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Protocols

# Development of a reverse transcription-loop- mediated isothermal amplification (LAMP) assay for the rapid detection of onion yellow dwarf virus

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ABSTRACT

Onion yellow dwarf virus (OYDV) is one of the most important viral pathogens of onion. In particular, on 'Rossa di Tropea' onion, granted with Protected Geographical Indication (PGI) trademarks, this pathogen represents the most limiting biotic stress in terms of spread, severity of symptoms and damage, and its detection is necessary to preserve high quality standards and avoid yield losses. A reverse transcription-loop mediated isothermal amplification (RT-LAMP) assay was developed for detection of OYDV. The specificity, sensitivity, repeatability and reproducibility of the assay were validated according to EPPO standard PM7/98 (2). Diagnostic specificity, diagnostic sensitivity and diagnostic accuracy were determined in both leaf and bulb tissues. To enhance the feasibility of a LAMP-based method for field diagnosis, several nucleic acid extraction methods were compared to simplify sample preparation. The results showed the reliability of the method for OYDV detection, with a limit of detection (LOD) comparable to real time reverse transcription polymerase chain reaction (RT-qPCR). The ease of sample preparation, and the more than acceptable LOD, indicated that the RT-LAMP assay could be used in plant pathology laboratories with limited facilities and resources, as well as directly in the field. This work was carried out in the frame of "SI.ORTO" project.

## 1. Introduction

Onion (*Allium cepa* L.) is the most widely cultivated species of the genus *Allium*, family *Amaryllidaceae.* Mostly used for human consumption as a vegetable and spice, raw or cooked, onion currently awakes the interest of the scientific community for its content of nutraceutical compounds. The major producer of onions worldwide is China, while Spain and the Netherlands are the leading countries in Europe. In Italy, onion production (419,000 tons, AGRISTAT 2017 http://agri.istat.it) is characterised by many ancient landraces/cultivars of high organoleptic quality. A large number of diseases and disorders affects onion crops, mostly caused by fungi but also by systemic pathogens including bacteria and viruses. In particular, *Onion yellow dwarf virus* - OYDV, genus *Potyvirus,* family *Potyviridae*, characterized by a ssRNA (+) genome, is one of the 14 viruses reported to infect onion, with a worldwide distribution in onion and *Allium* spp., inducing severe symptoms like yellowing, dwarfing and stem twirling and, in early infections, reducing bulb weight and size by up to 40%, with a seed loss up to 50% (Elnagar et al., 2011; Kumar et al., 2012). OYDV is naturally transmitted by several aphid species in a non- persistent manner, mainly by *Aphis craccivora* and *Myzus persicae,* (Drake et al., 1933; Abd El-Wahab, 2009; Kumar et al., 2011) and is considered one of the most limiting biotic stresses for onion, causing detrimental effects on crop yield and bulb quality (Katis et al., 2012). OYDV was first identified in 1932 in USA (Melhus et al., 1929), but it was not reported in Italy until 1993 (Marani and Bertaccini, 1983). In 2005, this virus was reported in Calabria (southern Italian region), on 'Rossa di Tropea' onion (Parrella et al., 2005), an Italian valuable crop, cultivated in the Calabria region under strict regulation.' Rossa di Tropea' onion is protected by a trademark at national and European level (Protected Designation Origin - IGP), to preserve its organoleptic, agronomic and nutraceutical features. Among over 10,000 onion accessions held in gene banks worldwide, 'Rossa di Tropea' is characterized by high nutraceutical compound content, in particular phenolics, flavonoids, fructoligosaccharides, alk(en)yl

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cysteine sulphoxides and saccharides, showing anti-inflammatory, anti-cholesterol, anticancer and antioxidant properties (Shon et al., 2004; Lanzotti, 2006; Grzelak et al., 2009; Benmalek et al., 2013).

In 'Rossa di Tropea' onion, OYDV has an alarming incidence rate according to different production phases and cycle. In particular, in the first year, when plants are derived from seeds, infection rates remain under 30% with symptom intensity directly related to infection time (early or late infection). In the second year, when plants are derived from bulbs for seed production, infection rates rise up to 95–100% in the field, with a severe symptomatology leading to product and plant losses (Manglli A., personal communication). The use of OYDV infected bulbs for second year production represents the main viral inoculum source, also for fresh bulb production, and the main phytosanitary risk for 'Rossa di Tropea' onion, leading to such high infection rates.

Recently a project named - *Study on Interaction between Onion yellow dwarf virus and nutraceutical compounds of "Rossa di Tropea" onion (SI.ORTO)* - was funded by Italian Ministry of Education, University and Research (MIUR), with the aim to identify and evaluate modulation and variation of onion secondary metabolites (*i.e.* flavonols, flavonoids, anthocyanines and diallylsulfides) caused by OYDV infection, by the use of standard and emerging approaches.

In the frame of the SI.ORTO project, a collateral activity was aimed at developing a fast and sensitive method for specific detection of OYDV based on a loop-mediated amplification under isothermal conditions (LAMP) (Mori et al., 2004). The reaction is promoted by the high strand displacement activity of *Bst* polymerase and does not need thermal cycling (Notomi et al., 2000; Nagamine et al., 2001). This technique can be used to detect different pathogens including bacteria and fungi and, with the addition of a reverse transcription step (RT-LAMP) for RNA conversion to cDNA, can be also applied to RNA viruses (Tomlinson et al., 2010a; Lei et al., 2014; Li and Ling, 2014; Amber et al., 2015).

LAMP enzymes are less affected by inhibiting compounds of PCR reactions, so LAMP-based methods are often easier and quicker than PCR-based methods as complex DNA/RNA extraction is not required. (Franco Ortega et al., 2018). Moreover, LAMP-based method does not require thermal cycling and can be performed in a water bath or heating block (He et al., 2016) or on a portable machine able to monitor fluorescence (Franco Ortega et al., 2018), allowing on-site testing.

