BIP proceeds in the same manner. After that, the outer primer (F3) anneals to the B3c region and starts the elongation. In the loop structure producing step, under the displacement activity of Bst enzyme, the strand made from FIP is replaced and released to then make the template for the next amplification cycle. Through two continuous amplification cycles, a DNA segment containing the target sequence is created that has a loop structure at its end that is used as the template for the cycling amplification step. The cycling amplification step is similar to the initial and loop structure producing steps, but with the loop structure as a single-strand template. Through each cycle, for each single-stranded DNA template, there will be two amplified products in which, one is identical to the template and the other is double the length of the template. This principle of LAMP was reviewed in detail by [36].

Strategy and resources for LAMP primer design

LAMP primers are designed specific to the target nucleotide sequence (DNA or RNA) of each pathogen. Due to the special structure, primers are often designed by PrimerExplore, a specialized free online software available on the site https://primerexplorer.jp/e/. Currently, four versions of PrimerExplore have been released, making this software the most popular tool for LAMP primer designing. A commercial one namely "LAMP Designer" by PREMIER Biosoft (USA) was also developed for this purpose.

In parallel with PrimerExplore and LAMP Designer, which were built by commercial entities, some extendable open-source resources for LAMP such as LAVA (LAMP Assay Versatile Analysis) and eLAMP (Electronic LAMP) have also been created for such purposes. These tools were developed as PERL scripts with graphical interface, satisfying most of the needs for scientists to identify appropriate targets for LAMP assays. These primer design tools enabled LAMP to become a more efficient technique for diagnosis [37, 38].

Detection of amplified LAMP products

LAMP amplification products can be recognized by the addition of indicators into the reaction mixtures before (hydroxynaphthol blue and calcein) or after (Ethidium bromide) the reaction takes place [35, 36, 39–41]. Hydroxynaphthol blue (HNB) indicates successful amplification by the alteration of reaction color caused by the pH change, while calcein and ethidium bromide emit fluorescence under UV light once the LAMP products have formed. The metal-ion indicators, HNB and calcein, are often used in because of their safety, in contrast to ethidium bromide, a known mutagen, carcinogen or teratogen. Another reason for their popularity is that by adding the indicator prior to reaction, cross = contamination can be avoided. Other indicators, such as EvaGreen dye, SYBR Green and Pico Green, are added for the analysis of the LAMP products. Their use is based on the time to positive (Tp) values of the fluorescence signal collection during every period time of amplification [41, 42].

