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Detection by Sensitive Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification of Olive Leaf Yellowing Associated Virus and Its Incidence in Italy and Spain

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Abstract: Olive trees (*Olea europea* L.) are constantly threatened by many viruses, such as the olive leaf yellowing-associated virus (OLYaV), that belong to the *Olivavirus* genus, family *Closteroviridae*. In this work, the OLYaV incidence in different regions of Italy and Spain, which represent the two most important European areas for olive production, was evaluated through the development of a real-time reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for reliable and sensitive OLYaV detection. The specificity and accuracy of the developed real-time RT-LAMP assay were determined; the assay showed that potential cross-reactivity with other viruses belonging to the *Closteroviridae* family was excluded. The LAMP assay detected OLYaV with a higher sensitivity than conventional end-point RT-PCR, detecting a total of 1.34×10^{-2} genome copies. A total of 80 and 120 plants of different olive cultivars from Spain (Comunitat Valenciana, Andalusia) and Italy (Sicily, Calabria, Apulia, Lazio, and Umbria) regions were tested, respectively. The percentage of infected plants was 46.25% and 30% for Spain and Italy, respectively, while the most susceptible cultivars were "Serrana Espadán" and "Villalonga" from Comunitat Valenciana and Andalusia regions (Spain) and "Ogliarola barese" from Apulia region (Italy). In addition, the survey demonstrated that the real-time RT-LAMP showed good sensitivity for OLYaV-positive sample detection, especially on asymptomatic olive trees. For this reason, the developed assay could be very suitable for phytopathological laboratories as a reliable and efficient method for a rapid and sensitive routine test on olive samples.

Keywords: RT-LAMP; OLYaV; *Closteroviridae*; *Olivavirus*; olive diseases; diagnosis

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1. Introduction

Olea europaea L. (*Oleaceae*) is the most widespread species of the *Olea* genus. In the Mediterranean basin, olive cultivation is considered one of the most economically important tree crops, as it has a combination of morphological and developmental characteristics suited to the climate conditions of these geographic areas [1,2]. The olive cultivation covers about 10 million hectares worldwide [3]; the Mediterranean area, in particular Spain, Italy, Greece, and Portugal, produces about 99% of European olive oil. The latest data from the European Commission reported that the European Union (EU) is the leading producer, consumer, and exporter of olive oil [4]. Over the past twenty years, olive cultivation systems have changed mainly with the aim of increasing yield [5,6] and production [7], and improving cultivation practices [8,9]. The human health benefits of olive oil and the increasing demand for olive products [10,11] have led to an increasing interest in

olive growing [12]. In fact, many traditional rain-fed olive groves have been converted to irrigation [6], and new orchards have been planted at super-high density (SHD), up to 2500 trees/ha [13]. However, these changes can lead to challenges related to the increasing incidence of olive tree pests and diseases [14]. Olive is susceptible to several diseases caused by fungi, bacteria, phytoplasmas, and viruses. In the last ten years, olive production has suffered a huge decrease due to biotic agents that have considerably undermined the Mediterranean olive tree and oil industry [15]. Furthermore, the vegetative propagation of olive plants using infected, but asymptomatic, material has represented a major means of systemic pathogen dissemination through olive orchards, particularly viruses [16–18].

To date, sixteen viruses belonging to eight genera (*Nepovirus*, *Cucumovirus*, *Tobamovirus*, *Alphanecrovirus*, *Oleavirus*, *Necrovirus*, *Marafivirus*, and *Olivavirus*) and one unassigned member for each *Secoviridae* and *Geminiviridae* families have been identified in olive [16,19–22]. Most viruses have been detected in Italy, Portugal, Spain, and, more recently, in other countries, such as the United States of America [23]. The infection's economic impact has not been really determined, although recent studies report that some olive viruses seem to affect the oil yield and quality [23–25], even if associated damages with viral infections are reported (e.g., lower yield, reduction of product quality, or market value due to defects in visual attraction) [26]. In particular, it was demonstrated that, depending on the cultivar and virus considered, the parameters that may decrease are as follows: oil yield (≈3.3% decrease), total phenols (≈400 mg/kg decrease), fatty acid composition modification, etc. Despite this, the impact of viruses on cultivated olive trees and production has rarely been studied [23].

In the past, olive leaf yellowing-associated virus (OLYaV) was an unassigned member of the family *Closteroviridae* [27], but, recently, thanks to the analyses of its full-length genome and its five broadly conserved proteins, it was demonstrated that OLYaV represents a new genus of the *Closteroviridae* family [28], named *Olivavirus* [29]. OLYaV has a monopartite positive-sense single-stranded RNA (ssRNA+) of 16,700 nucleotides (nts) that includes 11 open reading frames (ORFs) encoding proteins, some of which have homologies with those of other *Closteroviridae* members (ORF 1a, ORF 1b, ORF2-thaumatin-like protein, ORF4-HSP70h, ORF5-HSP90h, and ORF6-CP), while others have no homologous counterpart in the GenBank database, such as ORF3-p7, ORF7-p17, ORF8-p10, ORF9-p7, ORF10-p23, and ORF11-p10 [28].

Among olive viruses, OLYaV seems to be one of the most widespread [30]; it has been detected in southern Italy (including Sicily) in a large number of cultivars [31–35] and in high percentages also in Israel [36], the USA [37], Morocco [38], Tunisia [39], and Croatia [40]. Moreover, OLYaV has also been officially reported in Lebanon [41], Syria [42], Egypt [43], Greece [44], Albania [45,46], Palestine [47], Brazil [28], Slovenia [48], and Spain [49]. To date, no symptomatology has been clearly associated with OLYaV infection [49], as this virus has been detected often in symptomless trees [50]. The symptoms consist of foliar discolorations ranging from chlorosis to bright yellowing [50]. In addition, uncommon woody cylinder deformations such as stem pitting and woody gall symptoms were observed in olive trees (cv. Zarzaleña) [28]. It was also demonstrated that total twig length and number, trunk diameter, and leaf area were significantly reduced on olive-infected trees [51]. The OLYaV transmission occurs through the infected plant propagation material [27,52]. The vectors for olivaviruses remain to be identified [53], but it is strongly suspected that the OLYaV vectors are the psyllid *Euphyllura olivina* (Costa) (Homoptera: Psyllidae) [49] and unidentified mealybugs of the *Pseudococcus* genus [27].

In detail, the psyllid *E. olivina* is normally present in abundance during spring, causing up to 60% yield loss, mainly by fumagine formation with the waxy/sugary substance they emit during nymphal development. To date, it is present in Spain, France, the Canary Islands, Greece, South Africa, and the USA (California) [54]. Feeding occurs by penetration and suction of olive shoots, inflorescence, and flowers [55].