In this study, a diagnostic assay based on LAMP chemistry was developed and validated according to the European and Mediterranean Plant Protection Organization (EPPO) guidelines (PM7/98(2)). The evaluation of several methods for RNA extraction/sample preparation with different levels of complexity, was carried out to investigate the potential for this assay to be used for on-site OYDV testing and/or in laboratories without advanced facilities or instruments.

## 2. Material and methods

### 2.1. Plant material

Plant material (Supplementary Table 1) was derived from three experimental trials performed under protected conditions at CREA-DC in Rome, University *Mediterranea* of Reggio Calabria and at a farm in Campora (CS), located in the typical 'Rossa di Tropea' onion production area. In these experimental trials, leaves and bulbs from healthy and artificially OYDV-infected samples were produced. To obtain infected plants, mechanical inoculation of 3-months-old plantlets was performed by 10 punctures along two different leaves using a syringe needle soaked in OYDV-infected crude extract of leaves grounded in phosphate buffer (0.1 M). Additional material was obtained from leaf and bulb samples collected in several surveys carried out in different farms in Calabria in 2016/2017. OYDV infection was assessed by ELISA (BIOREBA, Switzerland), following manufacturer's instructions, and by RT-qPCR assay (Tiberini et al., 2018). In addition, four leaf samples of OYDV-infected garlic *(Allium sativum* L.) provided by CREA-DC were also included. In total, more than 50 samples were tested.

### 2.2. Total RNA extraction

The total RNA, used for the LAMP assay set-up and validation, was extracted using the RNA extraction Real Total RNA (tissue and cell) kit (Durviz, Valencia, Spain) following manufacturer's instructions with some modifications. Sample leaf or bulb tissue (from 1 to 3 g) was disrupted in a plastic bag (BIOREBA, Switzerland) in phosphate buffer (ratio 1:10), 100 µl of crude extract were added to 650 µl of lysis buffer provided in the kit and then the protocol instructions were followed. RNA was finally resuspended with 100 µl of RNase-free water and yield/quality was assessed by NanoDrop ND1000 spectrophotometer.

### 2.3. LAMP primers design and reaction conditions

A 500 bp nucleotide sequence elapsing from region coding for nuclear inclusion b (NIb) to coat protein (CP) genes of an OYDV Italian isolate (Calabria (IT) - Acc. No. KF623540.1) was chosen to design the LAMP primers. This sequence was first aligned with other NIb/CP sequences of representative OYDV isolates and then two external primers (F3 and B3), two internal primers (FIP and BIP) and two loop primers (F-loop and B-loop) were designed using PrimerExplorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). The possibility of predicted secondary structures and hairpins was checked by using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html). The RT-LAMP assay was carried out with 1 µl of RNA (1–30 ng/µl) or 1–3 µl of crude extract (obtained as above reported). The 25 µl reaction contained 5 pmol of each external primer (F3 and B3), 20 pmol of each internal primer (FIP and BIP), 10 pmol of each loop primer (LF and LB), 1x Isothermal Mastermix ISO-001 (OptiGene Ltd, UK) containing a fluorescence intercalating dye, and 0.25 units of AMV reverse transcriptase for isothermal amplification (OptiGene Ltd, UK). In order to better set up the assay, amplifications were performed in a StepOne instrument (Applied Biosystem, California, USA) selecting the settings for TaqMan reagents. In addition, in the preliminary setup procedures, to assess the applicability of the assay with less expensive and advanced instruments, the amplification was also performed by an incubation at 63 °C for 30 min in a thermocycler (MasterCycler – Nexus GX2, Epperndorf, Hamburg, Germany) and the products were run on agarose gel or directly stained with 0.5 µl of SYBR® Safe DNA gel stain (Thermofisher, California, USA) and visualized by UV-light. To determine the optimal reaction temperature, RT-LAMP was carried out at 60 °C, 63 °C, 65 °C with an amplification programme set up to perform an incubation of 30 min (corresponding to 30 cycles,1 min each cycle) with fluorescence measured every minute, and a melting curve following these steps: 95 °C for 15 s, 70 °C for 1 min and an increase of temperature C at 0.3 °C/s up to 95° to record the fluorescence. Samples from OYDV-infected leaves and bulbs (five for each matrix) were included. In each run, positive and negative controls were included, and reactions were performed in duplicate. The analytical specificity was assessed by testing other viruses which are either reported as naturally infecting onion or phylogenetically closely related to OYDV. In particular, to assess potential non-specific cross reactions, total RNA from Iris yellow spot virus (IYSV), Garlic common latent virus (GCLV) and Shallot yellow stripe virus (SYSV) belonging to *Tospovirus*, *Carlavirus* and *Potyvirus* genera respectively, were tested.

### 2.4. OYDV RT-LAMP assay limit of detection and comparison to ELISA, endpoint RT-PCR and RT-qPCR

In order to determine the OYDV RT-LAMP limit of detection (LOD) and to compare it with LODs of serological and molecular methods, tenfold dilution series of infected OYDV leaves and bulbs were analysed by ELISA, RT-LAMP, endpoint RT-PCR and RT-qPCR. For these assays, the samples were prepared by grinding 0.5 g of plant material (from infected and healthy leaves and bulbs) in 5 ml extraction buffer (phosphate buffered saline, 0.1% Tween 20). To obtain the dilution

series, 100 µl of infected extract were added to 900 µl healthy crude plant extract. From each diluted sub-sample, 200 µl were directly tested by ELISA and 200 µl were used for total RNA extraction using Real Total RNA (tissue and cell) kit (Durviz, Valencia, Spain) as above reported and assayed by endpoint RT-PCR, RT-qPCR and RT-LAMP.

ELISA was performed according to the manufacturer's instructions (BIOREBA, Reinach, Switzerland). Endpoint RT-PCR was performed using specific OYDV primer sets according to Manglli et al. (2014), and RT-qPCR was performed according to Tiberini et al. (2018) using previously developed OYDV primers/probe set, in a StepOne instrument (Applied Biosystem, California, USA) using TaqMan reagents. All primer sets were designed in the same coding region, from NIb to CP, used for the RT-LAMP assay.

