Plant Methods

METHODOLOGY

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Rapid real-time quantitative colorimetric LAMP methodology for field detection of *Verticillium dahliae* in crude olive-plant samples

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Abstract

Background *Verticilium dahliae* is the most important wilt pathogen of olive trees with a broad host range causing devastating diseases currently without any effective chemical control. Traditional detection methodologies are based on symptoms-observation or lab-detection using time consuming culturing or molecular techniques. Therefore, there is an increasing need for portable tools that can detect rapidly *V. dahliae* in the field.

Results In this work, we report the development of a novel method for the rapid, reliable and on-site detection of *V. dahliae* using a newly designed isothermal LAMP assay and crude extracts of olive wood. For the detection of the fungus, LAMP primers were designed targeting the internal transcribed spacer (ITS) region of the rRNA gene. The above assay was combined with a purpose-built prototype portable device which allowed real time quantitative colorimetric detection of *V. dahliae* in 35 min. The limit of detection of our assay was found to be 0.8 fg/μl reaction and the specificity 100% as indicated by zero cross-reactivity to common pathogens found in olive trees. Moreover, detection of *V. dahliae* in purified DNA gave a sensitivity of 100% (Ct < 30) and 80% (Ct > 30) while the detection of the fungus in unpurified crude wood extracts showed a sensitivity of 80% when multisampling was implemented. The superiority of the LAMP methodology regarding robustness and sensitivity was demonstrated when only LAMP was able to detect *V. dahliae* in crude samples from naturally infected trees with very low infection levels, while nested PCR and SYBR qPCR failed to detect the pathogen in an unpurified form.

Conclusions This study describes the development of a new real time LAMP assay, targeting the ITS region of the rRNA gene of *V. dahliae* in olive trees combined with a 3D-printed portable device for field testing using a tablet. The assay is characterized by high sensitivity and specificity as well as ability to operate using directly crude samples such as woody tissue or petioles. The reported methodology is setting the basis for the development of an on-site detection methodology for *V. dahliae* in olive trees, but also for other plant pathogens.

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Keywords *Verticillium* wilt, Loop mediated isothermal amplification, Molecular diagnostics, Field-based detection, Soilborne pathogens, Defoliating pathotype

Background

The development of diagnostic methods for detection of plant pathogens in the field is of high significance for the effective crop disease management. Verticillium wilt, caused by the soilborne vascular wilt fungus *Verticillium dahliae (V. dahliae)*, is considered to be the most important disease of olive trees worldwide, leading to devastating yield losses and even tree death [19, 29]. So far, no effective chemicals are available, as the pathogen is confined within the xylem vessels. Disease control is further hindered by the broad host range of the fungus (over 400 cultivated and weed species) and its long-term survival in the soil for up to 15 years by means of melanized resistant structures named 'microsclerotia' [46]. Management strategies have primarily focused on preventive measures such as the use of pathogen-free planting material, the use of tolerant/resistant cultivars and rootstocks and the early diagnosis through disease monitoring (Jimenez-Diaz et al. 2012). Despite its wide host range, *V. dahliae* displays genetic variability in the form of defoliating (D) and non-defoliating (ND) pathotypes, with the D-pathotype causing severe defoliation and increased wilting symptoms in specific hosts including olive [35].

Conventional time-consuming methods to identify plant-pathogens are based on symptom observation during field inspection, and/or transfer of samples to the laboratory to perform microbiological, biochemical, serological or pathogenicity tests. For the detection of culturable facultative fungal pathogens like *V. dahliae*, the pathogen is isolated in selective media then identified through microscopic observation [49]. In addition, more advanced molecular identification methods have been developed, with Polymerase Chain Reaction (PCR) being the gold-standard. Several PCR-based methods, including nested PCR, duplex nested PCR and real time PCR assays [17, 37–39, 47] have been applied for *V. dahliae* detection in olive tissues and soil, both before and after symptom development. The above PCR-based techniques display high sensitivity; in particularly, real time PCR is able not only to detect but also quantify the target pathogen and determine the colonization level of plant tissues by the pathogen. Nevertheless, commonly accepted drawbacks of this technology are the requirement of complex, expensive and lab-based equipment along with the fact that it is a laborious procedure with a high contamination-risk [4, 6]. Therefore, there is a growing need to develop portable diagnostic tools that can detect plant pathogens rapidly, in real-time and directly in the field from crude samples.

Recent advances in isothermal amplification have revolutionized the use of simpler, highly reliable and amenable to miniaturization techniques. Of the isothermal methods, the loop-mediated isothermal amplification (LAMP) technique, developed by Notomi et al. in 2000, employs 4 to 6 set of primers to carry out amplification at a constant temperature (~65 °C). To perform LAMP, *Bst*, a specific DNA polymerase capable of auto-cycling the target sequence, is required. LAMP's further advantages are the high amplification efficiency, ability to operate in crude samples since it is less affected by inhibitors and cost effectiveness due to the need of simple instrumentation [6]. For detection, LAMP is combined with fluorescence probes for lab-based quantitative testing or colorimetric dyes for qualitative eye observation in the field [16]. Detection through fluorescence probes can be quantitative but is restricted to a lab-environment; on the other hand, bare eye observation is simpler and does not require a trained user, however, it is qualitative and relies on the subjectivity of the user [51]. LAMP has been primarily applied for the detection of significant human pathogens, e.g. SARS-Cov-2 [45]; HIV [10] *Bordetella pertussis* [20], *Mycobacterium tuberculosis* [7], *Streptococcus pneumoniae* [50], *Klebsiella pneumoniae* [43], etc. For plant pathogens, LAMP assays have been developed for fungi (e.g., *Aspergillus nomius, A. parasiticus, A. flavus*, *Fomitiporia torreyae*, *Fusarium oxysporrum* f. sp., *lycopersici, Fulviformes umbrinellus*, *Ceratocystis platani* and *Alternaria solani*) [5, 14, 23, 32, 36]; oomycetes (e.g., *Pythium myriotylum*, *Phytophthora infestans* and *P. ramorum*) [15, 22],bacteria (*Xylella fastidiosa, Erwinia amylovora*) [1, 2, 8, 18], phytoplasmas [12, 21] and viruses/viroids (e.g., *ApNMV*, *ASPV*, *ASGV*, *ACLSV*, *ASSVd*, *TMV* and *TYLCV*) [11, 24–26, 31, 42, 52]. A common feature of the above methodologies is the use of a sample pretreatment purification step and mostly use of fluorescence probes for lab-based detection, with reported detection limits for plant pathogenic fungi and oomycetes in the range of 2 fg to 100.000 fg/μl reaction [5, 14, 23, 32]. Regarding *V. dahliae*, two studies employing LAMP have been reported [3, 40], both based on end-point naked eye observation with a detection limit of 50 and 500 fg per reaction of purified target DNA of *V. dahliae* D and ND

pathotypes, respectively [40]. Overall, the development of robust methods for sensitive detection for plant pathogens is lacking seriously behind similar reports for human pathogens at the point-of-care. This is despite the fact that the spread of plant-borne diseases and appearance of emerging ones call for urgent development and application of effective detection methodologies in the field.