As with other plant viruses, OLYaV is reported in many olive cultivars in different areas worldwide [49,56,57], and the implementation of preventive measures and selection of healthy and certified propagation material are appropriate strategies to control

olive viruses' dispersion [16,56]. The identification of OLYaV-free plants based on visual symptoms is not reliable because many infected olive trees remain asymptomatic [16]. For this reason, due to the high presence of latent infections, field selection must therefore be followed by molecular analyses [16,17,58]. In general, plant virus detection is based on immune-enzymatic assays (e.g., ELISA) [59] and molecular methods [60,61]. Currently, the available methods for OLYaV detection are based on conventional reverse transcription-PCR [27,31,49] and SYBR® Green real-time quantitative PCR (RT-qPCR) assay [62]; furthermore, high-throughput sequencing (HTS) has proven to be a robust tool for OLYaV detection [28,49]. These methods are valuable to identify latent infections and early disease stages; however, they require expensive equipment, sophisticated laboratory setup, and highly skilled personnel [63] and are impractical for large-scale use.

An alternative is represented by the loop-mediated isothermal amplification technique (LAMP), which allows the amplification of specific genome traits with high specificity, efficiency, and rapidity under isothermal conditions [64]. LAMP is a technique with different applications, such as multiplex LAMP (mLAMP), digital LAMP (dLAMP), lab-on-a-disc (LoaD), and LAMP and transposon-insertion site detection [65]. LAMP enzymes are less affected by PCR inhibitors; for this reason, LAMP methods are easier and quicker than PCR as, in some cases, difficult DNA/RNA extraction is not required [66,67].

In the present study, the OLYaV incidence was evaluated in different cultivars collected from Spain and Italy, which represent the major olive producers in Europe, through a new real-time reverse transcription LAMP (RT-LAMP) diagnostic protocol. The reasons for the LAMP methodology's development are based on the attempt to overcome different drawbacks of end-point RT-PCR (high-cost equipment, long times, inhibitor sensitivity, etc.). Conversely, LAMP provides high specificity, enzyme robustness, and inhibitor problem reduction [67]. Moreover, LAMP could represent a valid method for epidemiological studies thanks to its rapidity, reliability, and specificity. To our knowledge, the assay developed in this work is the first real-time RT-LAMP method for OLYaV detection.

2. Materials and Methods

2.1. Real-Time RT-LAMP Assay Development for Fast OLYaV Detection

2.1.1. Source of Viral Material

One characterized lyophilized OLYaV isolate from olive leaves, named V64 (GenBank Acc. No. MW056495) [49], stored in the "Bruno Rosciglione" plant virology laboratory of the University of Palermo (SAAF Department) (Palermo, Italy), was used as source material to develop the real-time RT-LAMP assay.

About 100 mg of olive leaf V64 isolate was previously rehydrated with 1 mL of extraction buffer (1.3 g sodium sulphite anhydrous, 20 g polyvinylpyrrolidone MW 24–40,000, 2 g chicken egg chicken albumin Grade II, and 20 g Tween-20 in one L of distilled water, pH 7.4) and subsequently used for total RNA extraction using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH & Co., Dueren, Germany) following the manufacturer's instructions, with minor modifications. Briefly, 350 µL of lysis buffer supplied in the kit was added to 350 µL of the extract; the manufacturer's protocol was followed after this preparation step. The eluted RNA was re-suspended in 60 µL of RNase-free water. The concentration of total RNA was measured in duplicate with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), subsequently diluted to ≈50 ng/µL, and stored at −80 °C until molecular analyses.

2.1.2. Primer Design and OLYaV Detection Using Canonical RT-PCR

New primer pairs for OLYaV detection by end-point RT-PCR were specifically designed in this work. In detail, the complete genome sequences available in GenBank (Acc. No. MT809205, MW056495, and OK569886) were aligned using the Clustal X2 program [68] to identify the nucleotide regions that show a high homology percentage at the nucleotide level within the reference isolates. A total of six primer pairs, targeting the nucleotide sequence elapsing region between 5′-UTR and ORF-1a, were designed using the OLYaV MW056495 as a reference sequence. The designed primers

were evaluated with the Nucleotide-BLAST algorithm (https://www.ncbi.nlm.nih.gov, accessed on 15 January 2023) to evaluate possible hybridization with other organisms, while the hairpins and secondary structures were verified using the OligoAnalyzer Tool (https://eu.idtdna.com/calc/analyzer, accessed on 15 January 2023). Moreover, the primers were also checked using Vector NTI Advance 11.5 software (Invitrogen, Carlsbad, CA, USA) with the complete genome sequences of other viruses belonging to the *Closteroviridae* family, including viruses belonging to the *Olivavirus* genus (Table 1), to evaluate their affinity percentages.

Table 1. Viruses belonging to Closteroviridae family, and their sequences used for *in silico* analysis.

Species	Genus	Family	GenBank Accession No.
Citrus tristeza virus (CTV)	*Closterovirus*	*Closteroviridae*	EU937521 AF001623 Y18420
Grapevine leafroll-associated virus 1 (GLRaV-1)			NC016509
Grapevine leafroll-associated virus 2 (GLRaV-2)	*Ampelovirus*		DQ286725
Grapevine leafroll-associated virus 3 (GLRaV-3)			AF037268
Tomato chlorosis virus (ToCV)	*Crinivirus*		RNA1: AY903447 RNA2: AY903448
Tomato infectious chlorosis virus (TICV)			RNA1: FJ815440 RNA2: FJ815441
Cucurbit yellow stunting disorder virus (CYSDV)			RNA1: NC004809 RNA2: NC004810
Actinidia virus 1 (AcV1)	*Olivavirus*		KX857665
Persimmon virus B (PeVB)			AB923924

The obtained primer sets were tested by a canonical RT-PCR assay using the OLYaV RNA positive control (PC) (≈50 ng/µL concentration), including a healthy olive plant RNA as a negative control (NC). Each sample was analyzed in triplicate in three independent, two-step end-point RT-PCR assays. In detail, the reverse transcription (RT) was carried out in a 20 µL final volume with 1 µL of total RNA, 0.4 mM dNTPs, 4 µL of 5X First Strand Buffer [50 mM Tris-HCl pH 8.3, 40 mM KCl, 6 mM $MgCl_2$] (Thermo Fisher Scientific, Waltham, MA, USA), 1 µM of reverse primer, 20 U of M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume. After an initial denaturation at 65 °C for 10 min, RT was performed at 42 °C for 45 min and 95 °C for 10 min. PCR was performed in a final reaction volume of 25 µL, containing 2 µL of the obtained cDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 µM of each primer, 2 U of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume (Table S1), according to the following cycling conditions: 95 °C for 3 min; 40 cycles of 30 s at 95 °C, 30 s at the specific annealing temperature of each primer pair, 45 s at 72 °C, and a final elongation of 10 min at 72 °C (Table S1). RT-PCR was carried out in a PCR System 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). RT-PCR products were verified by electrophoresis in a 1.5% agarose gel stained with SybrSafe™ (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by UV light. The primer pair that revealed the highest specificity and the absence of any non-specific products was chosen and used for subsequent tests. The obtained PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* One Shot™ Mach1™ competent cells (Invitrogen, Carlsbad, CA, USA). After ampicillin resistance selection of the transformants, the fragment presence was verified by colony-PCR with the specific primer pair previously used. The plasmid DNA was purified using the NucleoSpin Plasmid DNA Purification Kit (Macherey-Nagel

GmbH & Co., Dueren, Germany), quantified twice using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and sequenced in both directions using an ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems, Foster City, CA, USA). Finally, the sequence obtained was verified by the BLAST algorithm at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi, accessed on 30 January 2023).