In addition, to determine the absolute pathogen quantification, expressed as the number of starting RNA molecules, a standard curve for OYDV absolute quantification was performed using the OYDV RT-qPCR assay previously developed (Tiberini et al., 2018). An OYDV cDNA fragment slightly larger than the RT-qPCR primer/probe set annealing region (about 350 bp) was cloned into a plasmid and the obtained construct was used to transform *E. coli* competent cells according to the manufacturer's protocol (pGEM-T Easy Vector system II—Promega, Madison, WI). The plasmids were extracted using a Plasmid Mini-prep Kit (Bio-Rad, Hercules, CA) and cut using the *SpeI* restriction enzyme, and *in vitro* transcription was performed using the Riboprobe® Combination System-SP6/T7 RNA Polymerase kit (Promega), according to the manufacturer's protocol. DNA was removed with *DNase I* (Promega) and the RNA was recovered by extraction using phenol:-chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation. The obtained RNA was quantified using a NanoDrop™ spectrophotometer, the number of copies was determined as follows: [Number of copies = (amount of ssRNA in nanograms*6.022x$10^{23}$)/(length of ssRNA in basepairs*1x$10^{9}$*330], and the RNA was serially diluted and used for the construction of the standard curve.

### 2.5. RT-LAMP assay validation

The LAMP assay was validated according to EPPO PM7/98 (2). The selectivity, analytical specificity, analytical sensitivity, repeatability and reproducibility were assessed. The specificity was assessed using three technical replicates for healthy and infected samples listed in Table S2 (Supplementary Table S2), in three independent assays for each test, including viral pathogens reported in onion and phylogenetically closely related to OYDV and other potyvirus species. The analytical sensitivity was checked analysing the 10-fold dilution series, obtained as above mentioned for LOD assay, and repeated in three experiments. The repeatability was performed by analysing three replicates of the lowest concentration amplified. The reproducibility was verified running independent assays using different instruments in two different laboratories by different operators. The selectivity of OYDV RT-LAMP assay was checked on two matrices: onion bulbs and leaves, naturally and artificially infected by OYDV.

In addition to the above mentioned EPPO PM 7/98(2) validation, the LAMP assay was compared to RT-qPCR (Tiberini et al., 2018). The comparison was carried out and evaluated in terms of performance criteria as diagnostic sensitivity, diagnostic specificity and diagnostic accuracy. Results were classified, according to Table 1, as true positive (TP) or true negative (TN) if they were in agreement with the known status of the sample. Alternatively, they were classified as false positive (FP) or false negative (FN) if they disagreed with the status of the sample. The diagnostic sensitivity is defined as the proportion of known infected reference samples resulting positive in the assay, and was calculated as (D-SN) = [TP/(TP + FN)]The diagnostic specificity is defined as the proportion of uninfected reference samples that resulted negative in the assay, and was calculated as (D-SP) = [TN/(FP + TN)];the diagnostic accuracy is defined as the mean of diagnostic sensitivity and diagnostic specificity, which gives a complete evaluation of the protocol parameters, and was calculated as (D-AC) = [(TP + TN)/(TP + TN + FP + FN)].

**Table 1**
Performance criteria evaluation. Two ways tables indicating how diagnostic sensitivity, specificity, and accuracy were calculated on the basis of true, false positive and true, false negative results.

| Performance criteria | |
|---|---|
| + Obtained/+ expected (TP) | + Obtained/ − expected (FP) |
| − Obtained/+ expected (FN) | − Obtained / − expected (TN) |
| D-SN: TP/(TP + FN) | D-SP: TN/(FP + TN) |
| D-AC: (TP + TN)/(TP + FP + FN + TN) | |

TP = true positive; FP = false positive; FN = false negative; TN = true negative
D-SN = diagnostic sensitivity; D-SP = diagnostic specificity; D-AC = diagnostic accuracy

### 2.6. RNA extraction comparison

The total RNA extraction method above reported and hereinafter named as "*Extraction method n° 1*", used for the assay optimisation and validation, was compared to other methods based on different principles. In particular:

*Extraction method n° 2*: A crude extraction method was used in this study to extract plant material, based on the protocol of Tomlinson et al. (2010b) with some modifications. In a 2 ml tube, 100 mg of plant tissue were added to 200 µl of alkaline PEG buffer, pH 13.5 (50 g/l of PEG average Mn 4600; 20 mM KOH;) (Chomczynski and Rymaszewski, 2006) and four glass beads, then the tube was vigorously shaken either manually or by a homogeniser MM301 (Retsch, Germany) for 1 min at 300 $s^{-1}$. 10 µl of crude extract were transferred into a tube containing 90 µl of nuclease-free water, and used for immediate testing by RT-PCR, RT-qPCR and RT-LAMP.

*Extraction method n° 3:* As for method 2, but with PEG buffer replaced with phosphate buffer, pH 7.8. The neat extract was diluted as described for method 2.

*Extraction method n° 4:* This method is based on tissue printing on blotting paper. On a positively charged membrane (Amersham Hybond™− GE Whatman, USA), freshly cut tissue portions (leaves or bulbs) were gently pressed. The membrane spotted area was cut, immersed in 100 µl of glycine buffer (EDTA 1 M, NaCl 0.05 M, Glycine 0.1 M) in a 2 ml tube and incubated for 10 min at 100 °C. The 2 ml tubes were placed on ice and 10 µl of crude extract were transferred into a tube containing 90 µl of nuclease-free water for immediate testing by RT-PCR, RT-qPCR and RT-LAMP.

*Extraction method n° 5*: A sterile 200 µl tip was used to puncture leaf or bulb material 20 times, then directly soaked for 1 min in assay mixture for immediate testing by RT-PCR, RT-qPCR or RT-LAMP.