In the current work, we developed a methodology for the rapid and cost-effective detection of *V. dahliae* in the field using both DNA extracted form plant tissue and directly crude samples. The new LAMP assay targeted the multi copy internal transcribed spacer (ITS) region of the pathogen improving the sensitivity of the method compared to a previous reported work; Moreover, the assay was combined with an innovative portable device performing real-time quantitative colorimetric LAMP (qcLAMP), giving a sample-to-answer analysis time of less than 40 min. The high specificity and sensitivity of the assay is demonstrated using *V. dahliae* gDNA and DNA from artificially and naturally infected olive plants, with a detection limit in the former case of 0.8 fg/μl. Given that the main objective of the project was to detect the target DNA in plant tissue without the need for its extraction and purification, we adapted the methodology accordingly. Therefore, the amplification and detection of the target DNA can be carried out directly in plant tissues from branches of olive trees. The real time colorimetric detection methodology provides quantitative or semi-quantitative results, and is also demonstrated to be adaptable to field-based detection when combined with the portable qcLAMP platform developed for this purpose.

Results

Portable device and methodology for performing quantitative colorimetric LAMP

The qcLAMP methodology employed in this work comprises a new optimized protocol including sample collection and pretreatment, amplification of DNA via LAMP and data reporting (Fig. 1A); the above is combined with a recently developed prototype device compatible with a qcLAMP assay (Fig. 1B). Regarding the amplification assay, LAMP was chosen for its high amplification efficiency at a constant temperature of 67 °C, combined with phenol red dye inside the reaction mix for colorimetric real time detection. The LAMP assay was optimized to be compatible with purified DNA obtained upon an

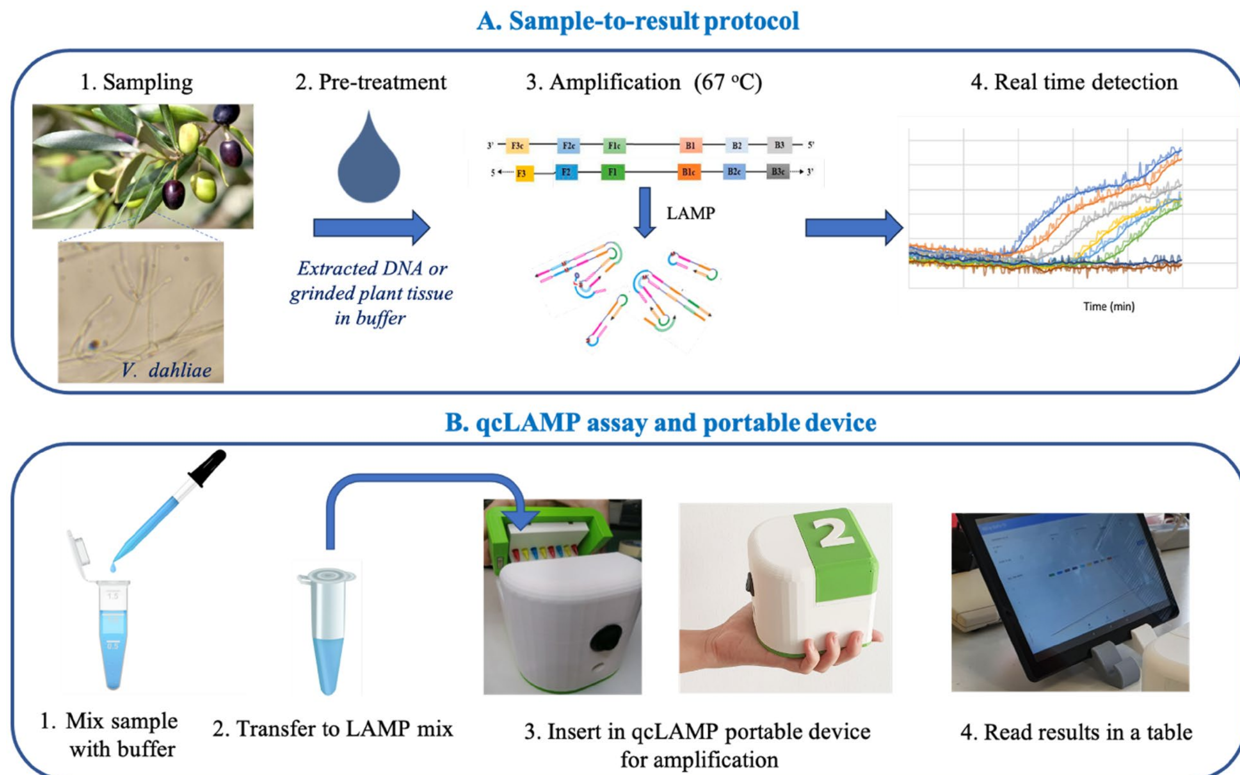


Fig. 1 Schematic representation of the (**A**) complete sample-to-result protocol and (**B**) qcLAMP assay and detection with a novel 3D-printed, purpose-built portable device

extraction step or DNA present in a crude plant tissue, both requiring sample pretreatement. For advanced field-based detection, a portable, lightweight, affordable and simple to operate device manufactured in the lab via 3D printing was employed (Fig. 1B); this device has already been demonstrated during the ultra-sensitive detection of human viruses (SARS-CoV-2 and influenza) and cancer mutations (BRAF) in saliva and tissue samples, respectively, during an extensive validation study [45]. Using a single temperature for LAMP amplification, instead of three in the case of PCR, simplified the device-construction which employed one instead of three resistors. Overall, the device consisted of mechanical parts including a 3D-printed case with a detachable top part where the 8 plastic tubes (eppendorfs) could be placed; the bottom part enclosed the electronic components, i.e., the main electronic board, a Raspberry Pi Zero, a mini camera and a temperature sensor connected to a PCB resistive micro-heater. For the real time monitoring of the amplification reaction, non-calibrated snapshots of the 8 tubes containing the LAMP mix were collected at predefined times by a camera placed opposite to the tubes. Upon the extraction of the red, green and blue (RGB) pixel values, a real time curve was displayed on the tablet. Real time graphs display on the x axis the time-course of the reaction and on the y axis the color index in pixels referring to the difference between the selected colors. A negative reaction produces a flat line parallel and close to the x axis while a positive one exhibits a change in the slope of the line, appearing at a specific time referred to as the time-to-positive (see step 4, Fig. 1A). The time-to-positive reflects the amount of the initial target: change of the slope at an early time-point is associated with a high concentration of the *V. dahliae* target while change at a later point indicates low amounts of the fungi in the initial sample. The ability to monitor the LAMP reaction in real time allows the performance of a quantitative colorimetric LAMP (qcLAMP) or semi-qcLAMP in a simple and rapid manner and with a device that can operate outside a lab-environment. More details on the manufacturing of the device and the software employed to collect and display data to a tablet can also be found in the reference of Papadakis et al., [45].

