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ORIGINAL ARTICLE

A novel and rapid loop-mediated isothermal amplification assay for the specific detection of *Verticillium dahliae*

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Keywords
loop-mediated isothermal amplification, nested-PCR, olive, RAPD marker, soilborne, *Verticillium dahliae*.

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Abstract

Aims: In this study, a loop-mediated isothermal amplification (LAMP) assay has been developed and evaluated for the rapid and sensitive detection of *Verticillium dahliae* Kleb., the causal agent of vascular wilts in many economically important crops.

Methods and Results: LAMP primers were designed based on a previously described RAPD marker, and the LAMP assay was applied for direct detection of *V. dahliae* grown on medium and from soil samples without DNA purification steps (direct-LAMP). Thirty-two agricultural soil samples from various olive orchards were collected, and the presence of pathogen was detected by LAMP, direct-LAMP and nested-PCR methods. The LAMP methodology could successfully detect *V. dahliae* with high specificity, and cross-reaction was not observed with different pathogenic and nonpathogenic fungi and bacteria. The LAMP assay was capable of detecting a minimum of 500 and 50 fg of purified target DNA per reaction of *V. dahliae* ND and D pathotypes, respectively. In contrast, nested-PCR could only detect 5 pg reaction^{-1} for both pathotypes. In artificially infested soil samples, the LAMP method detected 5 microsclerotia per gram of soil. Conversely, nested-PCR assay detected 50 microsclerotia g^{-1} soil. The detection ratios of LAMP and direct-LAMP protocols were better (26 and 24 positive samples out of 32 agricultural soils analysed, respectively) than that obtained for nested-PCR method (22 positive results). Moreover, direct-LAMP yielded positive detection of *V. dahliae* in agricultural soil samples within 60–80 min.

Conclusions: The newly developed LAMP method was proved to be an effective, simple and rapid method to detect *V. dahliae* without the need for either expensive equipment or DNA purification.

Significance and Impact of Study: This technique can be considered as an excellent standard alternative to plating and nested-PCR assays for the early, sensitive and low-cost detection of *V. dahliae* as well as other soilborne pathogens in the field.

Introduction

Verticillium wilts are devastating diseases caused by the soilborne fungus *Verticillium dahliae* Kleb. This pathogen has a worldwide distribution and substantially reduces crop production in a wide range of many economically important crops including woody (e.g., olive, cocoa, pistachio, stone fruits, avocado), herbaceous (e.g., cotton, tobacco, potato, artichoke) and ornamental plants (Pegg and Brady 2002; Klosterman *et al.* 2009). Verticillium wilt is one of the most important biotic constraints for olive (*Olea europaea* L.) cultivation in Iran, leading to significant yield losses and death of the trees (Sanei and Razavi 2012). Pathogen control is difficult and requires expensive

and complicated methods, due to, among other factors, the ability of the fungus to produce melanized structures (microsclerotia) able to endure for a long time in soils (Pegg and Brady 2002). Moreover, disease symptoms may develop a few years after planting seedlings in infested soil (Pegg and Brady 2002; Klosterman *et al.* 2009).

So far, there is no effective control measure that, when individually implemented, is able to prevent the spread of the disease to newly established olive orchards. Therefore, for successful prediction and efficient control of the disease, early, reliable and low-cost diagnosis tools are one of the most economically plausible measures for containment of Verticillium wilt of olive (López-Escudero and Mercado-Blanco 2011). In this regard, several approaches have been established for detection and identification of *V. dahliae* in soil such as plating, bioassays and immunoassays methods (Goud and Termorshuizen 2003). Although these methods are useful for the detection of *V. dahliae* in soil, they are time-consuming and usually involve equipments and skilled expertise (McCartney *et al.* 2003). Hence, to overcome shortcomings related to culturing-based diagnostic methods, nucleic acid (NA)-based techniques are greatly recognized as one of the most powerful for rapid and sensitive detection of *V. dahliae*.