Leaves and bulbs from artificially or naturally OYDV infected plants were divided into five portions. Each portion was extracted using one of the five methods described and the nucleic acid was serially diluted so as to obtain 10 orders of dilution. Each extract and the corresponding dilutions were tested by endpoint RT-PCR (Manglli et al., 2014), RT-qPCR (Tiberini et al., 2018) and RT-LAMP developed in this work.

## 3. Results

### 3.1. RT-LAMP primer design and assay optimisation

The six primers designed on OYDV NIb/CP nucleotide sequence (Supplementary Fig. 1), reported in Table 2, were synthesised and HPLC purified (BioFab Research, Rome - Italy). The optimum reaction temperature for RT-LAMP detection of OYDV was determined by testing temperature from 60 °C to 65 °C. After an incubation of 30 min, the optimal temperature was chosen on the basis of the average time to positive (Tp) or the time at which the fluorescence overcomes the

**Table 2**
Onion yellow dwarf virus - OYDV LAMP primer sequences. For each primer is reported 5' and 3' position (OYDV isolate Acc. No. KF623540.1), length, Tm and sequence.

| ID | 5'pos | 3'pos | Length | Tm | Sequence |
|---|---|---|---|---|---|
| F3 | 254 | 273 | 20 | 55.75 | GTGCATAGAAAATGGAACGT |
| B3 | 454 | 475 | 22 | 56.50 | TCTGTTAAATTTCGCTGAAGTC |
| FIP | | | 43 | | TGCATTATCCAGTACTGGTGCG-AAATTGGACTATGATGGACGG |
| BIP | | | 45 | | GACATTCAGACAAATTATGGCACAT-GGGCATGTATTTTTCTGTGG |
| LF | 311 | 334 | 24 | 61.09 | AGTGGATACTCAACTTGTTCCTCT |
| LB | 387 | 406 | 20 | 60.40 | TTCAGTGATGCAGCTGAAGC |

threshold level. This parameter, analogous to the threshold cycling time in qPCR (Tomlinson et al., 2013), was calculated for all the positive controls included in the reactions performed in the temperature range. No reaction for any sample included was detected at 60 °C. Good amplification curves were obtained at 63 °C and 65 °C, despite at the latter temperature not all the samples were detected. In view of above, the temperature of 63 °C was chosen as showing a good Tp value, ranging from 6 to 8 min (Fig. 1) regardless of sample matrix (bulbs or leaves). No amplification was observed in negative controls (healthy leaf and bulb samples and water), as well as, no reaction occurred with GCLV, SYSV, and IYSV infected samples, confirming the specificity of the assay (Fig. 3). In this preliminary setup phase, the amplification products were run on agarose gel (1.5%) or directly stained with SYBR® safe DNA stain (Supplementary Fig. 2).

### 3.2. Evaluation of OYDV RT-LAMP assay LOD and comparison with ELISA, endpoint RT-PCR and RT-qPCR

To determine the starting amount of target RNA, a standard curve plotting Ct values against log transformed concentrations of ten-fold dilutions of the target nucleic acid was determined (Fig. 2 ). The fluorescent signal was detected in RNA standard dilutions from 0.1 ng of RNA ($10^9$ copies) to 10 fg ($10^3$ copies). Efficiency and slope values were consistent with those reported in Tiberini et al. (2018). By the use of the standard curve, the starting number of RNA copies of the first ten-fold dilution series were also determined in parallel by ELISA, endpoint PCR, RT-qPCR and RT-LAMP, corresponding to 10 ng/µl, equal to $10^{11}$ copies of the virus.

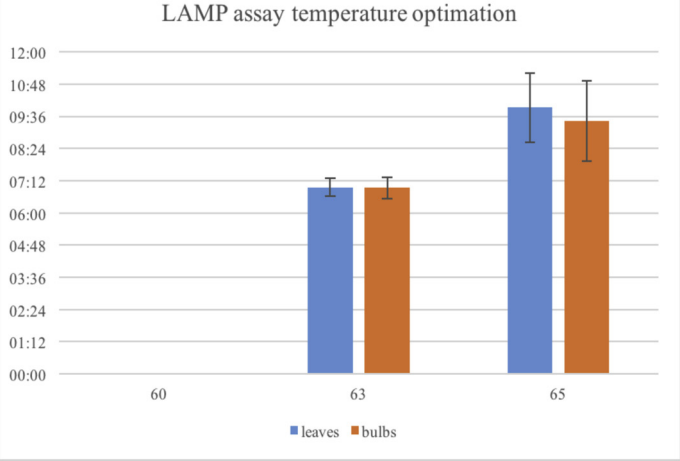
To evaluate the limit of detection (LOD), the tenfold dilution series were assayed in parallel by ELISA, end point RT-PCR and RT-qPCR. The results are reported in Table 3 and Supplementary Fig. 3. RT-LAMP showed an LOD approximately comparable to the RT-qPCR assay and was more sensitive than endpoint RT-PCR and ELISA ($10^{-4}$ and $10^{-2}$, respectively), as expected, allowing the detection of OYDV up to $10^{-5}$ diluted samples (corresponding to about $10^3$ copies) in both leaves and bulbs. ELISA assessed the virus presence up to $10^{-2}$ dilution limit, corresponding to about $10^7$ copies.