2.1.3. OLYaV Real-Time RT-LAMP Primer Design

A 429 bp nucleotide sequence elapsing from the 5′-UTR to the ORF1a genes of the OLYaV V64 isolate (GenBank Acc. No. MW056495) used as a reference sequence was chosen to design a specific LAMP primer set. In detail, a set of six primers was designed using the PrimerExplorer version 5 software (https://primerexplorer.jp/e/, accessed on 5 February 2023), including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). Primer set specificity was evaluated in silico using the nucleotide BLAST algorithm (https://www.ncbi.nlm.nih.gov, accessed on 5 February 2023) available at the NCBI website to evaluate possible cross-reactions with other organisms. In addition, each primer was tested against the full genomic sequences of other viruses listed in Table 1 to verify their affinity using the Vector NTI Advance 11.5 software (Invitrogen, Carlsbad, CA, USA).

2.1.4. OLYaV Real-Time RT-LAMP Assay Optimization

The OLYaV V64 isolate RNA was used as a template for the real-time RT-LAMP assay optimization, including healthy olive plant RNA (NC) as a negative control. The real-time RT-LAMP assay was performed in a volume of 25 µL, containing 0.2 µM each of OLYaV-F3 and OLYaV-B3, 1.6 µM each of OLYaV-FIP and OLYaV-BIP, and 0.4 µM each of OLYaV-LoopF and OLYaV-LoopB, 15 µL of LAMP Isothermal Master Mix (Optigene® Limited, West Sussex, UK), 1 µL of RNA (≈50 ng/µL), and nuclease-free water to reach the final volume (Table S1). Each sample was analyzed in triplicate in three independent real-time LAMP assays, performed at 65 °C for 60 min (fluorescence acquisition every 60 s), using a Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany). Additional steps for the melting curve were carried out to acquire the fluorescence using the following protocol: 95 °C for 1 min, 40 °C for 1 min, 70 °C for 1 min, and an increase in temperature at 0.5 °C/s up to 95 °C (Table S1). The fluorescence data were obtained in the 6-carboxyfluorescein (FAM) channel (450–495 nm excitation and 510–527 nm detection). The relative fluorescence units (RFU) threshold value was used, and the threshold time (Tt) was calculated as the time at which fluorescence was equal to the threshold value.

2.1.5. Sensitivity and Comparison of OLYaV Real-Time RT-LAMP Assay to Conventional RT-PCR

The plasmids obtained were used to determine the OLYaV real-time RT-LAMP sensitivity and to compare the results with an end-point RT-PCR assay. Ten-fold serial dilutions of the purified recombinant plasmid DNA diluted into healthy olive RNA extract were used as templates for both real-time RT-LAMP and end-point RT-PCR assays. The number of copies was determined as follows:

$$No.\,of\ copies = \frac{\text{DNA amount [ng]} \times 6.022 \times 10^{23}}{\text{DNA template lenght [bp]} \times 1 \times 10^{9} \times 650}$$

Additional steps for the melting curve were carried out, as described above (see Section 2.1.4). Each sample was analyzed in triplicate in three independent assays. The results obtained were compared, and the optimal reaction time of the real-time RT-LAMP was determined.

2.1.6. Specificity of OLYaV Real-Time RT-LAMP Assay

To verify the real-time RT-LAMP assay specificity and evaluate non-specific reactions with other viruses belonging to the *Closteroviridae* family, a real-time RT-LAMP assay was conducted using the OLYaV V64 isolate as a positive control and the RNAs of other viruses stored in the "Bruno Rosciglione" virology laboratory. In particular, the following viruses were tested: Citrus tristeza virus (CTV), grapevine leafroll-associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), tomato chlorosis virus (ToCV), tomato infectious chlorosis virus (TICV), and cucurbit yellow stunting disorder virus (CYSDV). In each run, total RNA from a healthy olive plant (NC) was included, and each sample was analyzed in triplicate in three independent LAMP assays. The experiment was performed with the conditions described above (see Section 2.1.4), reducing the reaction time to 45 min, including the melting curve steps.

2.2. Evaluation of OLYaV Incidence in Italy and Spain by Real Time RT-LAMP Assay on Symptomatic and Asymptomatic Olive Trees

To understand the effective OLYaV incidence in Italy and Spain, several samplings were carried out in the main Italian and Spanish olive-growing regions, and each sample was analyzed by the real-time RT-LAMP assay developed in this work.

The analyses were performed on 61 symptomatic and 139 asymptomatic field samples, for a total of 200 samples of different cultivars collected during spring 2022. In detail, 80 samples were collected in Spain, of which 40 were in the Valencia region (20 in the Valencia province and 20 in the Castellón province) and 40 were in the Andalusia region (20 in the Granada province and 20 in the Jaén province) (Figure 1). Regarding Italy, a total of 120 samples were collected, of which 40 were collected in Sicily (20 in the Agrigento province and 20 in the Trapani province), 20 in the Calabria region (Crotone province), 20 in the Apulia region (Trapani province), 20 in the Lazio region (Frosinone province), and 20 in the Umbria region (Terni province) (Figure 1). Table 2 reports the different olive cultivars collected.

Figure 1. Sampling regions in Spain (**left**) and Italy (**right**).

The Planthology mobile application was used to geo-reference all samples collected [69]; subsequently, the samples were stored at 4 °C and analyzed. Sampling was carried out, collecting eight branches per plant from symptomatic and asymptomatic olive trees. The hierarchical sampling scheme, with minor modifications to olive plants, was applied to collect the samples [70]. Total RNA was extracted, and the concentration was determined as described above. Subsequently, real-time RT-LAMP for easy detection of OLYaV was carried out. Each test included a positive control (PC) and RNA from a healthy olive plant

as a negative control (NC). Each sample was analyzed in triplicate in three independent real-time RT-LAMP assays.

Table 2. Olive cultivars collected from different Italian and Spanish regions.

Country	Region	Province	No. of Samples Collected	Cultivar
Italy	Sicily	Agrigento	20	Giarraffa
		Trapani	20	Nocellara del Belice
	Calabria	Crotone	20	Carolea
	Apulia	Taranto	20	Ogliarola barese
	Lazio	Frosinone	20	Leccino
	Umbria	Terni	20	Frantoio
Spain	Comunitat Valenciana	Valencia	20	Serrana Espadán, Picual
		Castellón	20	Serrana Espadán, Villalonga
	Andalusia	Jaén	20	Picual, Villalonga
		Granada	20	Arbequina

3. Results

3.1. Real-Time RT-LAMP Assay Development for Fast OLYaV Detection

3.1.1. Primer Design and OLYaV Detection Using Canonical RT-PCR

In Table 3, the six end-point RT-PCR primer pairs designed are reported. The in silico analysis results showed that no relevant matches with other organisms were identified.