Among molecular techniques, PCR-based procedures commonly show higher sensitivity and specificity compared to culturing-based methods, thus aiding in pathogen detection before symptom development and disease outbreak. Moreover, they can be implemented in certification schemes of pathogen-free planting material as preventive disease control measure (Goud and Termorshuizen 2003; López-Escudero and Mercado-Blanco 2011). Several PCR-based protocols have been successfully developed and applied for the effective detection of *V. dahliae* and its pathotypes (highly virulent, defoliating [D] and moderately virulent, nondefoliating [ND]) in both olive tissues (Mercado-Blanco *et al.* 2001, 2002, 2003), and infested soils (Pérez-Artés *et al.* 2005). Recently, thanks to the advent of real-time PCR technique, more rapid and high-sensitivity protocols have been made available for detection and quantification of *V. dahliae* in *planta* and in soil (Gramaje *et al.* 2013).

While PCR assays are commonly regarded as sensitive and reliable methods to detect and quantify *V. dahliae*, their implementation may require appropriate training, technical expertise and highly sophisticated and expensive equipment and reagents not fully affordable in ordinary laboratories (Francois *et al.* 2011; Rostamkhani *et al.* 2011; Moradi *et al.* 2012). Thus, there is a need to develop simple, rapid and cost-effective tests for the early diagnosis and screening of *V. dahliae* at large scale. This is particularly true for the proper evaluation of the presence of the pathogen in new soils expected to be devoted to olive cultivation. The aim is to prevent the spread of disease to new areas as well as to offer olive nurseries an appropriate tool to certify plant propagation material (López-Escudero and Mercado-Blanco 2011). Thus, development of a straightforward, rapid, sensitive and cost-effective method for detection of *V. dahliae* will be essential for implementing effective disease management strategies.

It is accepted that loop-mediated isothermal amplification (LAMP) (Notomi *et al.* 2000) can be an effective method to address deficiencies of PCR-based methods, overcoming common limitations of current diagnostic tools (Mori *et al.* 2013). A typical LAMP assay consists of one-step reaction that amplifies a target DNA sequence with both high sensitivity and specificity under isothermal condition (Notomi *et al.* 2000). LAMP has attracted a lot of attention as a potentially rapid, sensitive and cost-effective novel nucleic acid amplification method. Interestingly, LAMP is less influenced by inhibitors derived from blood, culture medium, plant tissues and soil compared with PCR (Ebbinghaus *et al.* 2012). This method can be even implemented without DNA extraction as the *Bst* polymerase enzyme is extremely resistant to many compounds inhibiting *Taq* polymerase enzyme (Francois *et al.* 2011). LAMP technique is growing in popularity, and many reports have been published concerning the application of this approach for detection of many infectious diseases in human and animals (Mori *et al.* 2013). Recently, a significant numbers of studies on the detection of bacterial and viral plant pathogens by implementing LAMP assays have been carried out (Almasi *et al.* 2012; Moradi *et al.* 2012). On the other hand, reports regarding detection and identification of fungal plant pathogens using this methodology are less frequent (Tomlinson *et al.* 2010; Almasi *et al.* 2013b; Niessen *et al.* 2013), particularly in the case of soilborne phytopathogenic fungi.

The main objective of the present study was to develop and evaluate a LAMP procedure for the rapid, highly sensitive, reproducible and specific detection of *V. dahliae* isolates directly from pure cultures and soil samples without the need for sophisticated equipment and in order to allow repeated analyses with high reproducibility. Based on our knowledge, this is the first study to develop a LAMP assay for detection of *V. dahliae* in agricultural soil samples. An additional aim of this study was to compare the LAMP protocol with currently available molecular detection methods in terms of sensitivity and specificity.

Materials and methods

Fungal isolates and inoculum production

A collection of 14 isolates of *V. dahliae* were used in this research (Table 1). The isolates were characterized by

Table 1 Isolates of *Verticillium dahliae* and different type of pathogenic and nonpathogenic fungi and bacteria used in the assessment of the specificity of the LAMP reaction