### 3.3. RT-LAMP assay validation and performance criteria

The RT-LAMP assay, following conditions reported in material and methods paragraph (using 63 °C for isothermal amplification), was validated following the criteria depicted in EPPO PM7/98 (2). Specificity was checked with the inclusivity and exclusivity panel of virus isolates shown in Table S2, with all RNA extracts in the range 10–50 ng/µl. No amplification was detected for the healthy controls and non-target species tested (Table S2). The average Tp for OYDV isolates (both bulbs and leaves) ranged from 6 to 7 min with an annealing temperature from 85.15 to 85.43 °C. Sensitivity was checked by testing the tenfold dilution series of extracts from bulbs and leaves as described above for the parallel comparison with ELISA and RT-qPCR assays, with an LOD corresponding to $10^3$ OYDV copies. By the same assays it was also possible to assess the selectivity, as healthy and naturally/artificially infected samples from both onion bulbs and leaves were included. In addition, OYDV was also detected in garlic samples. The repeatability and reproducibility were verified by running three independent assays using different instruments, as required by PM7/98, in laboratories at Università degli studi "*Mediterranea*" of Reggio Calabria and CREA-DC, by different operators on different days. Diagnostic sensitivity and diagnostic specificity were determined, for LAMP and RT-qPCR, on the basis of TP, TN, FP and FN values shown in Table 1. The results are summarised in Table 4. Both RT-LAMP and the RT-qPCR developed in a previous study (Tiberini et al., 2018), resulted in a D-AC from 97.14 to 100% for bulb and leaf samples respectively. In leaf samples, all positive and negative samples were correctly identified, resulting in D-SN, D-SP and D-AC of 100%. In bulb samples, 24 out 25 OYDV-infected samples were correctly identified, resulting in D-SP of 100%, D-SN of 96% and D-AC of 97.14%. (Table 4).

### 3.4. RNA extraction comparison

All the methods (endpoint RT-PCR, RT-qPCR and RT-LAMP) included in this analysis were able to detect OYDV, even if with different




| Sample | Temperature | | | | | |
|---|---|---|---|---|---|---|
| | 60 | | 63 | | 65 | |
| Matrices | leaves | bulbs | leaves | bulbs | leaves | bulbs |
| 1 | nd | nd | 06:54 | 07:01 | nd | nd |
| 2 | nd | nd | 06:45 | 06:15 | 10:51 | 09:33 |
| 3 | nd | nd | 07:32 | 06:54 | nd | 07:54 |
| 4 | nd | nd | 07:22 | 07:21 | nd | nd |
| 5 | nd | nd | 06:56 | 07:03 | 09:01 | 10:53 |
| Mean | nd | nd | 07:05 | 06:54 | 09:56 | 09:26 |
| SD | nd | nd | 0.0139 | 0.0169 | 0.0540 | 0.0622 |

**Fig. 1.** Lamp temperature optimisation. In y axis were reported the time to positive (Tp) values (equivalent of cycle threshold (Ct) in a qPCR) expressed in minutes, whereas in x-axis were reported the three different temperatures used to test the primer set. For each temperature (x-axis) selected (60 °C, 63 °C and 65 °C) the Tp mean value were reported (y-axis) for leaves and bulb samples, respectively, including standard deviation (nd = not determined – no reaction occurred).

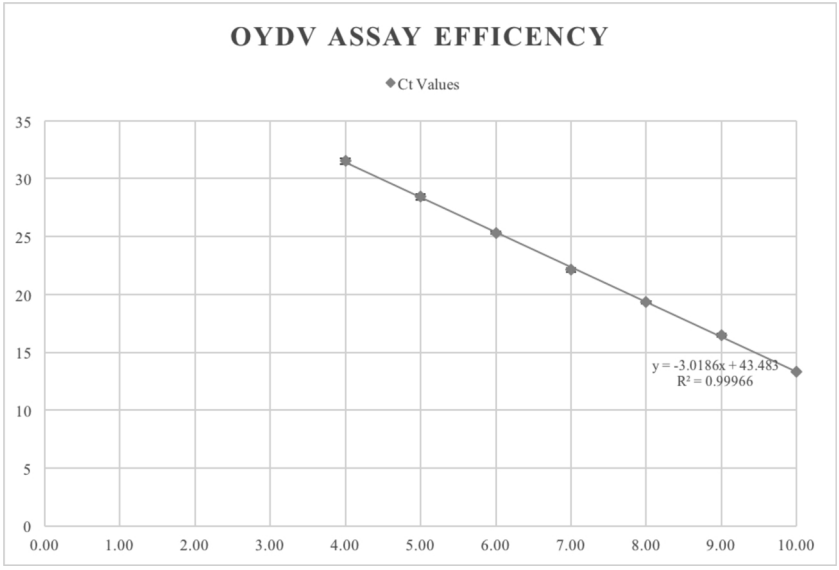



**Fig. 2.** Standard curve obtained for the Onion yellow dwarf virus - OYDV RNA quantification. The x-axis reports the logarithm of the number of copies of the in vitro transcript, while the y-axis shows the Ct values obtained in the reactions. Filled square represent the average Ct of the three replicates.