Table 3. OLYaV RT-PCR primer pairs designed in this work.

Primer Name	Binding Sites (Genbank Acc. No. MW056495)	Genomic Position	Nucleotide Sequence (5′-3′)	Annealing Temperature—Ta (°C)	Amplicon Size (bp)
OLYaV-21F	**21–44**	**5′ UTR-ORF1a**	**ATCAATTGAAGAAAACCACTCCC**	**59**	**450**
OLYaV-471R	**450–471**		**AGTACCTCCCACGACGTATTG**		
OLYaV-30F	30–54	5′ UTR-ORF1a	GAAAACCACTCCCTTCAATTCAAT	61	497
OLYaV-527R	505–527		GAAGAACTATTGATTGGCTTGGG		
OLYaV-36F	36–59	5′ UTR-ORF1a	CCACTCCCTTCAATTCAATAACAT	60	428
OLYaV-444R	444–464		CCCACGACGTATTGACCACTC		
OLYaV-46F	46–67	5′ UTR-ORF1a	CAATTCAATAACATAACATACC	60	345
OLYaV-371R	371–391		ATGTGACTTTTGACTGAGGTA		
OLYaV-495F	495–517	ORF1a	GAAGAACACTCCCAAGCCAATC	60	646
OLYaV-1141R	1120–1141		CACACTCTCTCTTGTAAGTCCC		
OLYaV-742F	742–763	ORF1a	GCGGCTTGGGTCATTAAGGGT	60	427
OLYaV-1166R	1147–1166		GGGCAAAAATAGTGAGAGCGAC		

All the obtained primer pairs were verified by end-point RT-PCR. Between the six primer pairs, OLYaV-30F/OLYaV-527R and OLYaV-46F/OLYaV-371R did not give the expected amplicon (), while the remaining primer pairs gave the expected amplicon (Table 3), but with non-specific bands, except for OLYaV-21F/OLYaV-471R, which showed the higher specificity without non-specific bands. Therefore, this primer pair was chosen as the best candidate for OLYaV detection by end-point RT-PCR. A 450 bp amplicon size was obtained, as expected PCR product, and the sequence showed a percentage identity of >99.9% with

the previously uploaded OLYaV sequences in GenBank, indicating that the assay was specific for OLYaV detection. The analysis using the Vector NTI 11.5 program against other viruses (see Section 2.1.2) has not demonstrated relevant matches.

3.1.2. OLYaV Real-Time RT-LAMP Primer Design

A set of six primers for OLYaV detection by a real-time RT-LAMP assay was designed between the 5′-UTR and ORF-1a coding regions, within the OLYaV-21F/OLYaV-471R amplified region. Table 4 and Figure 2 report the primer sequences and binding sites, respectively. Both the in silico analysis of LAMP primers and the hybridization analysis against other viruses showed no cross-reactions with other organisms and excluded relevant matches with other viruses, respectively.

Table 4. Primers designed for OLYaV detection by real-time RT-LAMP.

Primer Name	Nucleotide Sequence (5′-3′)	Amplicon Size (bp)
OLYaV-F3	CTGGTCTCTACGTACAGGGA	195
OLYaV-B3	GGTACTGCCTCAGTTCCCA	
OLYaV-FIP	CCGGTAACGACCTTCAGCCTTCCCCGCTGTG CAGAACAAC	-
OLYaV-BIP	AAGAAAACGGGCTGTAGCCCATGCCGTCAA TGTTACGAGC	
OLYaV-LoopF	TCTGTGCAGATCTAGATTTGGGA	-
OLYaV-LoopB	GAGGAAAGCAGCGACCACC	

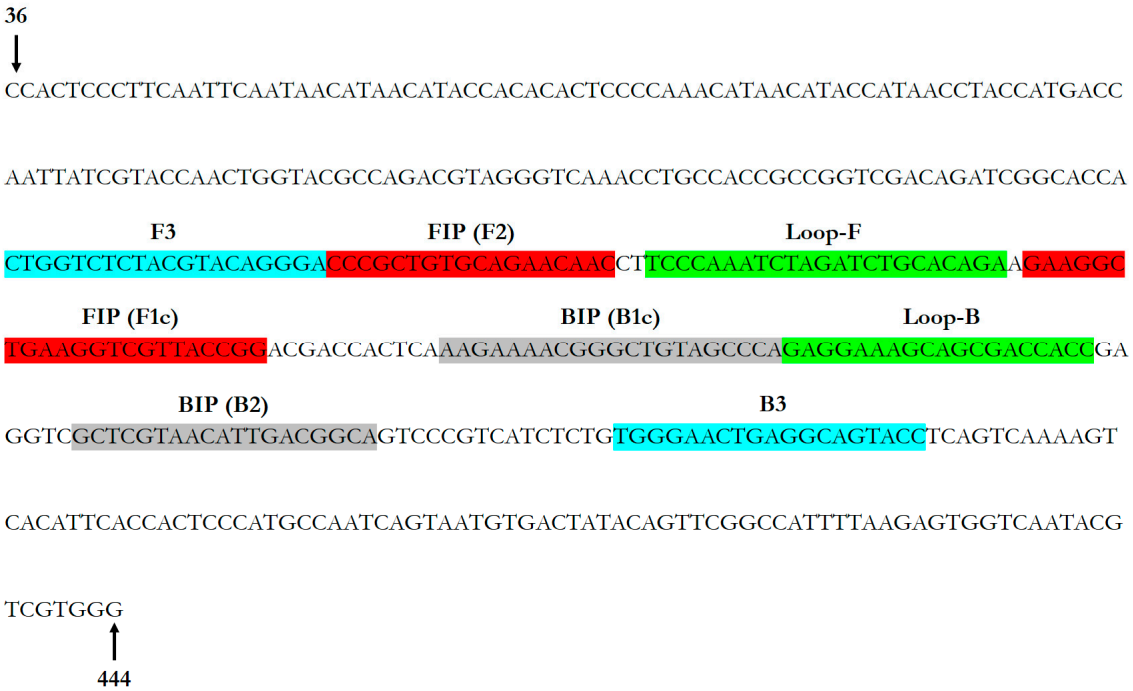

Figure 2. Genome position of the LAMP primer set designed in this work. F3 and B3 are shown in pale blue, FIP (F1c-F2) in red, BIP (B1c-B2) in gray, and the two loop primers LF and LB in green. Genomic position of the first and last nucleotide in the selected sequence is represented by the number at the beginning and end of the sequence (GenBank Acc. No. MW056495).

3.1.3. OLYaV Real-Time RT-LAMP Assay Optimization

As reported in Table 5 and Figure 3A, the amplification curve of the OLYaV V64 isolate showed an exponential trend at 14 min, reaching the reaction plateau in about 24 min,

while the melting curve displayed a peak temperature of approximately 90 °C (Figure 3B). No signal was obtained with the negative control (NC).

Table 5. Real-time RT-LAMP assay performance for OLYaV detection.