Design of qcLAMP assay for *V. dahliae – performance evaluation in the lab*

Genomic DNA Moradi et al. [40] designed a LAMP primer set targeting the RAPD-SCAR marker sequence for Spanish *V. dahliae* strains [40]. We tested these primers and, in addition, designed a new set targeting the ITS region of the *rRNA* gene. Figure 2A shows the qcLAMP amplification efficiency of the two sets of primers using genomic DNA extracted from pure cultures of representative isolates of *V. dahliae,* i.e., the non-defoliating reference strain SS4 (referred to as 25 V) from the USA (California) and a cotton defoliating isolate (63 V) from Greece. The real-time color change graph obtained with the portable cqLAMP illustrates efficient detection of the target-DNA when using the newly designed ITS primer set, with the strains 63 V and 25 V giving a time-to-positive response of 13.5 and 16.5 min, respectively. Zero amplification was observed in the case of the LAMP primers targeting the RAPD marker within 30 min of reaction. Following the selection of the primers targeting the ITS region, we tested the capability of qcLAMP to provide quantitative results. For this reason, we run several real-time qcLAMP reactions using, as staring material, serial dilutions (six tenfold dilutions) of the stock purified DNA. The highest template concentration (2 ng/reaction) showed a time-to-positive result at 18.2 min and the lowest (20 fg/reaction or 0.8 fg/μl) at 25.9 min (Fig. 2B), with the latter being the detection limit of *V. dahliae* purified gDNA. The good correlation ($R^2=0.99$) between the qcLAMP time-to-positive result and *V. dahliae* DNA amount confirms the quantitative nature of our experiments (Fig. 2C). Further comparative studies between LAMP, conventional PCR, nested PCR and SYBR qPCR showed that LAMP was more sensitive than the latter three techniques which gave a detection limit at 1 pg/μl (PCR), 100 fg/μl (nested PCR) and 10 fg/μl (SYBR qPCR) (Fig. S4). Last, the ITS LAMP primer assay was tested towards its specificity to *V. dahliae* by performing qcLAMP amplification in the presence of DNA from the non-target fungal pathogens *Stemphylium amaranthi*, *Fusarium lateritium, Colletotrichum boninense*, *F. solani*, *Alternaria sp*., *A. infectoria*, *Botryosphaeria dothidea* and *Colletotrichum gloesporioides.* No cross-reactivity was observed, as shown in Fig. 2D.

Purified DNA from naturally infected olive trees To validate further the assay, we used as starting material DNA extracted from both olive branches and petioles of naturally infected olive trees; the latter were obtained from commercial groves in two different locations in Greece, i.e., in the Fthiotida and Magnisia prefectures. As a first step, real time graphs were obtained from qcLAMP measurements with serial dilutions of extracted total DNA from a positive olive plant sample with a reported Ct value of 19.6 in real-time qPCR assays (Fig. 3A). The good correlation ($R^2=0.99$) observed between the fungal copies and qcLAMP time-to-positive result (Fig. 3B) once more verifies the ability of the newly developed method to extract quantitative information. Moreover, the reproducibility of the method was confirmed by testing 18 samples with a time-to-positive response of 25.3 ± 0.8 min (Fig. S1).