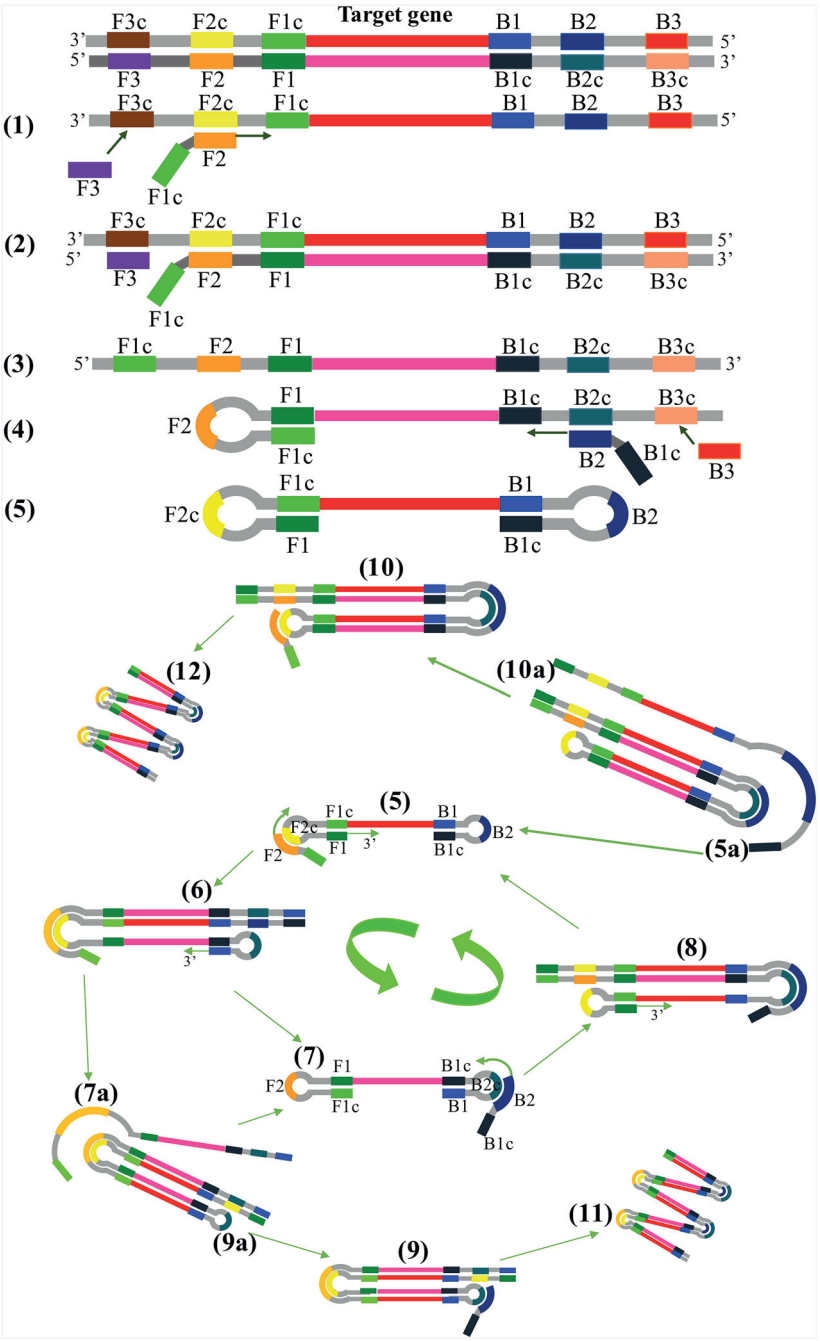

Fig. 3 Amplification principle of LAMP method. DNA synthesis starts with inner (FIP) primers, reaching their target at their specific complement sequence (B3c) (*step 1*). After that, the outer primer (F3) anneals to the B3c region and starts elongation (*step 2*). Under the displacement activity of the Bst enzyme, the strand made from FIP is replaced and released, becoming the template of the next amplification cycle (*step 3*). At the implement strand of target sequence, the DNA amplification process with BIP continues in the same manner (*step 4*). Through two rounds of amplification, a DNA segment containing the target sequence was created (*step 5*). FIP primers pair with the template (*step 5*) at the F2c site and start to elongate, making a new DNA strand that has the same length as the template (*step 6–7*), while the template elongates itself at the B1c sequence to make a new double-length DNA strand (*step 8*). Continuing in the same manner described above, each single strand of DNA creates two more strands by displacement activity of the Bst enzyme. One of those strands is of the same length as the template, while the second is of double length (*steps 6–11*)

The success of a LAMP reaction can also be confirmed basing on the turbidity of magnesium pyrophosphate in the tube bottom. This new compound is created during the LAMP amplification, as in Eqs. (1) and (2) [43]. The increased turbidity of magnesium pyrophosphate may be measured in real time or at the end of the reaction [43, 44]. Magnesium pyrophosphate precipitation is stable for only a short time, but enough for monitoring after the reaction [45]. LAMP products can also be visualized under ultraviolet light after electrophoresis on agarose gel and staining in ethidium bromide solution [46].

$$(\mathrm{DNA})_{n-1} + \mathrm{dNTP} \rightarrow (\mathrm{DNA})_n + \mathrm{P_2O_7^{4-}} \quad (1)$$

$$\mathrm{P_2O_7^{4-}} + 2\mathrm{Mg^{2+}} \rightarrow \mathrm{Mg_2P_2O_7} \quad (2)$$