Sample	Real-Time RT-LAMP Reaction Time (Min)		
	Assay #1	Assay #2	Assay #3
OLYaV V64 isolate	13.7	14.1	14.4
Negative control (NC)	-	-	-

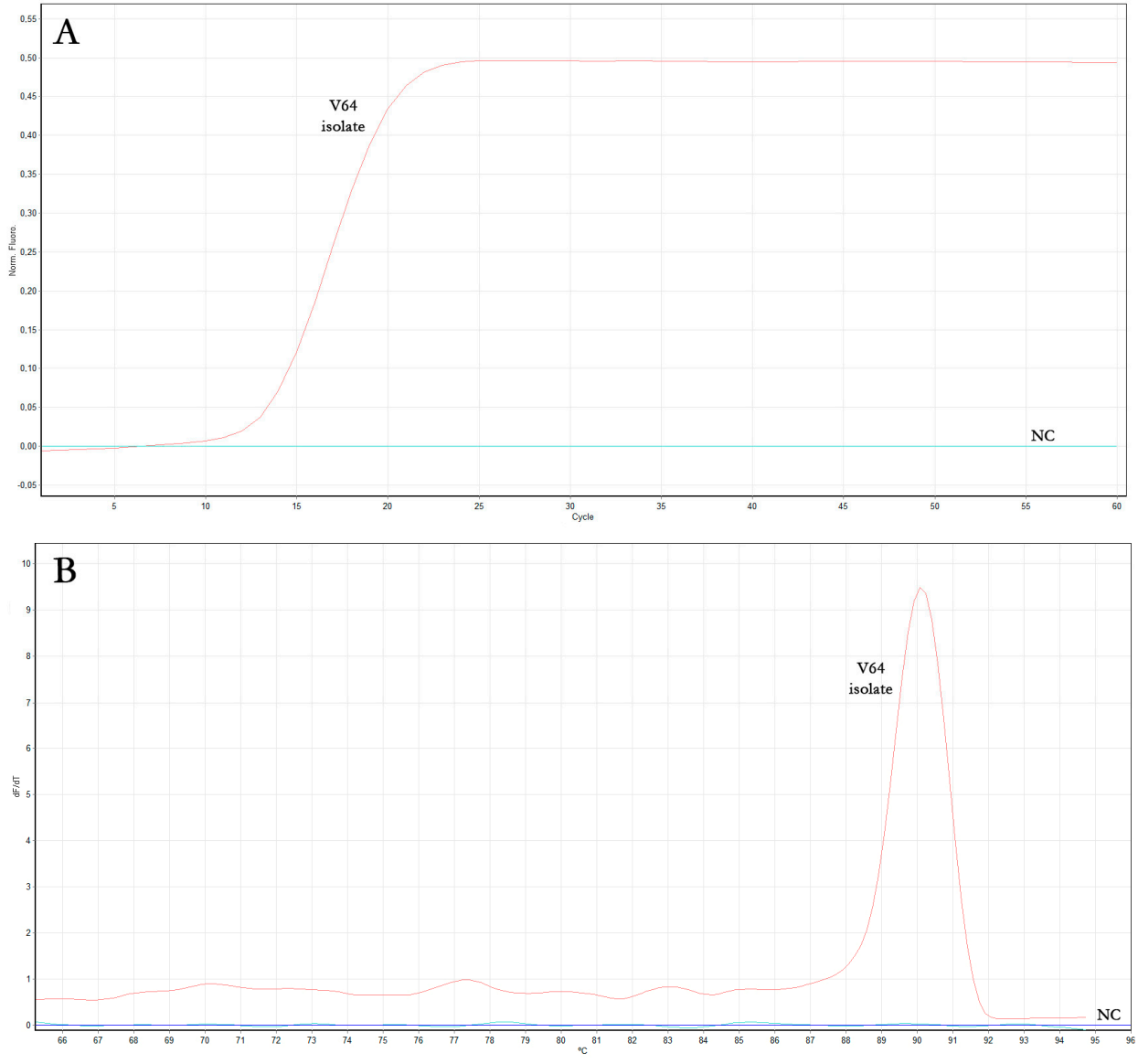

Figure 3. Real-time RT-LAMP assay results for OLYaV detection. Amplification (**A**) and melting (**B**) curves of OLYaV V64 isolate. **NC**: negative control.

3.1.4. Sensitivity and Comparison of OLYaV Real-Time RT-LAMP Assay to Conventional End-Point RT-PCR

To ascertain the sensitivity and efficacy of the real-time RT-LAMP assay, a comparative experiment was conducted using as a template ten-fold serial dilutions of a purified recombinant plasmid, starting from a concentration of ≈50 ng/µL (1.34×10^{10} copies). The results obtained in the three replicates for each test returned completely overlapping results.

The end-point RT-PCR was able to detect up to ≈50 × 10^{-11} ng/µL (Figure 4), while DNA concentrations up to ≈50 × 10^{-12} ng/µL were detected with the real-time LAMP assay developed in this work (Figure 5A). In detail, the LAMP assay was more sensitive and able to detect a total of 1.34 × 10^{-2} genome copies, whereas with the canonical RT-PCR, it was only possible to detect up to 1.34 × 10^{-1} genome copies. Moreover, even considering the lowest detectable concentration in real-time RT-LAMP (≈50 × 10^{-12} ng/µL), the results clearly showed that the time required to obtain reliable results was less than 45 min. The melting curves displayed the same peak temperature at 90 °C (Figure 5B), and the results of the RT-LAMP reaction time plateau were calculated as the mean values obtained from the three replicates (Table 6).

Table 6. Comparison of real-time RT-LAMP and end-point RT-PCR assays sensitivity using 10-fold serial dilutions of a purified recombinant plasmid (SD: standard deviation).

	Starting DNA Concentration (50 ng/µL) (1.34 × 10^{10} Copies)															
Assay	**10^{0}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**10^{-11}**	**10^{-12}**	**10^{-13}**	**10^{-14}**	**10^{-15}**
End-point RT-PCR	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–
LAMP reaction time plateau (min) (mean values ± SD)	13 ± 0.1	13.9 ± 0.2	15.2 ± 0.2	16.8 ± 0.2	17.4 ± 0.1	20.1 ± 0.4	22 ± 0.2	24.4 ± 0.4	25.1 ± 0.3	26.3 ± 0.4	31.2 ± 0.4	35.1 ± 0.3	43.4 ± 0.4	–	–	–