Isolate	Species and pathotypes*	Hosts	Geographic origin	LAMP signal
S407525†	*V. dahliae* ND	Olive	Zanjan, Iran	+
407525†	*V. dahliae* ND	Olive	Zanjan, Iran	+
S319971†	*V. dahliae* ND	Olive	Zanjan, Iran	+
319971†	*V. dahliae* ND	Olive	Zanjan, Iran	+
E_4‡	*V. dahliae* ND	Olive	Golestan, Iran	+
CS-56§	*V. dahliae* ND	Cotton	Fars, Iran	+
T1¶	*V. dahliae* D	Cotton	Golestan, Iran	+
D_5‡	*V. dahliae* D	Olive	Golestan, Iran	+
D-74§	*V. dahliae* D	Cotton	Fars, Iran	+
D-43§	*V. dahliae* D	Cotton	Fars, Iran	+
IRAN 1613**	*V. dahliae* D	Pistachio nut	Rafsanjan, Iran	+
IRAN 393 C**	*V. dahliae* D	Pistachio nut	Kerman, Iran	+
IRAN 434 C**	*V. dahliae* ND	Sweet almond	Azarbaijan, Iran	+
IRAN 667 C**	*V. dahliae* ND	Apricot	Shahrud, Iran	+
G††	*V. albo-atrum*	Alfalfa	Markazi, Iran	–
CBS 110218 (VD1)‡‡	*V. longisporum*	Oilseed rape	Sweden	–
CBS101220‡‡	*V. tricorpus*	*Brassica*	Israel	–
IMI 130213**	*V. nubilum*	Mushroom compost	Scotland	–
CBS 288.79‡‡	*V. biguttatum*	Unknown	Netherlands	–
PTCC 5180§§	*V. chlamydosporium*	Soil	France	–
F.g.N32**	*Fusarium graminearum*	Wheat	Iran	–
F-36¶¶	*F. oxysporum* f.sp. *tuberose*	Potato	Khorasan, Iran	–
Sch-F75***	*F. moniliforme*	Maize	Gorgan, Iran	–
Ss_{20}**	*Sclerotinia sclerotiorum*	Canola	Golestan, Iran	–
P6†††	*Aspergillus flavus*	Pistachio	Kerman, Iran	–
Ol.2·11‡	*Phytophthora palmivora*	Olive	Golestan, Iran	–
R17§	*Rhizoctonia solani*	Turf grass	Fars, Iran	–
ATCC 16404†††	*Aspergillus brasiliensis*	Blueberry	North Carolina, USA	–
ATCC 20423†††	*A. oryzae*	Soil	Japan	–
PTCC 5293§§	*Tolypocladium inflatum*	Soil	Norway	–
PTCC 5238§§	*Trichoderma* sp.	Soil	North of Iran	–
PTCC 5076§§	*Rhodotorula rubra*	Sour lemon	Iran	–
PTCC 5249§§	*Penicillium charlesii*	Soil	Japan	–
PTCC 5304§§	*P. citrinum*	Soil	Germany	–
PTCC 5292§§	*Mucor hiemalis*	Soil	North of Iran	–
ATCC 20034‡‡‡	*Mortierella vinacea*	Soil	Japan	–
PTCC 5182§§	*Lecanicillium muscarium*	Wheat	Germany	–
PTCC 1681§§	*Nitrobacter hamadansis*	Soil	Hamadan, Iran	–
PTCC 1659§§	*Nostoc ellipsosporum*	Soil	Babol, Iran	–
PTCC 1648§§	*Halobacterium karajensis*	Saline soil	Karaj, Iran	–
PTCC 1324§§	*Cellulomonas persica*	Forest soil	Iran	–
ATCC 21830†††	*Paenibacillus polymyxa*	Soil	Japan	–
ATCC 49337†††	*Bacillus atrophaeus*	Soil	USA	–
Ea 273 ATCC 49946†††	*Erwinia amylovora*	Apple	USA	–
Ea Z2¶¶	*E. amylovora*	Pear	Zanjan, Iran	–
IRIPP Abp2**	*Pantoea agglomerans*	Pear	Karaj, Iran	–
ATCC 11355†††	*Pseudomonas syringae*	Apple	USA	–
F7***	*Ps. fluorescens*	Apple	Karaj, Iran	–
DSM 1691¶¶	*Azospirillum lipoferum*	Bean	Karaj, Iran	–
Rb-133***	*Rhizobium leguminosarum*	Soil	Karaj, Iran	–

*Identification of D and ND isolates was carried out according to previously described protocols (Mercado-Blanco *et al.* 2003).

†Agricultural and Natural Resources Research Center of Zanjan, Iran.

‡Mycological collections, Dept. of Plant Protection, Gorgan University of Agricultural Sciences and Natural Resources, Iran.