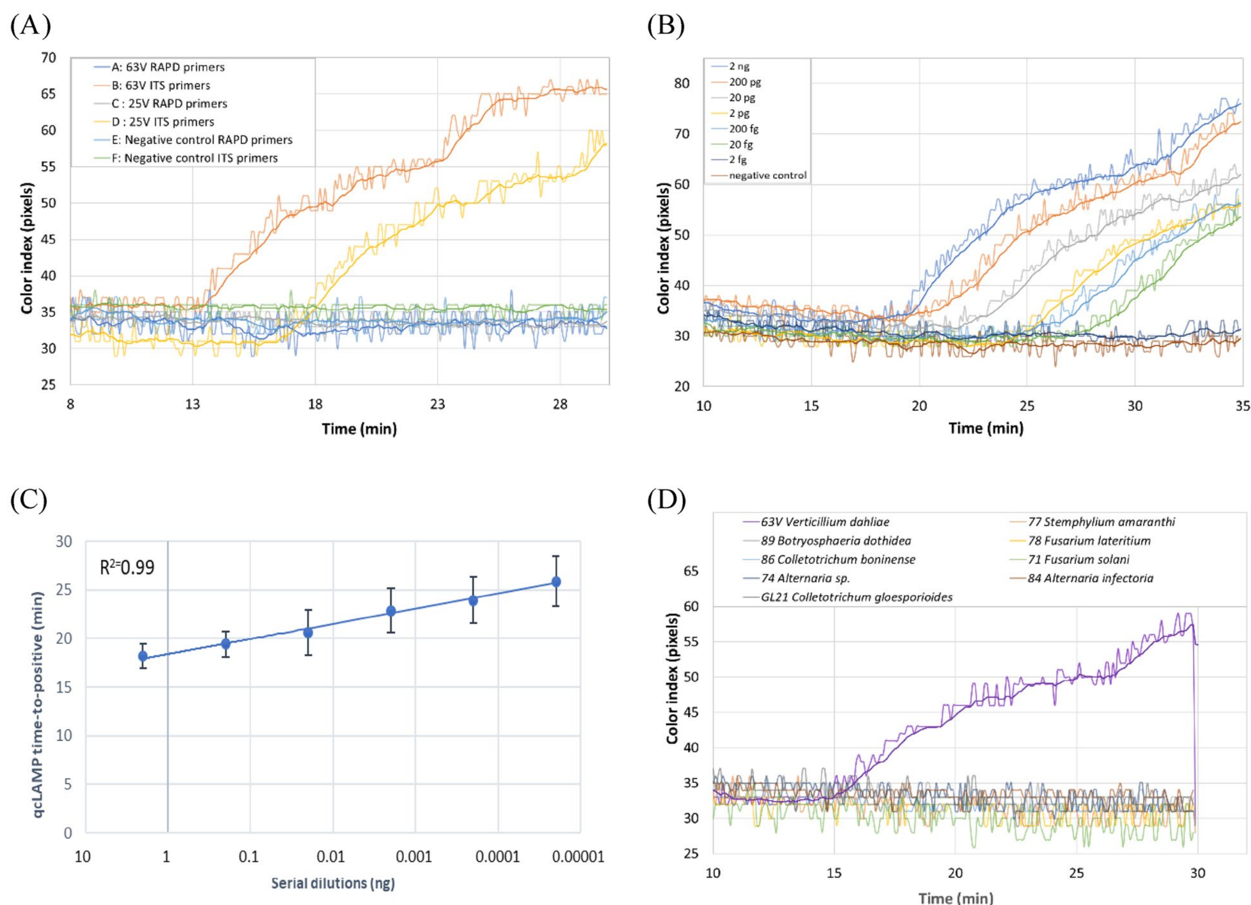


Fig. 2 **A** Real time colorimetric LAMP detection of 20 ng from two pathotypes of *V. dahliae* (63 V: defoliating isolate, 25 V: non-defoliating isolate), using the newly ITS-region designed primers compared to the RAPD-published primer set [40]. All experiments were performed in triplicates; **B** Real time colorimetric LAMP using as template serial dilutions of DNA extracted from *V. dahliae* culture ranging from 2 ng to 20 fg per reaction **C** Calibration curve derived from six tenfold dilutions starting from 2 ng of gDNA; the x axis depicts the amount of DNA in the reaction and the y axis the time-to-positive derived from the real time graph shown in **B**. Error bars represent deviation of at least triplicates; **D** Specificity of the real time colorimetric LAMP assay. At 20 ng fungal DNA only *V. dahliae* was detected at 16 min. No detection was observed when the same amount of DNA from other pathogens was used

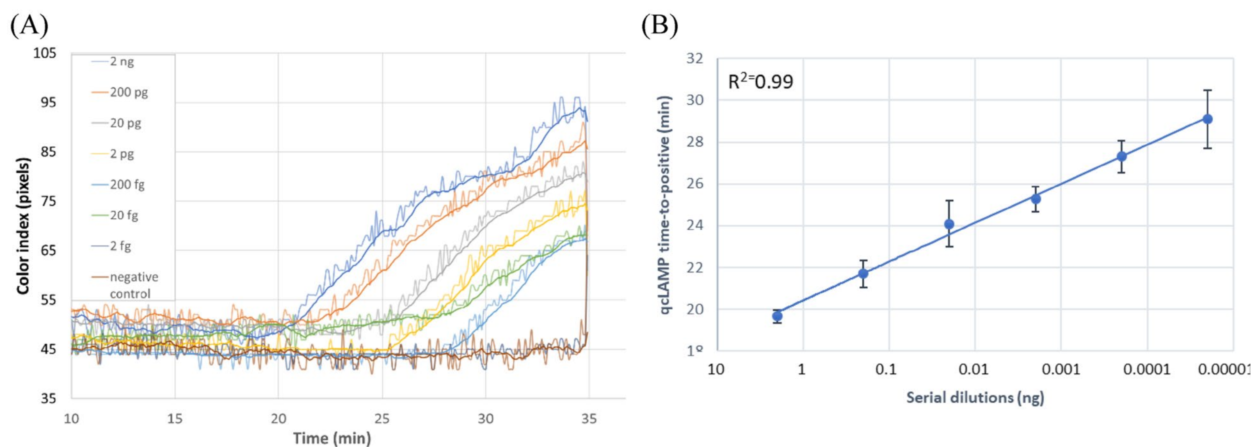


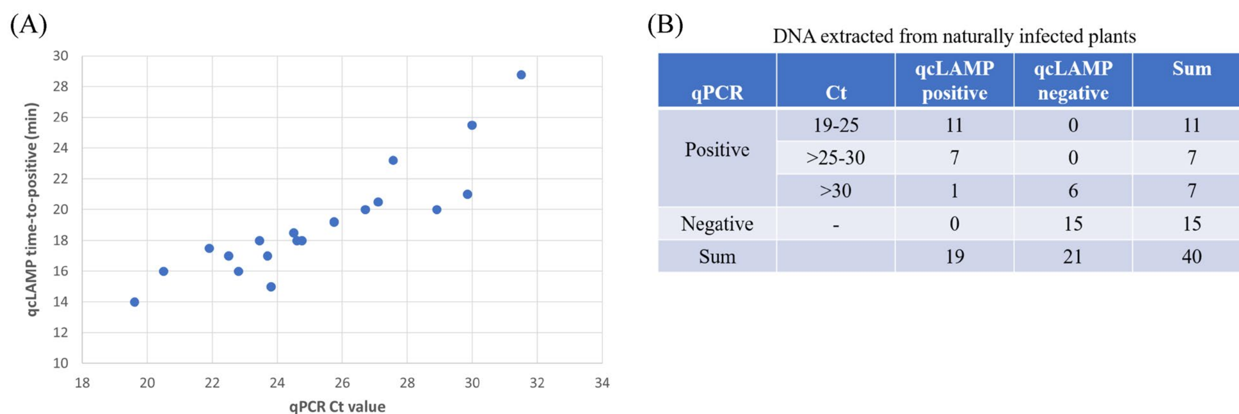
Fig. 3 **A** Real time colorimetric LAMP using as template serial dilutions of DNA extracted from an olive tree naturally infected with *V. dahliae* of a reported Ct value at 19.6 in real time PCR assays; **B** Correlation ($R^2 = 0.99$) between the qcLAMP time-to-positive results and six tenfold serial dilutions of infected plant using 20 ng of extracted plant DNA as template

Moreover, to evaluate the efficiency of the qcLAMP assay, we compared the method with the commonly used lab-based techniques. Overall, 20 branch samples were randomly collected from fields in Fthiotida and Magnisia, with *V. dahliae*-infected olive trees; specifically, symptomless branches (n = 20) and their respective leaf petioles (n = 20) were collected and analyzed by conventional PCR and qPCR assays in parallel with qcLAMP after DNA isolation. Prior to PCR, qPCR and qcLAMP analyses, fungal isolation on acidified potato dextrose agar (PDA) was performed from all branches collected, verifying the viability of *V. dahliae* endophytically. Both PCR methods were selected due to their universal application as the gold-standard end-point qualitative (PCR) and real-time quantitative (qPCR) methods, whereas fungal isolation on selective media is commonly used in plant pathology and mycology laboratories in routine diagnostic tests for vascular wilts. Furthermore, the qPCR was used to define the amount of target-load in the collected samples and investigate the quantitative nature of the qcLAMP. Of the 40 samples tested, 26 of them were positive and 14 negative according to qPCR results (Ct > 38) (Table 1). Of the 26 positive samples, qcLAMP accurately identified 19 of them as positive (sensitivity of 73%), while 7 gave a false negative reading (petiole of sample 2.3 and 9.2, petiole and branch of sample 3.2 and branch of samples 3.4, 4.1, 7.5–Table 1).