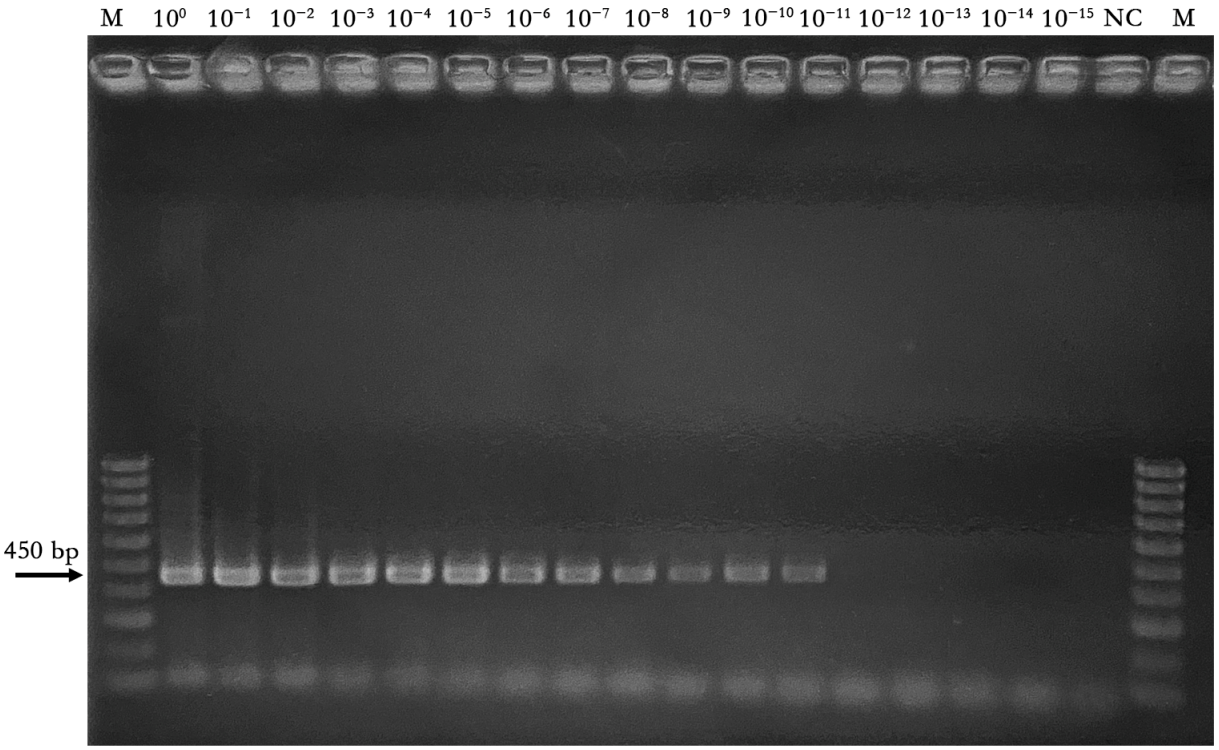

Figure 4. RT-PCR products electrophoresis on 1.5% agarose gel for OLYaV detection using 10-fold serial dilutions. **M**: Bioline HyperLadder 100 bp marker. From **10^{0}** to **10^{-15}**: 10-fold serial dilutions of a purified recombinant plasmid. **NC**: negative control.

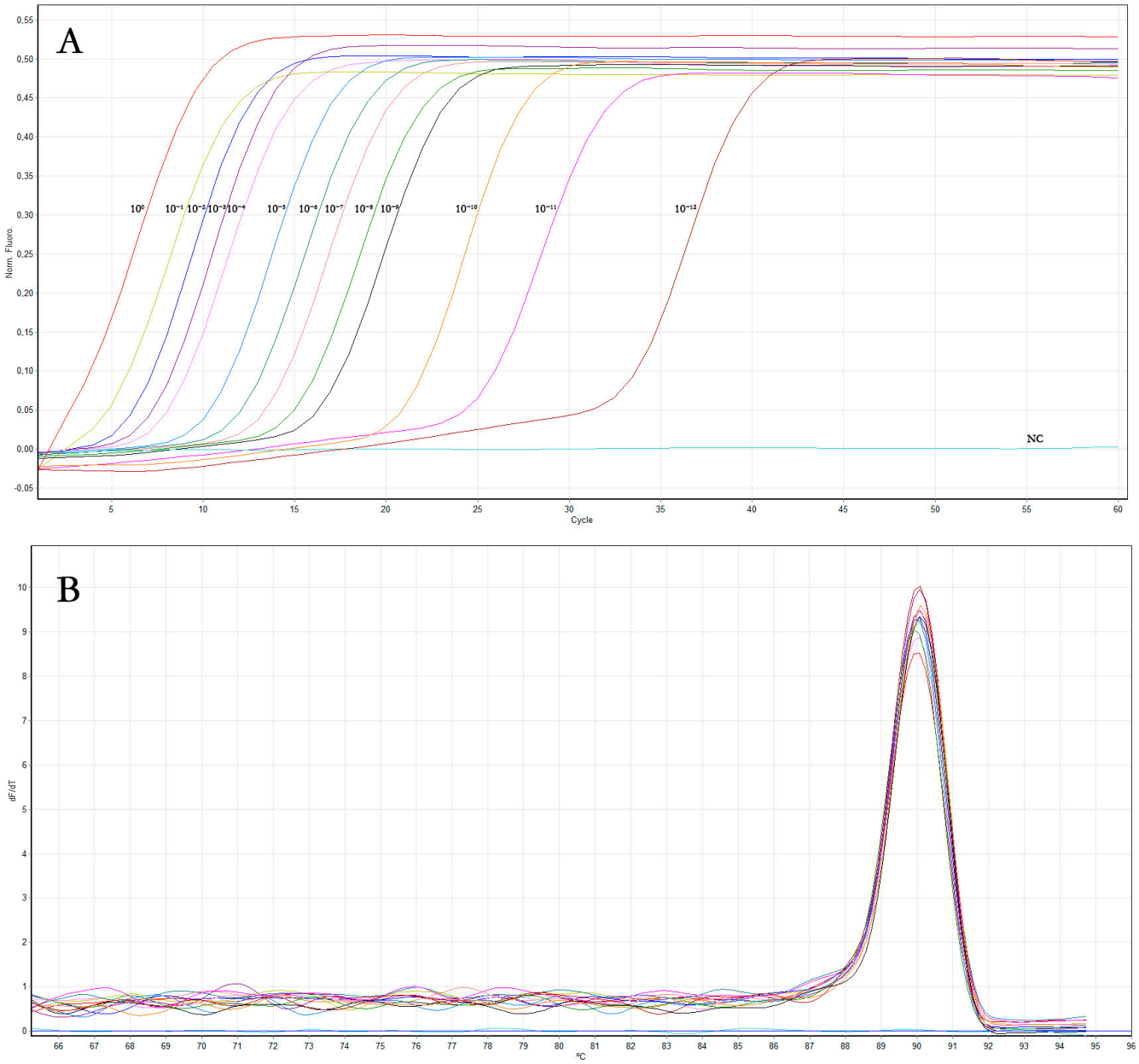

Figure 5. Sensitivity of real-time RT-LAMP assays for OLYaV detection using 10-fold serial dilutions. (**A**) Fluorescence of the 10-fold serial dilutions analyzed. Fluorescence increasing in positive sample curves (from ≈50 × 10^{-1} to ≈50 × 10^{-12} ng/µL) after 2 to 33 min. (**B**) Melting curves.

3.1.5. Specificity of OLYaV Real-Time LAMP Assay

To evaluate the LAMP assay specificity and to assess non-specific cross-reactions, a real-time RT-LAMP assay, using the outgroup reported in Section 2.1.6, was performed. A reaction time value of 15 min (Figure 6A) and a single peak at ≈90 °C of the melting curve (Figure 6B) were obtained with the OLYaV V64 isolate RNA, while no amplification was obtained with the other viruses used as outgroups.