§Culture collection of the Soil-borne Diseases Laboratory, Department of Plant Protection, Shiraz University, Shiraz, Iran.

¶Cotton Research Institute of Iran, Gorgan, Iran.

**Iranian Research Institute of Plant Protection, Tehran, Iran.

††Ghalandar *et al.* (2004).

‡‡Culture collections of micro-organisms (CBS), Utrecht, the Netherlands.

§§Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology, Tehran, Iran.

¶¶Seed and plant improvement institute, Karaj, Iran.

***Microbial collections, Dept. of Plant Protection, College of Agricultural and Natural Resources, University of Tehran, Iran.

†††American Type Culture Collection, Manassas, USA.

morphological, physiological and pathogenicity tests, and, finally, assignment to the olive and cotton D and ND pathotypes was performed by PCR according to Mercado-Blanco *et al.* (2003). Table 1 shows other pathogenic and nonpathogenic fungi and bacteria used for specificity tests. Storage and activation of *V. dahliae* isolates were conducted according to Pérez-Artés *et al.* (2000). Isolates were grown on potato dextrose agar medium (PDA, Sigma-Aldrich Co., Taufkirchen, Germany) for 9–11 days at 25°C in the dark. Inoculum density of microsclerotia was prepared based on the previous study (Pérez-Artés *et al.* 2005).

Soil sampling

Soil samples were collected during the spring of 2012 from 32 commercial olive orchards (4–20 years old; Zard, Roghani and Mari cultivars) located in one of the most important olive production areas of Iran (Tarom, Zanjan, Iran) where the presence of *V. dahliae* was previously reported (Sanei and Razavi 2012). Ten 100 g soil samples were taken at random from each orchard (trees showed Verticillium wilt symptoms) by shovel from a depth of 15 cm below the ground surface adjacent to the root system of the trees. Samples were kept in sterile polypropylene test tubes. The collected samples were then combined and mixed thoroughly. A 300 g subsample of mixed soil from each surveyed orchard (32) was divided into three biological replicates and used in subsequent tests (Kuchta *et al.* 2008). Soil samples were ground with a mortar and pestle, dried at room temperature, mixed well and sieved through a 2-mm pore-size sieve. To assess accuracy of the LAMP assay, half of each soil sample was sterilized by autoclaving for 1 h at 121°C on three consecutive days, maintaining the samples at room temperature between each autoclaving cycle, which was later used either as negative control or artificially infested with *V. dahliae* (Pérez-Artés *et al.* 2005). The medium NP-10 was applied for verification of the LAMP results for detection of *V. dahliae* in soil samples (Kabir *et al.* 2004).

Sensitivity of the LAMP assay in agricultural soil samples was evaluated by adding microsclerotia suspensions (isolates S407525 and T1 isolates, Table 1) to autoclaved soil samples. Samples (1 g) of each autoclaved soil were mixed with microsclerotia suspension to produce a range of $1–5 \times 10^5$ microsclerotia of *V. dahliae* per gram of soil. The infested soil samples were dried at room temperature and then stored at 4°C until used (Pérez-Artés *et al.* 2005). In all sensitivity tests, *Verticillium albo-atrum*, *Verticillium longisporum* and *Verticillium tricorpus* were considered as negative controls.

DNA preparation

Fungal total genomic DNA was extracted from 100 mg of powdered mycelia grown on PDB medium (7–10 days old) by the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). DNA purification was accomplished according to the manufacturer's instructions. DNA solutions were stored at −20°C until used. Quality and concentration of the DNA were determined by 1% agarose gel electrophoresis and also by spectrophotometer (260 nm). Final concentration of DNA samples was finally adjusted to 50 ng μl^{-1}. For all PCR and LAMP reactions, 1 μl DNA (10 ng μl^{-1}) samples were used as template (Sambrook and Russell 2001). DNA samples from nontarget species (Table 1) were also tested at this concentration.