Advantages of the LAMP method

LAMP has some salient advantages that make it competitive for diagnostics comparing to other techniques. First, LAMP is rapid, effective, specific and sensitive, amplifying its target up to 10^{10} times after 45–60 min of incubation [33]. The reaction uses four to six different primers to

recognize six distinct regions in the target sequence, including two external primers of similar length to the PCR primers and two internal primers having double the length of the outer primers. The combination of two pair of primers for a short target segment of DNA sequences (about 200–500 bp) makes the assay extremely specific, where the product of the reaction is unique. LAMP is 10–100 times more sensitive than PCR [46–48]. Even when adding two more loop primers, the sensitivity of the reaction further increased, and therefore, the time required can be shortened to 30–60 min [49]. The assay enables detections using only a few picograms or even fetograms of target DNA or RNA. The high specificity and sensitivity make LAMP effective in diagnostic functions.

LAMP is easily performed under isothermal conditions. With the displacement activity of Bst enzyme, the synthesis of a new DNA strand may occur in isothermal conditions at a temperature sufficient to denature a part of the double-stranded DNA template [34]. Furthermore, the LAMP enzyme shows less sensitivity to inhibitors in the amplification reactions and does not strictly require sophisticated equipment and expensive thermal cycler [50]. Instead, simple equipment like water baths or heated blocks are sufficient to maintain temperature for the reaction period, making LAMP an economical technique compared to PCR, especially real-time PCR [51]. These characteristics enable LAMP to be more useful; the assay can be implemented in a wide variety of experimental conditions, including in the laboratory or outside field tests [41, 52].

Drawbacks of LAMP

Despite its many advantages, some attention is required to avoid limitations of the LAMP technique. The target gene fragment is usually short, and the reaction products are series of DNA fragments which are not of the same size. Hence, they should not be used as material for other assays like PCR [33]. LAMP requires four to six primers which differ in length, causing difficulties for the experimental design. High specificity for detection of a short target segment makes LAMP unsuitable for studying new genes that have little known information. In addition, it is necessary to clearly understand the structural features of the target gene. An excess concentration of indicator (calcein, HNB, ethidium bromide) or other reaction components (including ionic forms of manganese or reaction cofactors) may inhibit polymerase reactions, break down the products or change the indicator color, which decreases the efficacy of LAMP [53].

Carryover contamination is another challenge to LAMP users. LAMP is very sensitive, and therefore, only a small amount of its target will still be detected, making it susceptible to contamination if the laboratory conditions are less than clean. The targets of LAMP are mainly bacteria, viruses and other microorganisms. These targets can exist freely in air, which may be carried over into test tubes, causing false positives. The contamination may occur in the master mix preparation or during DNA electrophoresis (where LAMP products may overflow from one well into another). To minimize these risks, handling needs to be done carefully, ideally using color indicators instead of electrophoresis. In addition, the amplification time required may affect the assay result. A suitable time is from 60 to 120 min, but it can be shorter if the LAMP uses loop primers. If the incubation time is too long, negative samples may become false positives. Moreover, the time taken for master mix preparation should be less than 30 min to get satisfactory results [54].

Application of LAMP for pathogens detection

More than 250 research articles about LAMP assays for plant diseases have been published in peer-reviewed journals in the decade following publication of the first one in the year 2000 [55]. More than 1200 other articles using LAMP can be found on PubMed (http://www.ncbi.nlm.nih.gov/pubmed) (Fig. 4A) for diagnoses in different scientific fields. Those fields include identification of the source of infection for plants, animals and humans, whether infected by fungi, bacteria or virus. LAMP also can be used to detect the microorganism agents causing plant disease. However, application of LAMP in plant disease diagnosis accounts for only 12.5% of the total publications. Since 2012, the number of articles on LAMP has increased exponentially, demonstrating LAMP's importance in recent research (Fig. 4B).

Assays for viral disease detection

Viruses are the most common agent causing diseases in plants, whether field crops, fruit trees, vegetables or industrial plants. More than 900 species of known plant viruses cause devastating diseases. Many have a wide range of hosts [11]. Yield losses due to plant virus diseases are estimated to be between 10 and 15%, causing losses of US$ 60 billion worldwide each year [56].