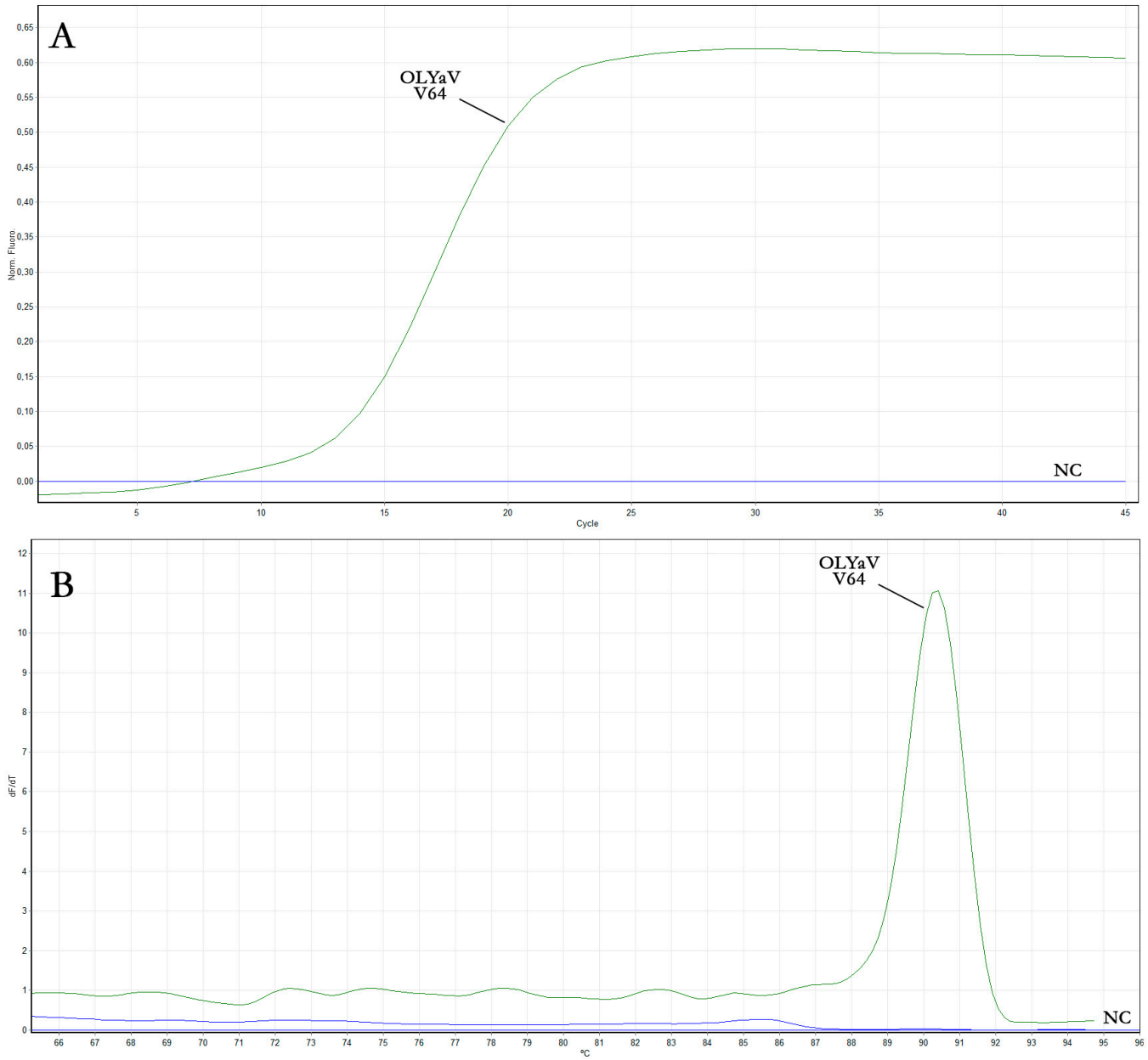

Figure 6. Specificity of the real-time RT-LAMP assays for OLYaV. Amplification (**A**) and melting (**B**) curves of OLYaV V64 isolate, and viruses used as out-group. **NC**: negative control.

3.2. Evaluation of OLYaV Incidence in Italy and Spain by Real Time RT-LAMP Assay on Symptomatic and Asymptomatic Olive Trees

To evaluate the OLYaV incidence in Italy and Spain and to confirm the validity of the real-time RT-LAMP assay developed, the analyses were performed on 61 symptomatic (Figures 7 and 8A) and 139 asymptomatic (Figure 8B) field samples, for a total of 200 samples, collected from different olive production sites in Italy and Spain.

Figure 7. Typical yellowing branches on olive tree infected by olive leaf yellowing-associated virus.

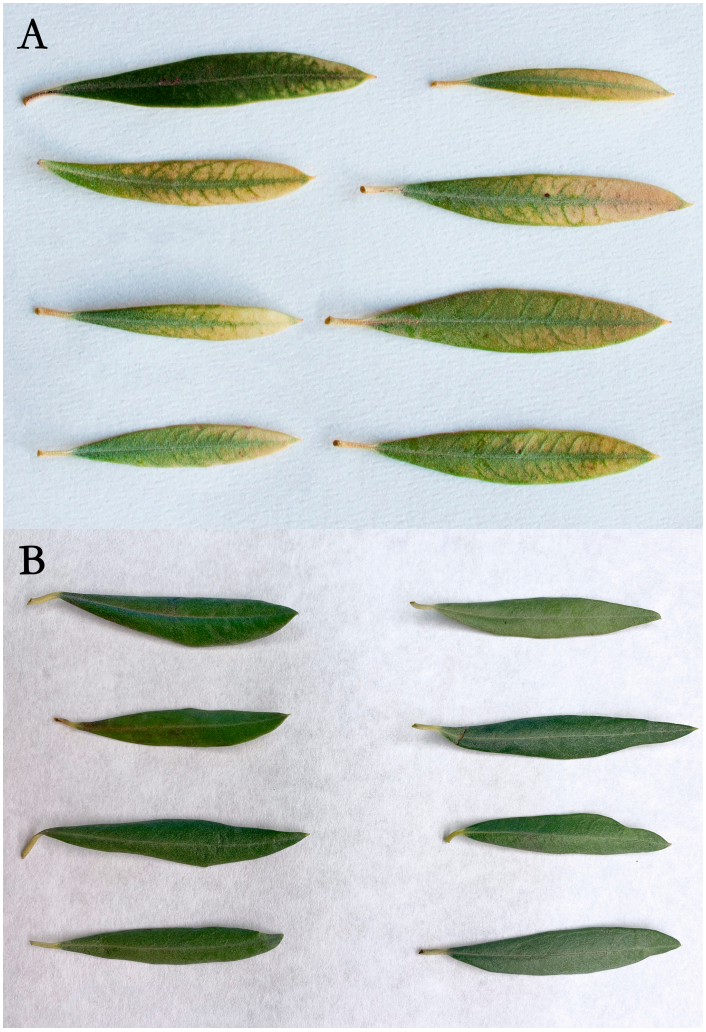

Figure 8. (**A**) Typical foliar symptoms caused by olive leaf yellowing-associated virus on collected samples. (**B**) Asymptomatic olive leaves sample.