DNAs were always extracted from samples (1 g) of agricultural, artificially infested and autoclaved soil samples according to the protocol described by Pérez-Artés *et al.* (2005). The soil samples were amended with 30% acid-washed autoclaved sand and ground to a fine powder. Then, 75 mg of this powder was transferred to a 1·5-ml centrifuge tube, which was then blended with 200 μl of 3·2% (w/v) skimmed milk (Nestlé, Nestlé Iran P.J.S. Co., Tehran, Iran), mixed by vigorous vortex and then centrifuged at 12 000 ***g*** for 30 min at 4°C. The supernatants were transferred to a fresh tube and thoroughly mixed with 8 μl of 10% SDS and an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1). This mixture was centrifuged at 12 000 ***g*** for 10 min at room temperature, and then, the upper phase was used as a template. Quality and concentration of the DNA were determined by 1% agarose gel electrophoresis and also by spectrophotometer (260 nm). DNA solutions were stored at −20°C and for using in subsequent PCR and LAMP reaction.

PCR assay

Single-round PCR was carried out for detection of D and ND pathotypes in medium, and nested-PCR was also used for identification of isolates in soil samples. The PCR assays were accomplished according to the previously described protocols (Mercado-Blanco *et al.* 2003; Collado-Romero *et al.* 2006), using specific primers (Table 2), with some minor modifications. The final volume of each PCR was 25 μl with the following reagents: 2·5 μl of 10× buffer [10 mmol l^{-1} Tris-HCl (pH 8·3), 50 μmol l^{-1} KCl and 1% v/v Triton X-100], 2·5 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} of dNTPs (CinnaGen Co., Iran), 100 nmol l^{-1} of each primer, 1 U of *Taq* DNA polymerase (CinnaGen Co., Tehran, Iran) and 1 μl of template DNA. Each reaction was carried out according to the following thermal cycling settings: initial denaturation step at 94°C for 4 min

Table 2 Details of nested-PCR (external and internal) and LAMP primers used in this study

Primer	Sequences (5′–3′)	Length	Amplicons (bp)	Target	References
Nested primers (nt) for ND					
NDf*	ATCAGGGGATACTGGTACGAGA	22-mer	1410	RAPD marker (accession	Mercado-Blanco
NDr*	GAGTATTGCCGATAAGAACATG	21-mer		no. AJ302675)	*et al.* (2001)
INTND2f†	CTCTTCGTACATGGCCATAGATGTGC	26-mer	824		
INTND2r†	CAATGACAATGTCCTGGGTGTGCCA	25-mer			
Nested primers (nt) for D					
D1*	CATGTTGCTCTGTTGACTGG	20-mer	548	RAPD marker (accession	Pérez-Artés
D2*	GACACGGTATCTTTGCTGAA	20-mer		no. AJ302674)	*et al.* (2000)
INTD2f†	ACTGGGTATGGATGGCTTTCAGGACT	26-mer	462		Mercado-Blanco
INTD2r†	TCTCGACTATTGGAAAATCCAGCGAC	26-mer			*et al.* (2002)
DB19*	CGGTGACATAATACTGAGAG	20-mer	523 for ND	RFLP marker accession	Carder *et al.* (1994)
DB22*	GACGATGCGGATTGAACGAA	20-mer	539 for D	no. DQ266249)	
espdef01†	TGAGACTCGGCTGCCACAC	19-mer	334 for D		Mercado-Blanco
					et al. (2003)
LAMP primers (nt)					
FIP‡ (F1c and F2)	CGTGATGCTCCGTTTAGGTGGA**TTTT** CGCCATGTTCGGTGCTAG	44-mer	181	RAPD marker (accession no. AJ302674)	This study
BIP‡ (B1c and B2)	TGGCACGTGTGGCGTAAGAC**TTTT** CGATGTCGAGTCTGACACTG	44-mer			
F3	TGGCAGCTTCTGATTCAGTT	20-mer			
B3	ACAGCGATTTGGATTCCCTC	20-mer			
LF	CTGACAACCAACGTCTAGATCTCA	24-mer			
LB	GGCTATTGAGTTCTGCACTCTGTC	24-mer			

*External primers.
†Internal primers.
‡T linker used (marked in bold).