LAMP assays were developed as effective tools to identify plant viral agents such as tobacco mosaic virus, viral spots on gold coffee and potatoes, *yellow leaf virus* (in wheat and tomato), *yellow dwarf virus* (in rice), as well as viruses on flowers, bananas, cauliflower and peaches, among others [46, 57–62]. *Banana streak virus* (BSV), a pathogenic virus on banana, was also identified through LAMP with a

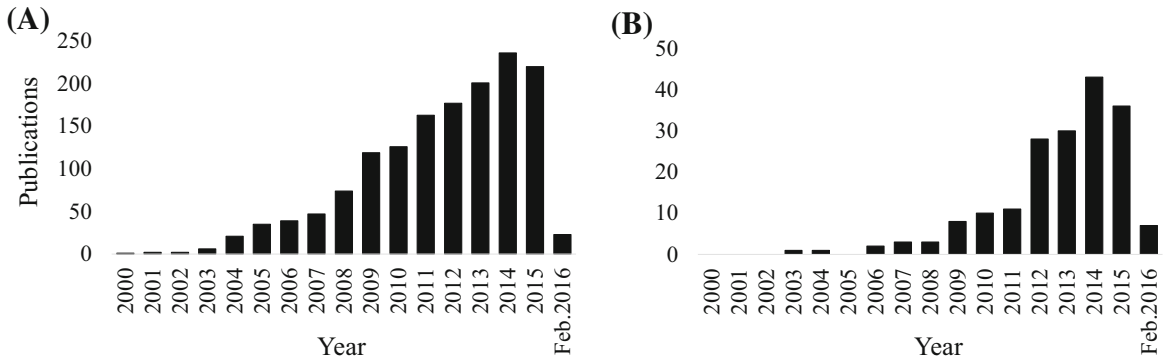

Fig. 4 Numbers of LAMP publications in all areas (**A**) and in plant disease diagnostics (**B**)

sensitivity of 1 pg DNA template, more sensitive than PCR for the same sample [60]. [62] also developed a LAMP assay that can identify mosaic virus on wheat at a sensitivity 100 times greater than PCR [62]. LAMP assays were available to detect *Plum pox virus (PPV)* (affecting stone fruits, including peaches, nectarines, plums, apricots, almonds and ornamental varieties) and various viral agents of Cassava brown streak disease, tomato, yam (Table S1, supplemental information).

LAMP coupled with a reverse transcriptase enzyme, known as RT-LAMP, has also been widely developed for the detection of retrovirus pathogens. For example, for the diagnostics of blight disease on soybean seed caused by *Bean pod mottle virus*, RT-LAMP provides high accuracy and high specificity, plus sensitivity between 100 and 1000 times greater than RT-PCR does [63]. RT-LAMP assays for the detection of 9 rice viruses were also tenfold more sensitive than RT-PCR [46]. One impressive RT-LAMP assay has been employed for detection of *Turnip mosaic virus*, the pathogen of at least 318 species in 156 genera of 43 families, mostly cruciferous plants. Recently, RT-LAMP assays are available to detect many more viral pathogens for plant diseases (Table S1).

Assays for bacterial disease detection

Bacteria are among the microorganisms which cause diseases on crop plants. More than 80 species of bacteria—each one consisting of numerous subspecies—have been found to be plant pathogens. For example, at least 392 different plants, including both monocots and dicots, can be infected by species of *Xanthomonas* [64]. LAMP is considered a useful tool to identify bacterial pathogens with the sensitivity about 10 pg target DNA for *Phytophthora sojae,* 10 fg or 5–18 CFU for cultured cells of *Xanthomonas*, 1 ng total DNA for *Candidatus* from infected citrus samples [65, 66]. The RT-LAMP assay can be 100 times more sensitive than RT-PCR, as shown in leaf samples of 76 sweet oranges (*Citrus sinensis*) [48].

Phytoplasma is a new group of plant disease pathogens with about 200 members distributed in 30 clades based on the sequence of 16S rRNA, which causes serious decrease in yields and productivity of crop plants. They have small genomes, lack cell walls and have no particular shape, although they are usually caught in the filamentous or elliptical shape with a diameter less than 1 μm. They quickly spread through the vascular system and then to other plants through the stomata system or insect sap consumption, in the Cicadellidae, Cixidae, Psyllidae, Delphacidae and Derbidae families [67–72]. LAMP has proven to be an economic, effective, rapid and reliable method in the diagnosis of *Candidatus Liberibacter solanacearum* in potatoes, as well as some other pathogens in papaya, coconut, cassava, Madagascar periwinkle (*Catharanthus roseus*), some of which have insect vectors [73–77] (Table S2).

