RESEARCH ARTICLE

Loop mediated isothermal amplification: an innovative gene amplification technique for plant diseases

MAGDALENA CARA1*, THAER YASEEN2, RENATA BAŽOK3, DARKO VONCINA3, ORGES CARA1

1Agricultural University of Tirana, Faculty of Agriculture and Environment/ Rruga “Paisi Vodica, Kodër-Kamëz, Tiranë, Albania
2C.I.H.E.A.M. – Istituto Agronomico Mediterraneo di Bari/ Via Ceglie 9, 70010 Valenzano (BA), Italy.
3University of Zagreb Faculty of Agriculture, Svetoslimunska 25, 10000 Zagreb, Croatia

*Corresponding author; E-mail: magdacara@ubt.edu.al

Abstract

Nowadays, molecular diagnostic methods of plant pathogens evolved in a fast way, the use of rapid and easy to use detection method is fundamental to prevent pathogens cross border and spread to enhance food quality and security. Real – Time Loop-mediated isothermal amplification (RT-LAMP) is a novel molecular detection method that specifically detects genomic DNA by using a set of six oligonucleotide primers specific to different regions of a target gene and Bacillus stearothermophilus (Bst) DNA polymerase protein. This method has been recently modified to be use as a RT-LAMP and then widely applied in many fields for on-site detection and ability to be used in cross border control of plant health, such as quarantine disease diagnosis. The application of rapid and simple DNA extraction method (10 min at 65°C) shortened the detection assay to less than one hour. During the period 2015–2017 are tested in Albania more than 100 samples for: Xylella fastidiosa in olive trees, Flavescence dorée in vineyards. Olive samples were taken in Vlora, Durrës, Saranda, etc. All the samples have been negative. Samples for the Flavescence dorée have been taken in Lezhë, Vlorë, Durrës in Albania and Istria in Croatia. The samples from Albania resulted negative while the samples from Croatia (17 samples) were 53% positive. Every procedure has been confirmed with positive and negative controls to use diagnostic kit denominated Xylella Screen Glow & FD from Enbiotech s.r.l., Italy. RT-LAMP method is more sensitive than conventional and RT PCR, advantage of LAMP is the isothermal reaction condition, hereby LAMP is affordable because of no need to have expensive thermal cycler. Although recommended reagent storage temperature is -20°C, reagents can be stored at environment temperature. Hereby there is no need to have cold chain for reagent distribution. The results indicate that the RT-LAMP assay is extremely rapid, cost-effective, highly sensitive and specific and has potential application in plant pathology surveillance. This new method is widely used nowadays by many laboratories and is recognized by EPPO as a standard method for some pathogens. Test performance study is being developed today in the circles of European scientists.

Keywords: Loop mediated isothermal amplification; Bst DNA polymerase, plant diseases.

1. Introduction

The Loop Mediated Isothermal Amplification (LAMP) is a unique nucleic acid amplification technique introduced 18 years ago by Notomi and his colleagues [13]. LAMP as a nucleic acid amplification technique operates under a unique amplification principle which involves two basic steps. They are the non-cyclical and cyclical phase [14,21]. The non-cyclical precedes the cyclical phase of the amplification process [14,21]. LAMP as a simple and cost-effective technique requires few and inexpensive materials to perform. The most crucial of all is the primers. LAMP uses four to six primers specially designed to target six to eight regions in a gene of interest [13,12]. They are Forward Inner Primer (FIP), Forward Outer Primer (F3), Backward Inner Primer (BIP), Backward Outer Primer (B3) and two optional loop primers. The first product is formed in the loop formation and DNA has incessantly amplified from the first products resulted in various sized DNA structures (Fig.1). The diagnosis, therefore, is possible although there is very small amounts of target gene [14,17]. The LAMP products from reaction can be detected by electrophoresis and observed a smear of multiple bands in a lane of positive
LAMP reaction [13,14,17]. The end result of strand displacement and loop formation and synthesis is the single-temperature amplification of a highly specific fragment from a DNA template at a much greater titre than that obtained with polymerase chain reaction. With LAMP, there are several methods to determine a positive reaction. In our study we have taken in consideration two pathogens: Xylella fastidiosa in olive trees, Flavescence dorée in vineyards. Xylella fastidiosa is a xylem-limited bacterium and the causal agent of a number of several diseases, among which Pierce’s diseases of grapevine, leaf scorch of almond, oleander and coffee, citrus variegated chloroses, and other disorders of perennial crops and landscape plants [8]. Survey on the spread of X. fastidiosa in Albania was organized for the first time from May 2014 [2], it was used ELISA, DTIBIA and PCR conventional tests. For the first time we have used Real – time LAMP in 2017 for this pathogen determination. In grapevine (Vitis vinifera L.), phytoplasmas are associated with grapevine yellows (GYs) diseases, that occur in the majority of grapevine growing countries worldwide (Constable et al. 2003). Flavescence dorée is associated with phytoplasmas belonging to the 16SrV group (i.e., Flavescence dorée phytoplasma; FDP). As such, FDP is listed in the EU2000/29 Council Directive on Harmful Organisms, and in the European and Mediterranean Plant Protection Organisation (EPPO) A2 quarantine list of pests [5]. Symptoms caused by FDP are not distinguishable by visual inspection and as the distribution of phytoplasma is uneven within a host that has a very low titre [15], only specific molecular approaches are suitable for the accurate and reliable detection of BNp and FDP. A protocol for the detection of FDP based on loop-mediated isothermal amplification (LAMP) assay was developed recently [9, 13]. LAMP is a highly specific and rapid technique, and it also circumvents the sensitivity of PCR and qPCR to inhibitors in plant extracts [6]; furthermore, its isothermal nature provides the potential for it to be deployed in the field [9, 18]. LAMP has shown a comparable or better performance to other detection methods and a wide applicability for the detection of plant pathogenic bacteria [11], viroids [10], fungi [19] and phytoplasmas [1, 3, 7, 9, 18].

