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Specific, Sensitive, and Rapid Diagnosis of *Xylella fastidiosa* from olive plant material by a new Loop-Mediated Isothermal Amplification (LAMP) system

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The gram-negative, insect-vectored, *Xylella fastidiosa* has recently been detected in Italian olive trees severely affected by Olive Quick Decline Syndrome (OQDS). The Italian isolate of this bacterium has been characterized and classified as "CoDiRO" strain of the subspecies *pauca* (Cariddi *et al.*, 2014; Elbeaino *et al.*, 2014; Loconsole *et al.*, 2016) and the spittlebug *Philaenus spumarius* L. (Aphrophoridae) was ascertained to be an effective vector in Italy (Saponari *et al.*, 2014). In Puglia region, a large-scale monitoring campaign was implemented by the Regional Plant Protection Service in order to demarcate the contaminated area boundaries and to implement adequate control measures. To this aim, Enzyme linked Immuno Sorbent Assays (ELISA) and Polymerase Chain Reaction (PCR) assays were largely used. Since the movement of large amounts of infectious materials to the laboratories for testing greatly exposes "*X. fastidiosa*-free areas" to the risk of contamination, the use of rapid and on-site detection methods was highly desirable. The suitability of a new on-site Loop-mediated isothermal amplification (LAMP) system (Enbiotech s.r.l., Italy), composed of a portable instrument (icgene mini) and a ready to use diagnostic kit denominated "Xylella Screen Glow", was therefore evaluated in this study for the detection of *X. fastidiosa* in host plants and insects. To this aim, its specificity and sensitivity were compared with those of PCR and real-time qPCR assays.

For PCR assay, *X. fastidiosa* RNA polymerase gene was amplified using RST31 and RST33 specific primers, which generate a PCR product of 733 bp (Minsavage *et al.*, 1994). The amplification reaction was conducted at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 45 sec, and a final elongation at 72°C for 7 min.

Quantitative Real-time PCR (qPCR) assay was performed to amplify the *X. fastidiosa* rimM gene using *XF*-F and *XF*-R primers together with a 6′FAM/BHQ-1 labelled probe *XF*-P (Harper *et al.* 2010). The assay was conducted in a Thermal Cycler (IQ™5, Bio-Rad Laboratories, Italy, at the following conditions: 50°C for 2 min and 94°C for 2 min, then 40 cycles of 94°C for 10 sec and 60°C for 40 sec. The threshold value was set automatically by the software (iQ™5 Optical System, V2.0). A cycle threshold (Ct) value below 35 was scored as a positive result.

Real-time LAMP assay was carried out using Enbiotech's LAMP system. The system envisages a rapid preliminary nucleic acid extraction from the sample, genetic amplification using LAMP technology, detection of the fluorescence emitted and automatic interpretation of results. The kit contains a strip with extraction buffer and another with dried primers and a LAMP mix stable at room temperature for the amplification and detection. Amplification conditions were set at 65°C for 25 min.

For the specificity tests through PCR, real-time qPCR and real-time LAMP methods, pure cultures of *X. fastidiosa* and a group of 19 non-target bacterial species and/or patovars were used. All bacterial species were grown on Nutrient Agar media while *X. fastidiosa* was grown on buffered cysteine-yeast extract (BCYE) agar medium (Wells *et al.* 1987) as a control. The bacterial DNAs were extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich s.r.l., Italy) and quantified at the spectrophotometer at 260 nm (A260).

Identical results were obtained for all three techniques adopted, as only *X. fastidiosa* DNA was amplified and no aspecific amplification was observed. Moreover, using the real-time LAMP system, *X. fastidiosa* was detected after only 15 min, while qPCR and PCR required about 30 min and 1.5 h, respectively.

Sensitivity tests were performed on DNA obtained from *X. fastidiosa* pure cultures harvested from BCYE agar medium and from artificially inoculated olive plants. Serial decimal DNA dilutions from 10 ng/µl to 1 fg/µl were prepared and analysed through all three diagnostic techniques.