followed by 30 cycles; denaturation at 94°C for 1 min; annealing at 64°C for 1 min; and extension at 72°C for 1 min. The final cycle involved 1 min annealing and 5 min extension. For the second-round PCR, 1 μl of the first-round PCR products was added to new master mix with internal primers, and amplification was followed under the same condition as the first round. Primer pair DB19/DB22 was used alone as well as jointly with primer espdef01, except the annealing temperature for the multiplex use of the three primers was reduced to 60°C. All PCRs were performed in a Bio-Rad thermal cycler (DNA Engine Model, New York, NY). 1 μl of 100-bp DNA ladder (Fermentas, St. Leon-Rot, Germany) served as a size marker. PCR products were separated by electrophoresis on 1% agarose gels in 1 × tris-acetate-EDTA (TAE) buffer at 85 V for 60 min. Gels were stained for 15 min in ethidium bromide (EtBr, Sigma, Madrid, Spain) (1 g ml^{-1}) and visualized on a UV transilluminator.

Design of primers for LAMP

To perform the LAMP assay, specific primers were designed from a previously reported *V. dahliae* RAPD (random amplified polymorphic DNA) genomic sequence (GenBank accession no. AJ302674; Mercado-Blanco *et al.* 2002) using the free online software Primer Explorer V4 (http://primerexplorer.jp/e/). Primers finally designed were outer primers (F3 and B3), inner primers (FIP and BIP) and additional loop primers (LF and LB) which accelerate LAMP reaction and therefore reduce the detection time (Table 2). Figure 1 shows the schematic position of LAMP and nested-PCR primers within the RAPD marker. Finally, the primers were tested for similarities with other sequences available in the GenBank databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN algorithm.

Optimization of LAMP reaction conditions

The LAMP assay was carried out in a reaction mixture (final volume 25 μl) containing 2·5 μl 10× thermopol buffer (New England Biolabs, Hertfordshire, UK), 1·6 mmol l^{-1} dNTPs (CinnaGen Co.), 1 mol l^{-1} betaine (Sigma-Aldrich, Munich, Germany), 1·6 μmol l^{-1} each of FIP and BIP, 0·2 μmol l^{-1} each of F3 and B3 and 0·8 μmol l^{-1} each of LF and LB primers, 6 mmol l^{-1} $MgSO_4$, 1 μl of purified template DNA (10 ng μl^{-1}), 1 μl (8 units μl^{-1}) of the *Bst* DNA polymerase (New England Biolabs) and sterile deionized water. To achieve further