2. Material and Methods

During the period 2015–2017 are tested in Albania more than 100 samples for: Xylella fastidiosa in olive trees and Flavescence dorée in vineyards. Olive samples were taken in Vlora, Durrës, Saranda, etc. Samples for the Flavescence dorée have been taken in Lezhë, Vlorë, Durrës in Albania and Istria in Croatia. Olive samples were tested in parallel with the ELISA and LAMP. Tests for ascertaining the presence of X. fastidiosa by DAS-ELISA using commercial kit of Loewe Biochemica GmbH and Agritest Italy. Since the Flavescence dorée is a quarantine pest and we have performed the tests in Albania, we have not obtained fresh plant material but DNA extractant from Croatia.

Figure 1. Principle of LAMP-PCR
While DNA denaturation step (the double stranded DNA into a single strand) is vital for conventional PCR, LAMP-PCR does not require that. There are 11 steps for LAMP-PCR. 1. After FIP (one of the LAMP primers) anneals to the complimentary sequence of target DNA conditioned around 65°C, DNA strand is synthesized from the 3’ end of the F2 in the FIP by DNA polymerase with strand displacement activity; 2. The F3 anneals to the F3c region on the target DNA and begins releasement of FIP-linked complementary strand synthesized at step 1; 3-4. The released single strand forms a loop structure at the 5’ end because the F1c is complementary to F1 and after BIP anneals to its complimentary, new DNA strand is synthesized from the 3’ end of the B2 in the BIP by DNA polymerase; 5. The B3 anneals to the B3c region, outside of BIP, on the target DNA and begins synthesis of DNA strand (BIP-linked complementary strand), forming dumbbell-like structure with stem-loop at each end due to complimentary of F1 and B1 to F1c and B1c, respectively. This structure serves as the starting material for the amplification; 6-12. FIP anneals to the stem-loop DNA and leads strand displacement DNA synthesis and, releasing the previously synthesized strand which forms a stem-loop structure at the 3’ end owing to B1c is complementary to B1. Later complementary strand with FIP is released. The released strand forms dumbbell-like structure with stem-loop at each end since F1 and B1 are complimentary to F1c and B1c, respectively. Because DNA synthesis continues, there are various sized structures [4].

ICgene is a new system for in situ genetic analysis, able to perform molecular analysis with LAMP technology that allows the execution of the tests by non-specialized personnel and outside of the molecular biology laboratories. LAMP technology allows rapid amplification of DNA at constant temperature and from small quantities of sample. Thanks to its characteristics it allows to perform genetic test directly on site and without the use of sophisticated laboratory instruments. The ICgene product line is composed of a portable instrument and a set of kits developed for specific applications. The kit foresees a rapid preliminary nucleic acid extraction from the sample, genetic amplification per test using LAMP technology, detection of the fluorescence emitted from the sample and automatic interpretation of the final result using the device ICGENE mini.