Seventy-three out of two hundred collected samples resulted positive for OLYaV by real-time RT-LAMP (Tables S2 and S3), with an incidence percentage of samples that resulted positive of 36.5%. Moreover, the obtained results showed an improvement in OLYaV-positive sample detection in asymptomatic samples. In Italy, out of 120 samples collected from the 6 different provinces investigated, 36 samples resulted positive for LAMP, with an infection percentage of 30%. In this case, the developed RT-LAMP assay was also able to detect the OLYaV presence in 7 olive asymptomatic plants (Figure 8B). Considering the cultivars analyzed, a different OLYaV incidence was observed. The higher OLYaV incidence was observed in the "Ogliarola barese" cv collected from the Apulia region, with a total of 11 out of 20 samples analyzed, followed by "Giarraffa" (Sicily) and "Carolea" cvs (Calabria), with a total of 8 and 7 samples resulted positive, respectively. A lower incidence was observed in "Nocellara del Belice" (Sicily), "Frantoio" (Umbria), and "Leccino" (Lazio) cvs, with a total of 5, 3, and 2 positive samples, respectively. Regarding the OLYaV incidence in Spain, 37 out of 80 samples resulted positive, with an infection percentage of 46.2%; in this case, 5 positive samples collected from asymptomatic plants resulted positive for OLYaV infection. The highest incidence was observed in Valencia (13 out of 20 samples) and Jaén provinces (11 out of 20 samples). Specifically, the following results were obtained for Spanish cultivars: 12 out of 20 samples of the "Serrana Espadán" and "Villalonga" cultivars, respectively, resulted positive for OLYaV, while a lower incidence was revealed in the "Picual" and "Arbequina" cultivars (8 and 5 positive samples out of 20, respectively).

Finally, to confirm the RT-LAMP assay results, the melting curve analysis, carried out on asymptomatic samples too, showed the same peak temperature (≈90 °C) in all samples, concordant with the OLYaV-positive control, including those that were asymptomatic and positive by real-time RT-LAMP.

4. Discussion

Olive trees are prone to several pathogens that can compromise product yield and quality, such as fungi, bacteria, and viruses [15]. Concerning viral diseases, infected plants generally exhibit morphological/physiological alterations, which usually result in lower production performance [71]. Viral symptoms on olive trees mainly consist of leaf yellowing, irregular fruit, streaking, and vein clearing [16,72]. However, some viruses can lead to symptoms only in some olive cultivars while remaining latent in others [16], as in the case of OLYaV; for this reason, viral diagnosis in the field and propagation material selection are impossible to perform without laboratory analyses [73]. Since there is no known source of genetic resistance to olive viruses, a key component of viral disease management is the use of virus-free plant materials; for this reason, the legislation currently in force for the exchange of olive trees or propagation material (Regulation (EU) 2016/2031) [74] requires the absence of several olive tree viruses, including OLYaV. The certification program's adoption demands the use of extremely sensitive diagnostic techniques for viral detection, which are the basis for obtaining virus-free olive plants, as reported in the certification scheme for olive trees and rootstocks (PM 4/17-3) by EPPO. The low viral titer in olive tissues and the presence of different inhibitors during the acid nucleic extraction, such as acidic polysaccharides, a variety of salts, secondary metabolites, and phytochelatins [75], are the major constraints of the diagnosis methods and do not always allow for successful, accurate, and reproducible detection [57]. Thus, sensitive, precise, and cost-effective detection assays for olive viruses are critical and of major importance to guarantee the sustainable production of the orchards. In this study, a real-time LAMP protocol was developed in order to overcome some of the limitations of the RT-PCR-based assays, e.g., reduced sensitivity to inhibitors [17,67], such as phenols and polysaccharides [76]. For OLYaV detection by real-time LAMP, a set of six specific primers, capable of recognizing a total of eight different regions targeting the ORF1a coding region, was designed. The technique developed has demonstrated a 10-fold higher sensitivity compared to the canonical RT-PCR; in addition, even considering the lowest detectable concentration of 50×10^{-12} ng/µL, the time required to carry out the experiment was less than 45 min, compared with the time

required for the conventional RT-PCR assay, which requires several hours to obtain the results due to the extended duration of the RT-PCR reaction protocol and results visualization by electrophoresis. The LAMP assay provides considerable sensitivity for OLYaV detection; similar results were previously reported by other researchers for various plant viruses [77–80]. Moreover, OLYaV was detected in olive orchards independently of the cultivar, crop management, or geographical location (Italy or Spain), probably due to a gene flow that occurred in the past between these two countries, which led to a wide spread of this pathogen over time and a distributed presence in cultivars across the countries. In this sense, OLYaV may represent an issue that should not be underestimated, both in terms of phytosanitary certification of propagation material and for new plantings, particularly new intensive and super-intensive olive orchards. For this reason, it is essential to monitor the commercial cultivars to date most widespread in Italy and Spain, including molecular diagnostic methodologies as reliable and sensitive as possible. The robustness and sensitivity of the methodology, suitable to be adopted under conditions of low symptom presence or low viral titer, could be a useful tool to be introduced in this context. Moreover, the incidence analysis showed that the developed assay was capable of detecting OLYaV in 12 asymptomatic olive plants from Spain and Italy, in 5 and 7 asymptomatic plants, respectively. OLYaV incidence was higher in the Spanish territory than in the Italian territory, out of a total of 80 and 120 samples analyzed, respectively. Probably, the higher OLYaV incidence in Spain could be due to the presence of the insect vector (*E. olivina*). Moreover, OLYaV appears to be more prevalent in the southern regions of Spain that are more involved in olive cultivation compared with the Italian regions.

5. Conclusions

To the best of our knowledge, this work represents the first OLYaV incidence study in the two most important European areas for olive production and reports for the first time the development of a LAMP methodology for the specific OLYaV detection that can be accomplished in 45 min. The real-time RT-LAMP methodology enabled substantial precision, sensitivity, and reproducibility for the OLYaV detection in infected olive trees. The real-time RT-LAMP developed is an alternative to the methods currently used for routine OLYaV detection and plant material certification programs, thus representing a potential contribution to improving virus diagnosis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9060702/s1, Table S1: Reaction mixtures, volumes, and protocols used for reverse transcription (RT), PCR, and RT-LAMP assays Table S2: Real-time RT-LAMP assay results for OLYaV detection of symptomatic and asymptomatic field samples collected from different Spanish provinces. Asymptomatic samples resulted positive are underlined in gray; Table S3: Real-time RT-LAMP assay results for OLYaV detection of symptomatic and asymptomatic field samples collected from different Italian provinces. Asymptomatic samples resulted positive are underlined in gray.

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