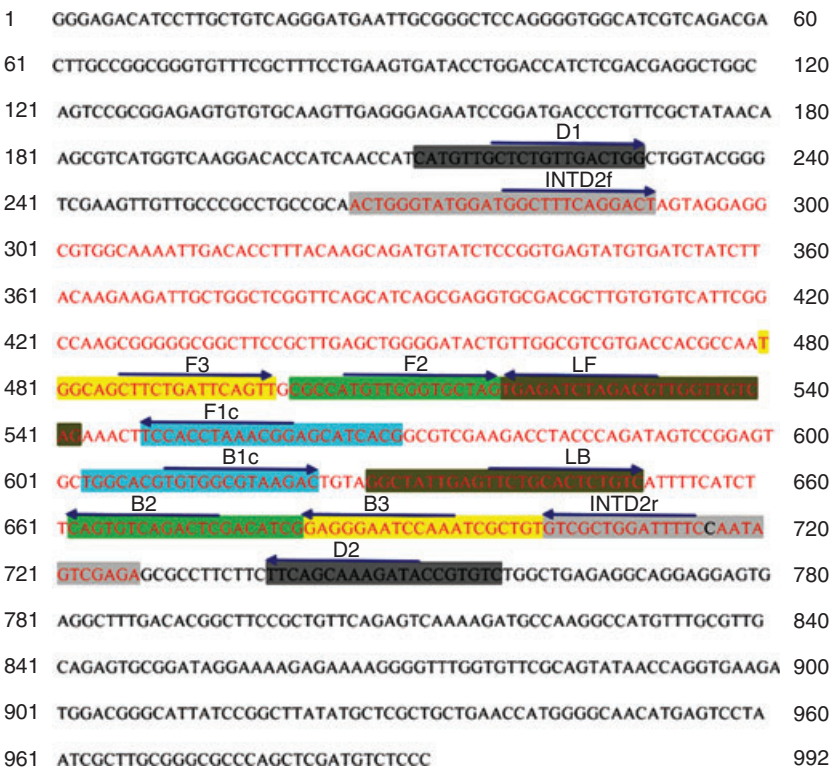

Figure 1 Schematic diagram of LAMP and nested-PCR primers binding sites within the *Verticillium dahliae* RAPD marker sequence (GenBank accession no. AJ302674; Mercado-Blanco *et al.* 2002) used in this study. FIP and BIP primers contain two distinct sequences: F1c plus F2 and B1c plus B2, respectively. *Note:* The red marked sequence was considered for designing LAMP primers as based on previous report (Mercado-Blanco *et al.* 2002) this region of RAPD marker (between 266 and 727 bp, except 716 bp position) is identical in both D and ND pathotypes.

turbidity in the LAMP reaction, 10 mmol l^{-1} $CaCl_2$ was also used (Almasi *et al.* 2013a). The mixture was incubated at 64°C for 60 min using a thermo block (simple heating block); the mixture was then heated at 85°C for 2 min to terminate the reaction. Finally, all LAMP products were separated on 1·5% (w/v) agarose gel.

Visual detection and confirmation of LAMP products

To visually detect the LAMP products, several dyes were added to the LAMP reaction. Prior to amplification, 1 μl of the hydroxynaphthol blue (HNB) dye (3 mmol l^{-1}, Lemongreen, Shanghai, China) or 1 μl of GeneFinder™ (diluted to 1 : 10 with 6 × loading buffer; Takara, Dalian, China) was added to the master mix. After amplification, 0·5 μg EtBr or 2 μl of 1/10-diluted original SYBR® Premix Ex Taq™ II dyes (Takara Bio Co., Ltd., Otsu, Japan; RR081A) was added to LAMP reaction. The tubes containing GeneFinder™ and HNB dyes were easily monitored for colour change by simply looking at them in daylight, while those containing EtBr and SYBR® II were examined under UV light (302 nm) after a short vortex. After visualization, the LAMP products were monitored using 1·5% agarose gel electrophoresis stained with EtBr.

PCR assay was performed to verify whether the LAMP reaction amplified the correct DNA target. PCR amplification was performed by using the outer LAMP primers pair (F3 and B3, Fig. 1). The PCR assay was carried out in 25 μl reaction mixture containing 2·5 μl of 10× buffer (Tris-HCl [pH 8·3] and KCl), 0·5 μmol l^{-1} each of F3 and B3, 2·5 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} each dNTP (CinnaGen, Co.), 1 U of *Taq* DNA polymerase (CinnaGen, Co.) and 1 μl of template DNA. Initial denaturation was conducted at 94°C for 5 min, followed by 35 cycles of denaturation (30 s at 94°C), annealing (45 s at 62°C) and extension (30 s at 72°C) and a final extension cycle at 72°C for 5 min. 5 μl aliquots of PCR products were subjected to 1·5% agarose gel electrophoresis. Afterwards, the PCR product was extracted from agarose gel using Qiaex Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into pMD18-T vector (Takara, Bio Inc.) and then transformed into NEB 5-alpha *Escherichia coli* cells (New England Biolabs, Beverly, MA). The positive clones (white colonies) were subjected to sequencing using F3 primer (Bioneer Co., Daejeon, South Korea). These procedures were performed for both S407525 (ND) and T1 (D) isolates.

Direct detection of *Verticillium dahliae* in PDA medium and soil

To demonstrate the robustness of the LAMP technique against inhibitors, direct detection of *V. dahliae* grown

on PDA plates and infesting soil was accomplished without DNA purification. A simple, rapid and inexpensive procedure was applied to directly detect *V. dahliae* in PDA. Thus, 0·1 g of fungal mycelia (7- to 10-day-old cultures of S407525 and T1 isolates) was removed from PDA plates with a sterile needle and then suspended in 300 μl distilled water. The suspension was then centrifuged at 12 000 ***g*** for 5 min at 4°C, and 2 μl of supernatant was directly added to the LAMP and PCR mixtures as a template.