Closer look at the measured Ct values reveals that when considering the various cut off values, a sensitivity of 100% is calculated for samples within the range of 19 < Ct < 30 (18 samples) while the sensitivity drops significantly (14%) for samples with a high Ct value (> 30). The time-to-positive result observed for the 19 positive samples varied between 14.0 min for the lowest Ct-value sample (19.6) and 28.8 min for the highest Ct-value (31.5). Figure 4A shows the correlation between qPCR Ct values and qcLAMP time-to-positive results. In addition, all qPCR-confirmed negative to *V. dahliae* samples were also identified as negative with the qcLAMP method (Fig. 4B). It is noted that PCR measurements performed for all the above samples gave the same results to those obtained with our qcLAMP method (Table 1). However, PCR is a time consuming (1.8 h/assay) lab-based technique that requires expensive instrumentation as opposed to the hand-held portable qcLAMP device and 30 min relevant assay-time. In addition, looking at the relative *V. dahliae* DNA quantity in the samples tested, the detection limit of the qcLAMP was assessed at 0.4–0.15 (2^{-dCt}) for

Table 1 Detection of *Verticillium dahliae* in DNA samples extracted from olive tissues (branches and petioles) collected from naturally infested olive orchards, using our newly developed colorimetric LAMP assay along with 2 conventional methods, PCR and qPCR. Symbols '+' and '-' represent successful and unsuccessful pathogen detection, respectively; qPCR-confirmed negative samples are indicated in red

Olive orchard	Sampled tree cultivar	Sample code	PCR (branch/petiole)	qPCR-Ct value (branch/petiole)	qcLAMP (branch/petiole)	qcLAMP time-to-positive (min)
# 1	Kalamon	1.1	–/–	38.2/38.8	–	–
	Kalamon	1.3	+/+	24.5/26.7	+/+	18.5/20.0
	Kalamon	1.4	–/–	40.0/40.0	–	–
	Kalamon	1.6	–/–	40.0/40.0	–	–
	Kalamon	1.8	+/+	24.7/25.7	+/+	18.0/19.2
# 2	Megaritiki	2.1	+/+	20.5/23.4	+/+	16.0/18.0
	Megaritiki	2.2	±	23.7/40.0	±	17.0/-
	Megaritiki	2.3	-/+	31.5/31.8	±	28.8/-
	Megaritiki	2.4	+/+	19.6/30.0	+/+	14.0/25.5
	Megaritiki	2.7	+/+	21.9/27.6	+/+	17.5/23.2
# 3	Amfissis	3.1	+/+	22.8/27.1	+/+	16.0/20.5
	Amfissis	3.2	–/–	31.7/30.5	–/–	–/–
	Amfissis	3.3	–/–	40.0/40.0	–	–
	Amfissis	3.4	–/–	33.3/40.0	–/–	–/–
	Amfissis	3.5	+/+	23.8/28.9	+/+	15.0/20.0
# 4	Chalkidikis	4.1	–/–	35.7/40.0	–/–	–/–
	Chalkidikis	4.2	±	24.8/36.9	±	18.0/-
# 5	Amfissis	7.3	+/+	22.6/29.9	+/+	17.0/21.0
	Amfissis	7.5	–/–	35.2/39.2	–/–	–/–
# 6	Megaritiki	8.1	–/–	40.0/40.0	–	–



DNA extracted from naturally infected plants

qPCR	Ct	qcLAMP positive	qcLAMP negative	Sum
Positive	19-25	11	0	11
	>25-30	7	0	7
	>30	1	6	7
Negative	-	0	15	15
Sum		19	21	40

Fig. 4 **A** Scatter plot of the Ct values (ranging from 19.6 to ~ 31.5) for 19 positive samples of DNA extracted from naturally infected trees versus the qcLAMP time-to-positive (ranging from 14.0 to 28.8 min) using 20 ng extracted plant DNA as template; (**B**) comparison of qcLAMP to qPCR for various Ct cut off values

sample 2.3 (branch—petiole) using SYBR qPCR. Finally, the isolation frequency of the pathogen on media correlated positively with qcLAMP, as it was isolated from all branch samples that were tested positive for qcLAMP with the exception of sample 3.2 (Table S1).

qcLAMP assay for *V. dahliae* testing in crude samples

For developing a field-based test, the capability to directly detect *V. dahliae* without nucleic acid extraction, was examined. For this reason, several fast nucleic acid extraction methods were evaluated, initially in the lab: the use of grinded wood tissue with sandpaper had contamination problems, probably related to dispersal of contaminated dust in tools and media. Moreover, when we used a needle to transfer a plant sample from vascular tissue of branches or petioles in the LAMP reaction, this resulted in false positive reactions due to pH change. The finally selected isolation protocol included the homogenization of wood tissue-slices in TE buffer or water followed by transfer of 1 μl into the LAMP reactions containing phenol red indicator and placed in the qcLAMP prototype device for 35 min. Regarding the type of plant tissue used, we focused on the detection of *V. dahliae* in woody tissue from branches, because higher biomass of the pathogen was assessed in branches compared to petioles (Table S1). The latter was confirmed by observing zero amplification of the olive actin gene in SYBR reactions (data not shown). As a first evaluation, we showed that the above simple sample-pretreatment method is able to detect *V. dahliae* with qcLAMP in leaves (petioles) of artificially infected olive plants (10-month-old) and naturally infected trees and in wood of artificially infected olive in 30.0, 27.0 and 28.0 min, respectively, and discriminate between healthy plant tissue, where no detection was observed (Fig. S2).

In a follow up step, the detection of *V. dahliae* was demonstrated using as starting material crude plant extracts derived from wood-samples from 20 naturally infected olive trees from the Kalamon cultivar (5 trees in a field situated in Attica region) and Amfissis cultivar (15 trees in a field situated in Amfissa region) (Greece). Prior to qcLAMP testing, all samples were confirmed to be infected to *V. dahliae* by obtaining a positive SYBR qPCR response using extracted DNA (Table 2). Regarding the number of samples tested per tree, in the case of trees from the Kalamon cultivar, we performed multi-sampling (2–8 pieces) from the same tree while in the case of trees from the Amfissis cultivar, each tree was tested once, with the exception of one tree (Amfissa 1) that was tested twice. Based on Table 2, qcLAMP detected successfully 70.0% of the total positive trees from the two orchards; looking more closely in the results, we observe that we detected 80% of the Attica trees and 67% of the Amfissa trees, starting in both cases from water-homogenized crude samples. Interestingly, when we compared the PCR-obtained Ct values of the tested samples, we noticed that the higher detection capability (80%) of the Kalamon trees was corresponding to low infection levels ($30 < Ct < 33$), while the lower detection capability (67%) of the Amfissa trees included both low (6 samples with a $Ct > 30$) and medium (9 samples with a $Ct < 30$) pathogen-loads. As expected, SYBR qPCR or nested PCR analysis was not able to detect *V. dahliae* in unpurified homogenized samples (Table S2).