ICgene kit features and device
Complete with all components necessary for the analysis, from extraction to detection. Ready-to-use extraction system that allows for total DNA extraction in only a few minutes and without the use of sophisticated laboratory instruments. Ready-to-use amplification and detection system composed of strips that contain freeze dried primers and a Master Mix with all the reagents necessary for the execution of the test. Ready-to-use components eliminate the possibility of environmental contamination or human error. Speed and simplicity of the test (about 30 minutes). Possibility of performing genetic tests directly on site, stable at room temperature, transportation at room temperature, stored at +4°C. Device that performs 3 steps necessary for genetic testing (nucleic acid extraction, gene amplification and detection and interpretation of results), user interface through a custom android app, automatic interpretation of results, automatic detection of the kit through contactless badge, integrated browser experiments, ready to connect to the Internet and to a remote database, compact instrument with a modern and innovative design [16].

2.1. LAMP Protocols

Kit Xylella Screen Glow & FD Glow are produced from Enbiotech S.r.l. – Italy. We have skipped the first step extraction for samples that we have DNA template. Preparing the LAMP reaction: Use one Primer Mix tube per sample (2X) one positive control and one negative control. 22 µl of LAMP mix (ZLFDG), 30 µl of mineral oil (ZMOFDG) and 3 - 5 µl of the extracted DNA to the Primer Mix tube. The amplification program in 65 Celsius degree into the device ICGENE mini – Enbiotech S.r.l. – Italy.

2.1.1 Extraction of DNA of FDp in Croatia samples:
The extraction is done in Department of Plant Pathology in Faculty of Agriculture University of Zagreb from main vein of symptomatic leaves (where applicable). It was used commercial isolation kit: DNeasy Plant Mini kit (QIAGEN) according to manufacturer's instructions Elution volume: 100 µl Volume in tubes: ca 85 µl.

2.1.2 ELISA for Xylella fastidiosa

Tissue extracts were obtained from leaf petioles and midveins excised from 6–8 mature leaves and macerated in plastic bags in the presence of extraction buffer (polyvinyl pyrrolidone 20 g/l, Tween-20 - 0.05%, BSA 2g/l in 1
X PBS buffer, pH = 7.4) using a semi-automated apparatus. Samples were tested using specific antibodies to X. fastidiosa (Loewe Biochemica GmdH, Germany and Agritest Italy), following manufacturer’s instructions and using the controls supplied with the kit. Plates were coated with 200 µl/well of anti-X. fastidiosa IgG diluted 1:200 in coating buffer and incubated at 37°C for 4 h. Test samples were loaded in microplates and kept overnight at 4°C before the addition of alkaline phosphatase-conjugated anti-X. fastidiosa IgG diluted 1:200. Plates were incubated at 37°C for 4 h before the addition of the substrate (1mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8). Absorbance was recorded at 405 nm using a microplate reader (ELx800 Absorbance Reader). Samples were considered positive if their absorbance was three times higher than the mean absorbance of healthy control samples [20].

3. Results and Discussion

Every procedure has been confirmed with positive and negative controls to use diagnostic kit denominated Xylella Screen Glow & FD from Enbiotech s.r.l., Italy. The sigmoid curve indicating that the sample is positive for Xylella fastidiosa or Grapevine Flavescence Doree phytoplasms and the appearance of the symbol “+” on the left of the samples name. A straight line indicating that the sample is negative for Xylella fastidiosa or Grapevine Flavescence Doree phytoplasms. Olive samples tested with ELISA and LAMP resulted as the following: from Vlora (25), Durrres (15) and Saranda (10) were negative with both tests. Vineyard samples collected in Albania; Vlore (10), Durrres (10), Lezhe (13) and Istria, Croatia (17) have been all negative for samples taken in Albania and 9 positive samples from Istria Croatia. Table 1.

<table>
<thead>
<tr>
<th>Sampling place</th>
<th>Number of the samples</th>
<th>ELISA test</th>
<th>RT – LAMP</th>
<th>RT – LAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Xylella</td>
<td>Xylella</td>
<td>Flavescence doree</td>
</tr>
<tr>
<td>Vlora</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Durrres</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Saranda</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Vlora</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Durrres</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lezhe</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Istria (Croatia)</td>
<td>17</td>
<td>9 samples positive</td>
<td>8 samples negative</td>
<td></td>
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</tbody>
</table>

4. Conclusions

LAMP is highly sensitive and specific DNA/RNA amplification method. Advantage of LAMP is isothermal reaction condition, hereby LAMP is affordable because of no need to have expensive thermal cycler. Although recommended reagent storage temperature is -20°C, reagents can be stored at ambient temperature. Hereby there is no need to have cold chain for reagent distribution. Crude DNA preparation can be used as LAMP template DNA. This new method is widely used nowadays by many laboratories and is recognized by EPPO [5] as a standard method for some pathogens. Test performance study is being developed today in the circles of European scientists.

5. Acknowledgements

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6. References