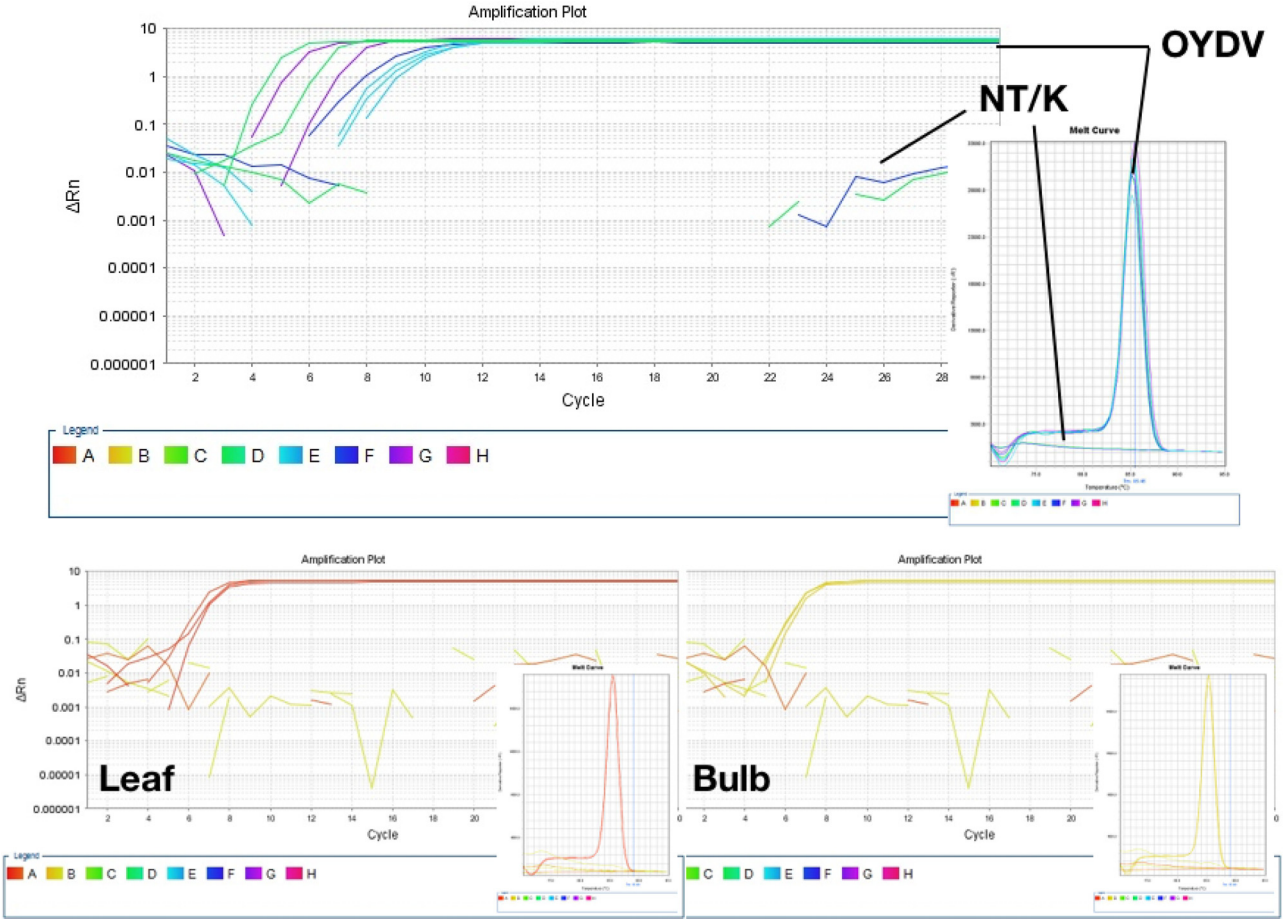



**Fig. 3.** Amplification plot and melting curves obtained from RT-LAMP assay including positive samples (OYDV), healthy samples, negative control (K) and not target viruses (No targets/NT) included in this study: Iris yellow spot virus (IYSV), Garlic common latent virus (GCLV) and Shallot yellow stripe virus (SYSV) belonging to *Tospovirus, Carlavirus and Potyvirus* genus, respectively. In the bottom part, are reported the amplification plots and melting curves for leaf and bulb OYDV infected samples, respectively. In any amplification plot in y-axis are reported the ΔRn value while in x-axis the amplification cycle. In any melt curve graph in y-axis is reported the derivate reporter, while in x-axis the temperature (°C).

**Table 3**
Analytical Sensitivity in leaves and bulbs comparing ELISA, EndPoint RT-PCR, RT-qPCR and RT-LAMP. The starting number of RNA target copies was assessed by qPCR, with the undiluted extract corresponding to $10^9$ copies of OYDV.

| Assays on Leaves | 1 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ELISA | + | + | + | – | – | – | – | – | – | – | – |
| EndPoint RT-PCR | + | + | + | + | + | – | – | – | – | – | – |
| RT-qPCR | + | + | + | + | + | + | + | – | – | – | – |
| RT-LAMP | + | + | + | + | + | + | – | – | – | – | – |
| **Assays on Bulbs** | **1** | **$10^{-1}$** | **$10^{-2}$** | **$10^{-3}$** | **$10^{-4}$** | **$10^{-5}$** | **$10^{-6}$** | **$10^{-7}$** | **$10^{-8}$** | **$10^{-9}$** | **$10^{-10}$** |
| ELISA | + | + | + | – | – | – | – | – | – | – | – |
| EndPoint RT-PCR | + | + | + | + | – | – | – | – | – | – | – |
| RT-qPCR | + | + | + | + | + | + | – | – | – | – | – |
| RT-LAMP | + | + | + | + | + | + | – | – | – | – | – |

**Table 4**
Performance criteria evaluation for leaf and bulb samples, comparing RT-LAMP to RT-qPCR. In the table for each sample type, and each assay, is indicated true, false positive and true, false negative results and the diagnostic sensitivity, specificity, and accuracy expressed in percentage value.

| | RT-LAMP | | | | RT-qPCR | | | |
|---|---|---|---|---|---|---|---|---|
| Sample type | TP | FP | TN | FN | TP | FP | TN | FN |
| Leaves | 25/25 | 0/10 | 10/10 | 0/25 | 25/25 | 0/10 | 10/10 | 0/25 |
| Bulbs | 24/25 | 0/10 | 10/10 | 1/25 | 24/25 | 0/10 | 10/10 | 1/25 |

| | D-SN (%) | D-SP (%) | D-SN (%) | D-SP (%) |
|---|---|---|---|---|
| Leaves | 100 | 100 | 100 | 100 |
| Bulbs | 96 | 100 | 96 | 100 |

| | D-AC (%) | D-AC (%) |
|---|---|---|
| Leaves | 100 | 100 |
| Bulbs | 97.14 | 97.14 |

TP = true positive; FP = false positive; FN = false negative; TN = true negative
D-SN = diagnostic sensitivity; D-SP = diagnostic specificity; D-AC = diagnostic accuracy

efficiencies and not for all sample matrices. The results are summarised in Table 5 and Fig. 4. As expected, extraction method n° 1 was the most efficient, confirming RT-qPCR and RT-LAMP LOD up to $10^{-6}$ and $10^{-5}$ dilution series in leaf and bulb samples, respectively, as well as an endpoint RT-PCR based amplification method with an LOD $10^{-4}$ and $10^{-3}$ for leaf and bulb samples respectively (Table 5). Extraction methods n° 2 and 3 resulted in lower RNA yield (number of RNA molecules was estimated as about $9 \times 10^5$ copies), but RT-qPCR and RT-LAMP assays were able to detect OYDV up to $10^{-2}$ dilutions series close to LOD of $10^3$ copies, in both leaf and bulb samples. Endpoint PCR analytical sensitivity was limited to undiluted samples ($10^0$ dilutions series), corresponding to about $10^5$ OYDV copies. The higher feasibility of RT-LAMP was highlighted also using the extraction method n° 4, detecting the target RNA in both leaf and bulb sample up to $10^{-1}$ dilutions series, whereas endpoint RT-PCR did not give any amplification. The extraction method n° 5 gave an amplification only at $10^0$ dilution series for leaf sample and bulb and in RT-LAMP (with a very high Tp of over 23 min), but not in endpoint RT-PCR or RT-qPCR.