For comparison with the qcLAMP method, we took representative samples from Table 2 and analyzed them using the same LAMP assay in parallel with end-point naked eye colorimetric detection and gel electrophoresis (Fig. S3). Obvious advantages of the qcLAMP methodology are the real time (semi-) quantitative nature of the

Table 2 Colorimetric LAMP assay reaction time for detection of *Veticillium dahliae* on crude branch wood of olive trees collected from naturally infested olive orchards. Infection of olive trees was first determined with qPCR on purified DNA of olive stems, as shown by the Cycle threshold (Ct) of sampled trees

Sample info			Crude sample	Purified DNA
Olive orchard	Tree cultivar	Tree code	qcLAMP (min)	SYBR qPCR Ct
# 1	Kalamon	A1_AUA	undetected	32.8
	Kalamon	A2_AUA	25,5[a]	31.6
	Kalamon	A6_AUA	22,75[b]	31.6
	Kalamon	B6_AUA	24	30.7
	Kalamon	G8_AUA	22	31.2
# 2	Amfissis	Amfissa 1	30	23.9
	Amfissis	Amfissa 3	undetected	26.3
	Amfissis	Amfissa 4	20	23.8
	Amfissis	Amfissa 5	24	25.1
	Amfissis	Amfissa 6	30	31.8
	Amfissis	Amfissa 7	28	26.1
	Amfissis	Amfissa 8	undetected	25.1
	Amfissis	Amfissa 9	25	18.7
	Amfissis	Amfissa 10	undetected	25.1
	Amfissis	Amfissa 11	24	27.8
	Amfissis	Amfissa 15	30	31.8
	Amfissis	Amfissa 16	undetected	31.0
	Amfissis	Amfissa 18	25	31.2
	Amfissis	Amfissa 19	undetected	30.2
	Amfissis	Amfissa 20	31	30.1

detection of the target pathogen compared to both the naked eye observation and gel-electrophoresis and the faster response time and field-based detection capability compared to the gel electrophoresis.

Discussion

The aim of this study was to develop a *V. dahliae* detection assay for olive trees and for field-based diagnostics using the isothermal LAMP method and a portable simple-to-use device. Classical *V. dahliae* detection requires fungal isolation methods followed by microscopic recognition or PCR-based molecular techniques, both lab-based and time-consuming techniques. Early detection of *V. dahliae in planta* is of paramount importance for testing plant material on-site in nurseries and orchards in order to prevent the disease spread and contribute to production of pathogen-free plant material. However, two are the challenges to achieve the above: first, to design a sensitive assay that is compatible with crude plant extracts and, second, to achieve the required specificity and sensitivity without the need for a multi-step protocol and use of complex, sophisticated and expensive equipment. Regarding the assay, unlike the previously published LAMP test targeting the RAPD-SCAR marker sequence [40], the assay reported here designed to target the ITS region was capable of detecting *V. dahliae* defoliating and non-defoliating pathotype strains within 35 min of the qcLAMP reaction using purified DNA. The higher sensitivity of the new assay is possibly attributed to the new ITS region targets, as up to 125 copies have been reported in ascomycetes [28] compared to the single copy RAPD –SCAR target. Indeed, the high copy number of the nuclear ribosomal gene clusters in the genome targeted by the ITS primers displays high amplification sensitivity facilitating also *in planta* detection of fungal pathogens [28, 35]. LAMP assays for fungal pathogen detection using the ITS region as target have already been developed for *Rhizoctonia, Macrophomina* and *Ascochyta* [9, 30]. Moreover, the non-target pathogens *Stemphylium amaranthi*, *Fusarium lateritium, Colletotrichum boninense*, *F. solani*, *Alternaria* sp., *A. infectoria*, *Botryosphaeria dothidea* and *Colletotrichum gloesporioides* frequently present in olive branches and leaves [33] were not amplified proving the specificity of the present assay (Fig. 2d). This is an additional advantage of using the ITS region, since it displays high intergenic and low intragenic variability (White et al. 1990), and, thus, provides specificity on target organisms. Overall, the demonstrated detection limit of 20 fg/reaction (Fig. 3B) is among the lowest reported for plant pathogen detection using LAMP [5, 14, 15, 22, 23, 32]. In addition, our newly developed LAMP protocol was proven more reliable and faster in detecting *V. dahliae* strains of different pathotypes within 35 min, compared to previous LAMP tools [40].

One of the main advantages of the current study is the ability to perform rapid on-site detection of the pathogen with our newly developed portable qcLAMP platform. The apparatus is easy to carry, due to its low weight, small size and ability to connect to a tablet to view the results [45]. Currently, most of the LAMP assays developed for plant pathogens are based on laboratory-restricted equipment for the isothermal reactions, such as water baths, heat blocks, real-time turbidity meters or real-time qPCR instruments [5, 9, 14, 15, 22, 30, 32]. In few studies the portable fluorometer Genie ® II (OptiGene Limited) was used, which is a bench-top equipment of higher weight, more expensive and with no ability for connection to a mobile device [1]. The handheld device used herein was combined with a simple sample pre-treatment methodology starting form crude wood-slices homogenized in water without the need for DNA extraction. This is a significant advancement for field-based detection compared to previous studies which required time-consuming DNA extraction protocols [3, 22, 30, 40]. The superiority of our

method for field-based application was shown when only LAMP was able to detect *V. dahliae* in naturally infected trees, even with very low target fungal biomass /infection levels (relative *V. dahliae* DNA quantity lower than 1, as evaluated by SYBR qPCR, see Table S2). In addition, based on results shown in Table 1, it seems that the detection capability of the method depends on the No of samples tested per tree. The distribution of *V. dahliae* within olive plants varies according to plant tissue and sampling time [35]; however, multiple sampling seems to improve significantly the sensitivity of the assay as shown by the high detection capability from the Kalamon cultivar infected trees (Table 2). On the contrary, both highly infected (Amfissa 3, 8 and 10) and plants with low fungal biomass / low infection load (Amfissa 6 and 19) were not detected when a single sample was collected and tested per tree (Tables 2 and S2).