Assays for fungal disease detection

There are about 15,000 species of disease-causing fungi in plants, which make up 40–60% of total losses due to pests and diseases [78]. Therefore, early detection of fungal diseases attracts a great deal of interest from the research community. LAMP assays have been developed to identify the genus Leptosphaeria (*Leptosphaeria* spp.) in oilseed rape; *Fusarium oxysporum* f. sp. *Lycopersici*, which causes vascular wilts disease; and *Pythium aphanidermatum,* which causes tomato root rot disease, and *Bursaphelenchus xylophilus*—the causal agent of pine wilt disease [79–82]. LAMP is proven to be faster and much cheaper than real-time PCR in accurately determining the change of *Leptosphaeria* populations which cause phoma leaf spotting and stem canker disease of oilseed rape. In this assay, the enzyme GspSSD DNA polymerase—the fastest enzyme in isothermal amplification of DNA and RNA—was used instead of Bst polymerase, allowing maximum enhancement of amplification efficiency when compared to conventional LAMP.

For the detection of *F. oxysporum* f. sp. *lycopersici*, the pathogen causing vascular wilts of tomato and more than 100 other cultivated plant species, a colorimetric LAMP was developed and found to be 100-fold more sensitive and 4 times faster than PCR. Reagents hydroxynaphthol blue, GeneFinder^{TM} and SYBR Green I showed long stability in color change and also prevented cross-contamination together, making them reliable color indicators for LAMP. There is promise for more direct LAMP assays where the DNA purification steps are omitted without affecting amplification efficiency, opening a new approach to shorten time requirement for LAMP [79]. One such assay has been developed to detect *F. oxysporum* f. sp. *lycopersici* in tomatoes infected with vascular wilts disease.

To identify the causal agents of root rot in many crop species, 86 strains of *Pythium* spp. from various hosts were collected and cultivated for detection by LAMP reaction using a real-time turbidity meter. LAMP assay results showed high specificity from soil borne with the detection limit of about 10 fg DNA, equivalent to that of real-time PCR and 10 times as sensitive as PCR [80].

Meanwhile, real-time fluorescence LAMP for rapid detection of plant pathogens also shows excellent detection efficiency. A real-time fluorescence LAMP assay (RealAmp) was developed for quantitative detection of *F. oxysporumf.* sp. *cubense (Foc)*, which causes Fusarium wilt (Panama disease) in banana. The limit of detection of this assay was 0.4 pg/µl plasmid DNA mixed with extracted soil DNA or 10^3 spores/g of artificially infested soil in more than 90% of field samples, giving it almost the same efficiency as real-time PCR [42]. The use of LAMP and real-time LAMP has been reported to successfully detect *Botrytis cinerea* for rot root of flowers, fruit and vegetables [83]. The application of LAMP for detection of plant fungal diseases is shown in Table S3.

Lyophilized LAMP and combination of LAMP with other techniques

The ability to rapidly and efficiently amplify small and specific amounts of DNA makes LAMP a powerful tool for the diagnosis of plant diseases and pathogens. Besides LAMP, PCR-based techniques have been used as references for plant diseases diagnosis. Among recent reports, the most commonly used PCR techniques are qPCR, qRT-PCR or nested PCR. This arsenal of techniques has proven to be highly reliable and highly sensitive, so they could be used to confirm the usability (accuracy and reliability) of LAMP.