## 4. Discussion

PCR-based methods are often used in well- equipped laboratories, to carry out identification in routine diagnosis. Further, most of these molecular methods are required assays in a certification scheme for virus-free germplasm production and trade. Despite the high reliability and sensitivity of PCR-based methods, they have the drawback of being quite complex and time-consuming. Since these methods are commonly known to be sensitive to inhibitors, such as phenols and polysaccharides (Tian et al., 2004; Schrader et al., 2012), extraction methods are required to obtain pure nucleic acids from sampled material (Boonham et al., 2008). A a simple, easy and on-site assay is needed when rapid detection is required for establishing prompt phytosanitary measures limiting virus damage to the crop. LAMP shows optimal features, as it allows rapid, sensitive, specific and easy field detection and is potentially less sensitive to inhibitors (Kaneko et al., 2007). Further, it has been used for viral pathogens in humans, animals and plants (He et al., 2016).

In this study we developed a diagnostic method based on LAMP to detect OYDV in 'Rossa di Tropea' onion.

The designed assay successfully discriminated OYDV from GCLV, SYSV, and IYSV, other common onion pathogens and phylogenetically closely related virus species (reported in Table S2). A comparison with other diagnostic methods commonly used for OYDV detection,

**Table 5**
Comparison between the three amplification protocols on RNA extracted with the five methods. Analytical sensitivity was evaluated comparing the last dilution in which the amplification signal was detected. In details: method 1: Real Total RNA (tissue and cell) kit (Durviz, Valencia, Spain); method 2: PEG buffer extraction; method 3: $PO_4$ extraction; method 4: tissue printing/glycine buffer extraction; method 5: no extraction tips soaked in reaction buffer.

| Extraction method | Tissue type | No. of starting RNA molecules | Endpoint RT-PCR | qRT-PCR | RT-LAMP |
|---|---|---|---|---|---|
| Extraction 1 | Leaves | $6.44 \times 10^8$ | $10^{-4}$ / ≅$10^4$ mol. | $10^{-6}$ /≅ $10^2$ mol. | $10^{-5}$ / @$10^3$ mol. |
| | Bulbs | $5.85 \times 10^8$ | $10^{-3}$ / ≅$10^5$ mol. | $10^{-5}$ / ≅$10^3$ mol. | $10^{-5}$ / ≅$10^3$ mol. |
| Extraction 2 | Leaves | $9.43 \times 10^5$ | $10^0$ / ≅$10^5$ mol. | $10^{-2}$ /≅ $10^3$ mol. | $10^{-2}$ / ≅$10^3$ mol. |
| | Bulbs | $8.76 \times 10^5$ | $10^0$ / ≅$10^5$ mol. | $10^{-2}$ /≅ $10^3$ mol. | $10^{-2}$/ ≅$10^3$ mol. |
| Extraction 3 | Leaves | $8.87 \times 10^5$ | $10^0$ / ≅$10^5$ mol. | $10^{-2}$/ ≅$10^3$ mol. | $10^{-2}$/ ≅$10^3$ mol. |
| | Bulbs | $8.68 \times 10^5$ | $10^0$ / ≅$10^5$ mol. | $10^{-2}$/ ≅$10^3$ mol. | $10^{-2}$/ ≅$10^3$ mol. |
| Extraction 4 | Leaves | $1.38 \times 10^5$ | nd* | $10^{-}$/ ≅$10^4$ mol. | $10^{-1}$ / ≅$10^4$ mol. |
| | Bulbs | $1.6510^5$ | nd* | $10^0$/ ≅$10^5$ mol. | $10^{-1}$/ ≅$10^4$ mol. |
| Extraction 5 | Leaves | nd* | nd* | nd* | $10^0$ / nd* |
| | Bulbs | nd* | nd* | nd* | $10^0$ / nd* |

* nd: not determined.