Conclusions

To conclude, we have developed and demonstrated a field-deployable method for real-time detection of *V. dahliae* in olive trees based on a quantitative colorimetric LAMP protocol integrated with a portable device. Starting from crude olive tree wood and with minimal sample preparation, we demonstrated that our method can detect trees infected with *V. dahliae* with high sensitivity and in just 35 min, without the need for technical expertise and specialized equipment. The method is highly simplified incorporating just one pre-amplification step before transfer to the qcLAMP device; the ability to obtain real-time results on a tablet combined with the capability for connectivity and wireless data transfer to a decision-making center provides promising alternative to currently used laboratory-based diagnostic methods. To the best of our knowledge, this is the first report on the detection of a plant pathogenic fungus with LAMP coupled with a portable prototype real-time colorimetric LAMP platform without the need to extract purified DNA from the samples, as evaluated in the important wilt pathogen *V. dahliae* in olive, setting the grounds for development of devices for on-site detection of plant pathogens.

Materials and methods

Fungal isolates and DNA extraction

In order to evaluate the LAMP primers, DNA was extracted from culture of representative isolates of *V. dahliae*. Specifically, the reference non defoliating cotton strain from USA SS4 (designated as 25 V), a Greek cotton defoliating isolate (63 V) and a Greek non defoliating race 1 tomato isolate (70 V) [48]were used. The fungal isolates were reactivated from 25% glycerol stocks stored in the deep freezer (− 80 C), in Potato Dextrose Agar (PDA) and subsequently grown for 7 days. Afterwards they were transferred to liquid Sucrose Sodium Nitrate medium (SSN) and incubated for 7 days by rotation at 150 rpm; mycelia were collected by filtration and lyophilized. For DNA extraction, the standard phenol based isolation protocol [13] (Leach et al. 1986) was applied.

LAMP primer design

The new LAMP primers were designed targeting the ITS region, based on the differences between *V. dahliae* and other non-target fungi (Figure S6). PrimerExplorer5 was used to design a set of six LAMP primers, using the default parameters and the proper sequence of the region. The sequences of the exported primers are shown:

F3: 5′ CTTTGAACGCACATGGCG 3′

B3: 5′ GGGTTTAGAGGCAAGCGC 3′

FIP: 5′ CGTAGATCCCCAACACCGGGTTCCAGTATCCTGGGAGGC 3′

BIP: 5′ CCTTAAAAGCAGTGGCGGACCCACTCCGATGCGAGCTGTA 3′

LF: 5′ GGGCTCGAGGGTTGAAAC 3′

LB: 5′ GCGTGGCCCTTCCTTG 3′

LAMP assay

The primers were resuspended and a 10×concentrated mix was prepared which contained: 2 μM F3, 2 μM B3, 18 μM FIP, 18 μM BIP, 6 μM LF, 6 μM LB. Each LAMP took place in a 25 μl reaction mix containing: 12.5 μl Warmstart colorimetric LAMP kit (NEB, Ipswich, Massachusetts, USA), 2.5 μl 10×primer mix, 1 μl DNA template, 9 μl dH_20. Warmstart colorimetric LAMP kit contains: the key enzyme of LAMP, *Bst* DNA polymerase; all the necessary solutions (dNTPs, MgSO4) for the reaction; and the pH indicator phenol red in a low-buffer reaction solution that changes color from pink to yellow upon LAMP amplification (due to the release of a proton per nucleotide incorporated). The reactions were performed at 65 °C for 30 min in a lab prototype real-time colorimetric LAMP device (IRIS). In each run, 20 ng / reaction fungal DNA were included as positive control. According to samples that were analyzed, water (non-template control), DNA from young (10-month-old) olive plants or crude extracts of olive wood slices from adult olive trees with absence of *V. dahliae* infection were included as negative controls to exclude contamination of reagents or non-specific amplification of LAMP primers.

Assessment of sensitivity and specificity of the LAMP assay

Initially, the new ITS LAMP assay was compared to the published RAPD LAMP primers [40]. Three LAMP reactions with the RAPD primer set and 3 LAMP reactions with new ITS primer set were performed. In both cases, 20 ng of extracted DNA from the defoliating strain 63 V,

20 ng from the non-defoliating strain 25 V and 1 μl dH_2O (negative control) were added as template.

Sensitivity of the new improved ITS LAMP primer set was assessed by qcLAMP reactions using as starting material serial dilutions of the stock solution of DNA extracted from the Greek race 1 tomato strain 70 V with non-defoliating genetic profile. In order to check the specificity of the primers towards *V. dahliae* DNA amplification, the assay was tested using DNA from the non-target fungi *Stemphylium amaranthi*, *Fusarium lateritium*, *Colletotrichum boninense*, *F. solani*, *Alternaria* sp., *A. infectoria*, *Botryosphaeria dothidea* and *Colletotrichum gloesporioides* at a concentration of 20 ng DNA per 25 μl reaction.

Collection of olive plant samples

In May 2019, diseased and symptomless branches from naturally infested olive trees (20 samples) were collected. In particular, olive orchards of the cultivars Amfissis (7), Chalkidikis (2), Kalamon (5) and Megaritiki (6) with severe Verticillium wilt symptoms, located in Fthiotida and Magnisia prefectures, were surveyed and branches from diseased and visibly healthy trees (one branch per tree) were collected. All these orchards were established in land previously planted with cotton and are adjacent to cotton fields with severe incidence of Verticillium wilt. These samples were used in our studies with extracted DNA.

To test LAMP reactions directly on crude samples, olive trees naturally infected with *V. dahliae* and healthy control trees were sampled in 2022 from two fields located in Attica and Amfissa. In April 2022, 2 branches from 5 naturally infected olive trees of the Kalamon variety in the Attica region were gathered. Additionally, in the period of May–June 2022, 16 branches from 16 trees from the Amfissis cultivar were sampled in the Amfissa region.

Verticillium dahliae isolation on acidified PDA medium

To isolate the fungus, branches were surface-sterilized by spraying with 95% ethyl alcohol and passing them quickly through a flame, thrice. Prior to isolation, leaves were removed and leaf petioles from each branch were collected and stored at -20 °C. For each branch, 6 to 10 xylem chips (approximately 4×2×1 mm in size) were aseptically removed and placed onto acidified potato dextrose agar (PDA) in Petri dishes after the removal of the phloem. Plates were incubated at 24 °C in the dark for 3 weeks and the emerging fungal colonies that grew out of tissue excisions were examined under a light microscope and identified as *V. dahliae* based on their morphological characteristics [46]. Pathogen isolation ratio was expressed as the frequency of positive *V. dahliae* isolation of each branch. The wood tissues from the remaining branch were cut to 2–3 mm long pieces and stored at − 20 °C.