Sometimes, LAMP can be combined with other techniques to increase the usability of the assays. One such example is lyophilized LAMP, wherein assay mixtures are lyophilized and therefore compatible with field applications. As mentioned above, the LAMP reaction was conducted by displacement activity of the Bst enzyme. However, Bst activity is only preserved at −20 °C. Enzyme activity is reduced and the amplification becomes less specific as the temperature reaches 45 °C. This is a limitation for using in the field. The lyophilization of the LAMP reaction mixture can maintain activity of Bst DNA polymerase at ambient temperatures, enabling the use of the assay at remote locations where low-temperature transport and storage is not possible. In this case, a mix of Bst enzyme (and reverse transcriptase for RNA targets), pathogen-specific primers and dNTPs are lyophilized together. An assay consists of 80 µl of the aqueous reaction buffer containing $MgSO_4$ and 20 µl of the sample. When added to the lyophilized LAMP reaction mixture, turbidity is exhibited after incubation at 60° C for 1 h. Dialysis of the Bst DNA polymerase before lyophilization helps remove the glycerol preservative, facilitating the drying process while maintaining the strand displacement activity [84, 85]. This approach has been successfully applied for the detection of *Phytophthora ramorum* and *P. kernoviae*, which cause sudden oak death and dieback/leaf blight in a variety of trees, shrubs and herbaceous species [86].

New innovations combining LAMP with other methods promise to improve the effectiveness and usefulness. For instance, the combination of LAMP with a lateral flow dipstick (LFD) enables the assays to be more easily and widely applied for field diagnosis [87]. Indeed, the standard for LAMP detection is using DNA electrophoresis to visualize the amplified products on agarose gel. However, this has its disadvantages: it is not compatible with field diagnosis because of the equipment required for electrophoresis. In addition, the staining chemical used for visualization may be unsafe for people and the environment. Meanwhile, LFD based on the principle of lateral flow assay (LFA), which applied the combination between biotin with FITC-labeled DNA probes and mix with chromatography on a thin membrane, allows observation of results by the naked eye [88]. LAMP-LFD uses specific biotinylated primers to amplify the target DNA, reducing the time needed while maintaining the specificity and sensitivity. The amplified product can be visualized on a dipstick instead of an agarose gel [89]. This combination of technologies is applied for plant disease diagnosis as a perfect replacement for conventional LAMP in point-of-care testing [85, 86, 89]. Another combined approach is the IC-LAMP, which combines immunocapture and LAMP. This technique maintains high reliability, sensitivity and specificity of LAMP assay while reducing the time required cutting the cost of each essay and eliminating the step for DNA or RNA extraction [58, 90, 91].

Remarks and future directions

LAMP is a rapid and accurate technique for the diagnosis of plant pathogens. By using only four or six primers for one unique nucleic target sequence and the strand displacement activity of Bst DNA polymerase. LAMP requires minimal investment on equipment, and its high amplification efficiency technique offers sensitivity at least tenfold higher than PCR in most applications. Furthermore, the diverse methods to identify end products make LAMP more convenient than other conventional molecular diagnostic methods.

LAMP assays are not only for detecting DNA targets; they are now capable of detecting RNA targets. Reverse transcriptase LAMP (RT-LAMP) was developed for detection of retroviruses, which cause plant diseases, based on their RNA sequences. The usability of LAMP has been improved by being used in combination with other techniques. The lyophilized reaction mixture extended the laboratory use of LAMP to a field-compatible technique. The lateral flow dipstick offers an additional option for LAMP product visualization, while IC-LAMP shortens the duration of the assay by omitting the steps for preparation of pathogen DNA or RNA without compromising the assays' effectiveness. New reports continue to surface for products that make LAMP easier, more efficient, better at avoiding contamination, and safer for the operators.

LAMP has proven to be an effective tool for plant disease diagnostics. Over only a decade, LAMP has become an important method in diagnostic science, including the diagnosis of plant diseases. In the context of continuously changing environments due to the adverse impact of drought, floods, climate change and greenhouse gas emission, plant diseases caused by new or newly evolved pathogens are affecting crop production more and more. Thus, the role of LAMP is crucial for early diagnosis to protect crop plants and improve crop productivity. Continuous improvements to LAMP assays enhance efficiency for highly adaptable tests that meet a variety of demands for plant disease diagnosis, including point-of-care testing. Besides its uses in plant disease diagnostics, LAMP assays for plant pathogen detection have huge potential in the certification of non-disease status of nursery plants and disease-free seeds and seedlings production.

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