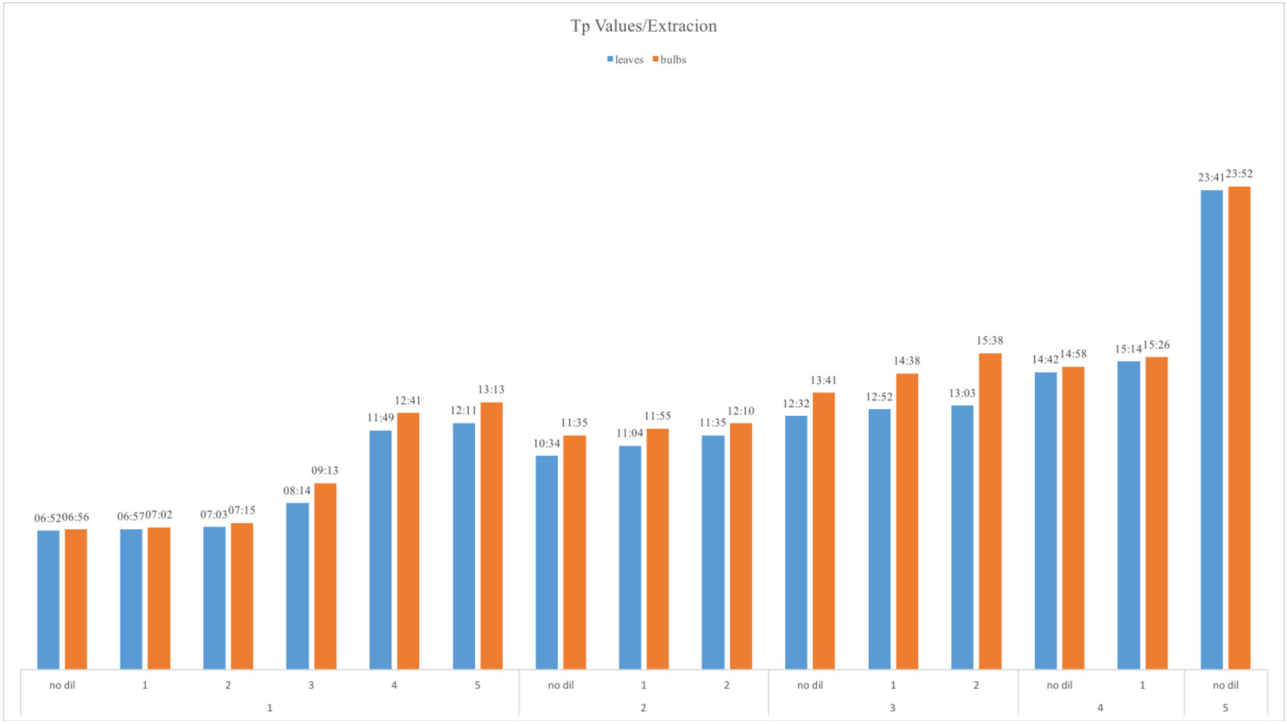



**Fig. 4.** OYDV RT-LAMP time-to-positive (Tp) mean values, for leaf and bulb samples, respectively, determined in each dilution series assayed for all the extraction method from 1 to 5. Tp values were reported up to the lowest dilution determined by RT-LAMP. In y-axis were reported the Tp value, while in x-axis were reported the limit of dilution detected and the different extraction methods: 1: Real Total RNA (tissue and cell) kit (Durviz, Valencia, Spain); method 2: PEG buffer extraction; method 3: $PO_4$ extraction; method 4: tissue printing/glycine buffer extraction; method 5: no extraction tips soaked in reaction buffer.

including serological and PCR-based methods, showed that the performance of RT-LAMP was similar to RT-qPCR. Absolute quantification of the number of starting RNA molecules in infected samples allowed direct comparison of commonly used serological and PCR-based diagnostic methods for OYDV. The RT-LAMP was observed to have an LOD of $10^3$ copies of target RNA in both leaf and bulb samples, corresponding to 1 fg RNA, $10^4$ fold more sensitive than ELISA and up to 100 fold more sensitive than endpoint RT-PCR. These data were in accordance with literature both on LAMP LOD (Franco Ortega et al., 2018; Wilisiani et al., 2019) and comparison to other diagnostic methods usually applied in vegetable crop virus detection (Luo et al., 2016). In particular, the OYDV RT-LAMP LOD could be comparable to data reported for OYDV TaqMan-based RT-qPCR (Tiberini et al., 2018), both allowing the virus detection up to $10^3$ RNA copies. As RT-qPCR is considered the most commonly used and sensitive detection technique for plant viruses (Liu et al., 2013), these results highlighted the feasibility in the use of this diagnostic tool in routine testing.

The LAMP-based assay was validated according to EPPO guidelines reported in PM7/98 (2), and was found to have good specificity, sensitivity, selectivity, repeatability and reproducibility. In addition, RT-LAMP was compared to RT-qPCR (Tiberini et al., 2018) and was shown to have similar diagnostic accuracy, sensitivity and specificity.

Since PCR-based methods are reported to be affected by several inhibitors (Franco Ortega et al., 2018), and ease of sample preparation is a crucial requirement for future application as an on-site detection method using portable instruments, five different extraction methods were compared. The conventional method used for RT-LAMP optimisation and validation (extraction method n° 1) was compared with methods that could be easily used directly in field. The five extraction methods showed significant differences in terms of yield of RNA molecules, and no amplification occurred in RT-qPCR for method 5. Using this method, OYDV was detected in the undiluted sample by RT-LAMP only. Since RT-qPCR and RT-LAMP showed a similar LOD, the PCR-based method was affected by the presence of inhibitors, whereas RT-LAMP could be generally assumed to be less sensitive to inhibition. Data obtained by this method comparison underlined that the LAMP-based detection method, being easy and robust, could be easily used directly in field. The extraction method n° 4 could be useful when applied to test a large number of samples, reducing the time-consuming sampling procedures with a limited sensitivity up to $10^{-1}$ dilution.

In particular, it is important to highlight that the OYDV RT-LAMP assay developed in this study gave optimal results in naturally infected samples, independently on the extraction method. OYDV was detected in leaf samples from field survey even by the extraction method where no sample preparation was performed (extraction method n° 5) (Table 5). The ability to detect the target at low titer and in natural infection conditions using RT-LAMP clearly demonstrates the potential for the herein developed method to be used on real samples in the field. In addition, the possibility to visualize the amplification products easily on agarose gel and/or by direct staining in the tubes (Supplementary Fig. 2), confirm the robustness of the assay without the need of advanced and expensive instruments.

## 5. Conclusions

In conclusion, to our knowledge this is the first diagnostic assay based on LAMP for a viral pathogen in onion. The RT-LAMP developed in this work was found to be robust, easy, rapid and sensitive enough to have the potential to be used in disease management and OYDV control. The optimal results obtained from samples derived by field surveys demonstrate the potential of the method and its applicability; in addition, the assay robustness was ascertained by validation according to EPPO standard PM7/98 (2) with a more than acceptable diagnostic accuracy. The reduction in delay between sampling and diagnosis could lead to a more effective and reliable defence measures and a qualitative recovery and maintenance of the onion crop.

## Compliance with ethical standards

Ethical statement. This research did not involve any animal and/or human participant.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.orG/10.1016/j.jviromet.2019.113680.

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