DNA extraction from plant tissues

Plant tissues (branches and petioles) stored at − 20 °C were freeze-dried and ground to a fine powder by using an autoclaved mortar and pestle, in the presence of liquid nitrogen. Total DNA was isolated according to the Cetyltetramethyl ammonium bromide (CTAB) method [41] with slight modifications. Wood powder (100 mg) was transferred in a 1.5 ml Eppendorf tube and 500 μl of 2X CTAB extraction buffer (100 mM Tris–HCl, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 0.5% v/v β-mecraproethanol) were added and homogenized. The samples were incubated at 65 °C for 45 min with periodical vortexing and centrifuged at 10.000 rpm for 10 min. The supernatant (~250 μl) was transferred to new tubes and equal amounts (~250 μl) of phenol:chloroform:isoamyl alcohol (25:24:1) were added and mixed by vortexing. Samples were centrifuged at 13.000 rpm for 15 min. The aqueous phase (~200 μl) was transferred into a new tube where an equal amount of chilled isopropanol was added, followed by quick and gentle inversion, and final incubation at − 20 °C overnight. The DNA pellet was precipitated at 13.000 rpm for 20 min, washed with 500 μl of 70% ethanol and precipitated at 13.000 rpm for 5 min. The DNA pellet was then suspended in 40 μl of Tris–HCL (10 mM, pH=8). Then, 2 μl RNAse A (5 mg ml^{-1}) were added followed by incubation at 50 °C for 15 min. Purity and quantity of DNA were determined using a Q5000 UV–Vis Spectrophotometer (Quawell, San Jose, CA, USA). The DNA of each isolate was adjusted to a concentration of 20 ng $μl^{-1}$ and stored at − 20 °C until use.

PCR and real-time quantitative PCR (qPCR) assays

Real time SYBR qPCR assays were applied to determine the detection limit of *V. dahliae* on serial dilutions of extracted DNA from a concentration of 20 ng / μl from each isolate as assessed by measurement on a specialized photometer (nanodrop). qPCR reactions were performed with the primers Vd-F (5′-CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3′) and Vd-R (5′-CGCCTGCGGGACTCCGATGCGAGCTGTAAC-3′), designed in the ITS region (with submission code to the genbank Z29511 database) of *V. dahliae* [44] in a thermocycler of Applied Biosystems, step one plus (Waltham, Massachusetts, USA). The same DNA dilutions were used as template to determine the LAMP limit of detection.

Furthermore, PCR and real-time quantitative PCR (qPCR) assays were conducted for detection and quantification of *V. dahliae* DNA in olive tissues according

to Markakis and co-workers (2009, 2010) [34, 35]. For conventional PCR assays, the *V. dahliae* ITS region was amplified with primers Vd-F / Vd-R [44]. All PCR assays were carried out in an FG-TC01 FastGene® Gradient thermocycler (NIPPON Genetics EUROPE), by the use of BK 1003 KAPA Taq PCR kit (KAPABIOSYSTEMS, Wilmington, Massachusetts, USA). PCR performance included an initial denaturation at 95 °C for 3 min; followed by 35 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C, and 1 min of extension at 72 °C; and a final extension step at 72 °C for 10 min. In real-time PCR assays, the relative DNA quantity of *V. dahliae* was determined by using the $2^{-\Delta\Delta CT}$ method [27]. Likewise, the primer pair Vd-F/Vd-R was used to amplify *V. dahliae* DNA, whereas the olive actin gene was used as an internal standard to normalize small differences in total DNA template amounts. The level of *actin* was determined using the primer pair OeACT-F 5′-ATCCTCACAGAGCGTGG -3′ and OeACT-R 5′- CGA TCATTGAAGGCTGG -3′ [34, 35]. The real time qPCR reactions were performed in duplicate and the absence of nonspecific products and primer dimers was confirmed by the analysis of melting curves. The qPCR amplifications were performed by using the PowerUp™ SYBR® Green Master Mix (ThermoFisher, Waltham, USA) in a QuantStudio 3 Real-Time PCR System (ThermoFisher, Waltham, USA).

For quantification of *V. dahliae* DNA in tissues of naturally infected olive trees that were used for LAMP detection in crude samples, a similar SYBR qPCR assay was applied with KAPA SYBR Fast Master Mix (2x) ABI Prism kit in StepOnePlus™ Real-Time PCR System of Applied Biosystems. For conventional PCR, primers Vd-F/ Vd R were used and abovementioned PCR conditions were applied. For nested PCR, a PCR reaction with universal primer ITS5/ITS4 was performed in a first round using crude extracts as a template. PCR conditions included an initial denaturation at 95 °C for 3 min; followed by 35 cycles of 1 min of denaturation at 94 °C, 1 min sec of annealing at 55 °C, and 1 min of extension at 72 °C; and a final extension step at 72 °C for 10 min. In the second round, 1 μl out of 25 of the first reaction was used as template for PCR with the *V. dahliae* specific primers Vd-F/ Vd R.

Direct *V. dahliae* detection in olive trees

For the direct detection of *V. dahliae* in olive trees, olive branches from infected and healthy trees were sampled. The initial optimized sample preparation procedure for LAMP included grinding of branch wood with sandpaper. About 10 mg of grinded wood tissue or 2–3 leaf petioles were suspended in 50 μl of Tris–EDTA solution (TE buffer) with pellet pestles. The samples were kept at room temperature for 5 min. Then centrifugation of samples for 10 min at 14.000 g was applied and afterwards 1 μl sample was diluted to 19 μl dH_2O. Finally, 1 μl diluted sample was introduced into the LAMP mixture with phenol red. To further simplify the sampling protocol, the centrifugation step was replaced by filtration of stem-water solution. 1 μl of the filtered solution was then added to the reaction mixture. When the protocol was evaluated on naturally infected trees of the Kalamon and Amfissis cultivars located in Attica and Amfissa regions, respectively, the extraction protocol was further simplified by squeezing with a sterilized pestle soaked-wood slices (80–200 μl ddH2O), followed by a 5 times dilution in water (without centrifugation) and use of 1 μl in the LAMP reaction.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-024-01251-x.

Additional file 1.

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Author contributions

M.M. carried out part of the experimental work and contributed to the acquisition of the data A.P. carried out part of the experimental work and contributed to the acquisition of the data G.P. carried out part of the experimental work and contributed to the acquisition of the data G.P. (papad.) designed / manufactured the new measuring platform A.K.P. designed /manufactured the new measuring platform E.J.P. contribution to the concept of the work A.K.T. contributed to the design and implementation of the experimental work, analysis of the results and drafting of the paper E.A.M. contributed to design and implementation of the experimental work, analysis of the results and drafting of the paper E.G. contributed to the concept, design and implementation of the experimental work, analysis of the results and writing and revising of the manuscript.

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Availability of data and materials

All data generated in this study are included in the publication. All materials are available through the corresponding authors.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

Dr G. Papadakis and Prof E. Gizeli are co-founders of BIOPIX DNA TECHNOLOGY, a spin-off of IMBB-FORTH that commercializes the prototype device shown in this work. The other authors declare no competing interest.

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