Healthy olive extract used for diluting the bacteria suspension was obtained following two different procedures. In the first one, excised petioles and midribs (0.3 - 0.5 g) were extracted in the presence of Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer and homogenized. The serial diluted extracts were heated at 65°C for 30 min and centrifuged at 10.000 rpm for 5 min. Then the DNA was extracted from the supernatant by mixing in an equal volume of chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol, after incubation at -20°C for 1h. In the second procedure, sap was extracted from olive cuttings by injecting with a syringe 100µl extraction buffer through the plant shoot vessels. The serial dilutions of *X. fastidiosa* were directly used for the real-time LAMP reaction, after incubation at 65°C for 10 min. The remaining part of the extract was purified through a QIA shredder mini spin column (DNeasy Plant Mini kit, Qiagen, Milan, Italy), for using in the Real-time PCR and PCR sensitivity assays.

X. fastidiosa DNA extracted from pure culture successfully amplified by PCR and qPCR until a DNA concentration of 0.1 pg/µl; only real-time LAMP was able to detect up to 10fg/µl concentration.

Also in this test, the real-time LAMP system allowed to detect the presence of *X. fastidiosa* DNA at a very low concentration within a shorter reaction time (20 min instead of 40 and 90 min required by qPCR and PCR assays, respectively), using the crude extract from the plant or from insect vector. Using the crude extract qPCR and PCR assays were not able to detect the DNA of the bacterium.

Using saps extracted from plant tissue, real-time LAMP method allowed the detection of *X. fastidiosa* DNA from a concentration of 105 up to 10 CFU/ml with both purified and non-purified sap extracts, similarly to qPCR analysis. Conversely, the PCR assay showed to be highly influenced by the extraction method adopted, since it was unable to detect *X. fastidiosa* DNA from olive sap extracts in the samples containing less than 104 CFU/ml.

Unlike the other two techniques, the sensitivity of Enbiotech's real-time LAMP system showed to be not affected by the grade of purity of DNA samples and required shorter amplification times. In addition, the Enbiotech's real-time LAMP system did not require laborious sample preparation and expensive equipment, thus being applied also by non-specialized personnel.

These results, together with the simplicity of the extraction procedure and the brief reaction time required, make the Enbiotech's real-time LAMP system highly suitable for *X. fastidiosa* detection directly in the field, thus minimizing the risk of carrying infectious plant material (and vectors) in pathogen-free areas.

The Protocol of Real time LAMP for *Xylella fastidiosa* is reported in Table 1.

Table 1. Protocol of Real time LAMP for *Xylella fastidiosa*

DNA extraction

This method of DNA extraction could be applied for insect vectors/potential vectors or for plant material.

Insect: stored insect at 80% ethanol should be removed from ethanol and dried on tissue paper for 5 min; the extraction could be done for a single insect or for a pool up to maximum 5 insects.

Plant material: a) olive leaves from different parts of the plant (4-5 peduncles with a diameter of 2-3 mm) could be used for DNA extraction; b) for other host plant species, 5µl of ELISA crude extract could be used; ELISA extract prepared using 0.5-1 gr of midveins and petioles excised + 1-2 ml of extraction buffer, homogenized using the semi-automated homogenizer or by using a hummer.

- Collect a number of tubes equal to the number of specimens that will be tested.
- Add 200 µl of extraction buffer (1% Triton x-100, 20 mM Tris-HCl, 20 mM EDTA) in 0,2 ml tubes.

Open the tubes and add the specimens for DNA extraction (4-5 peduncles 1-2 mm of peduncle with a diameter of 2-3 mm immediately after cutting or an intact insect), or 5µl of ELISA extract or animal specimens (integral insect up to 5 insects in one single tube).

Vortex.

Place the tubes directly into LAMP (device or any heating device) and start the DNA extraction program (65°C for 10min).

Use 2.5 µl of DNA extracted for each tube for amplification process in the next step.

Preparing the Real time LAMP reaction

Prepare 0.2ml safe-lock tube with the same numbers of extraction procedure; add 5ul of primer mix (1 µM of each internal primer (FIP and BIP), 0.1 µM of each external primer (F3 and B3), 0.5 µM of each loop primer (LF and LB). To simplify the assay Primer mix generally provided in ENBOTECH Xylella screen Glow dried in a separate strip as ready to use Primer Mix tubes.

For each tube, add 17.5 µl of LAMP master mix, 30 µl of mineral oil and 2.5 µl of extracted DNA to the Primer Mix tube (If you are using Xylella screen Glow you should add 22.5 µl of LAMP master mix).

It is recommended to use positive control and negative control for each run.

Place the tubes directly into LAMP device, associate a number of each sample in the tablet RT LAMP software.

Perform the amplification program and wait 25 min for the results.

Results will be automatically viewed in tablet screen at the end of amplification.

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