The collected soil samples were directly used in the LAMP reaction after some minor treatment. At first, 0·5 g fine powder of soil sample (amended with 30% acid-washed autoclaved sand) was thoroughly mixed with 300 μl of 3·2% (w/v) skimmed milk (Nestlé, Nestlé Iran P.J.S. Co.); the solution was then mixed and centrifuged at 15 000 ***g*** for 10 min at 4°C. The harvested supernatant was incubated for 10 min at 95°C in a simple heating block, after which 2 μl of the solution was directly added to LAMP and nested-PCR master mixtures. Three replicates were accomplished for each sample at PDA and soil samples for direct detection of *V. dahliae*.

Results

Detection of LAMP products

The LAMP primers could successfully amplify the defined segment of RAPD marker (Fig. 1) for the both pathotype of *V. dahliae*. This is because the RAPD marker (GenBank accession no. AJ302674) is present in both pathotypes, as previously suggested by Mercado-Blanco *et al.* (2002). However, LAMP primers were previously assessed by BLASTN, and no significant similarity was found neither with closely related fungal species nor remotely related ones.

The LAMP assay was run using purified DNA and yielded positive results for all *V. dahliae* isolates evaluated in this study (Table 1). To verify LAMP products, several steps were taken. For instance, visual observation of reaction tubes containing calcium pyrophosphate formed a cloudy, white precipitate (Fig. 2a), which was more stable and dense than that obtained by amending the reaction mixture with magnesium pyrophosphate (Fig. 2b). Likewise, after adding EtBr, the colour of positive samples turned into ochraceous yellow when observed under UV light (Fig. 2c), while addition of SYBR® Premix Ex Taq™ II turned the colour of positive sample from red to green (Fig. 2d). HNB changed violet to sky blue colour in positive samples, an observation clearly visible with naked eye (Fig. 2e), whereas GeneFinder™ created an obvious green pattern in positive LAMP products (Fig. 2f).

Verification of LAMP results

The LAMP products from purified fungal DNA were monitored by naked-eye observation (Fig. S1b) and using 1·5% agarose gel electrophoresis. The typical ladder-like pattern on agarose gel was observed in all isolates of *V. dahliae,* but not in the negative control (Fig. S1a). The sequencing of PCR products obtained showed 98–99% identity between amplicons sequenced and the *V. dahliae* RAPD marker sequence (AJ302674) used for the design of primers here reported. These results confirmed that the specific gene target was amplified (Data set S1, Fig S2).

Sensitivity and specificity of the LAMP assay

To evaluate sensitivity of the LAMP and nested-PCR assays, a 10-fold serial dilution of DNA (extracted from *V. dahliae* S407525 and T1) was prepared from 50 ng to 50 fg of purified target DNA per reaction. The results indicated that the LAMP assay could detect as little as 500 fg DNA reaction^{-1} for ND pathotype (S407525). GeneFinder™ dye was added to LAMP products, and positive reactions were directly detected by visual inspection (Fig. 3a). Similarly, the LAMP assay detected 50 fg DNA reaction^{-1} for D pathotype (T1), which is a lower level relative to ND pathotype (Fig. 3c). On the contrary, the nested-PCR protocol could only detect 5 pg DNA reaction^{-1} of *V. dahliae* ND pathotype (Fig. 3b), the same results were obtained for D pathotype as well (data not shown).

The artificially infested soil samples with microsclerotia were used as template. The inoculum densities were generated from 1 to 5 × 10^5 microsclerotia per gram of soil of the *V. dahliae* ND and D pathotypes (S407525 and T1 isolates), and extracted DNA (see Material and methods) was used in nested-PCR and LAMP assays. Furthermore, the direct-LAMP assay was carried out based on the described procedure. The results demonstrated that the LAMP assay could detect 5 microsclerotia g^{-1} soil for D pathotype (T1 isolate, Fig. 3d) and 50 or less microsclerotia g^{-1} soil for ND pathotype (S407525 isolate). However, the direct-LAMP assay could detect 5 and 50 microsclerotia g^{-1} soil for D and ND pathotypes, respectively, the detection time slightly increased (8–10 min). The nested-PCR showed the same limit of detection (50 microsclerotia g^{-1} soil) for both D and ND pathotypes by specific primers (data not shown). DNA extracted from infested soils with *V. albo-atrum*, *V. longisporum* and *V. tricorpus* did not show any results in LAMP, direct-LAMP and nested-PCR methods. These results indicated that the LAMP assay was approx. 10 times more sensitive than nested-PCR procedure for

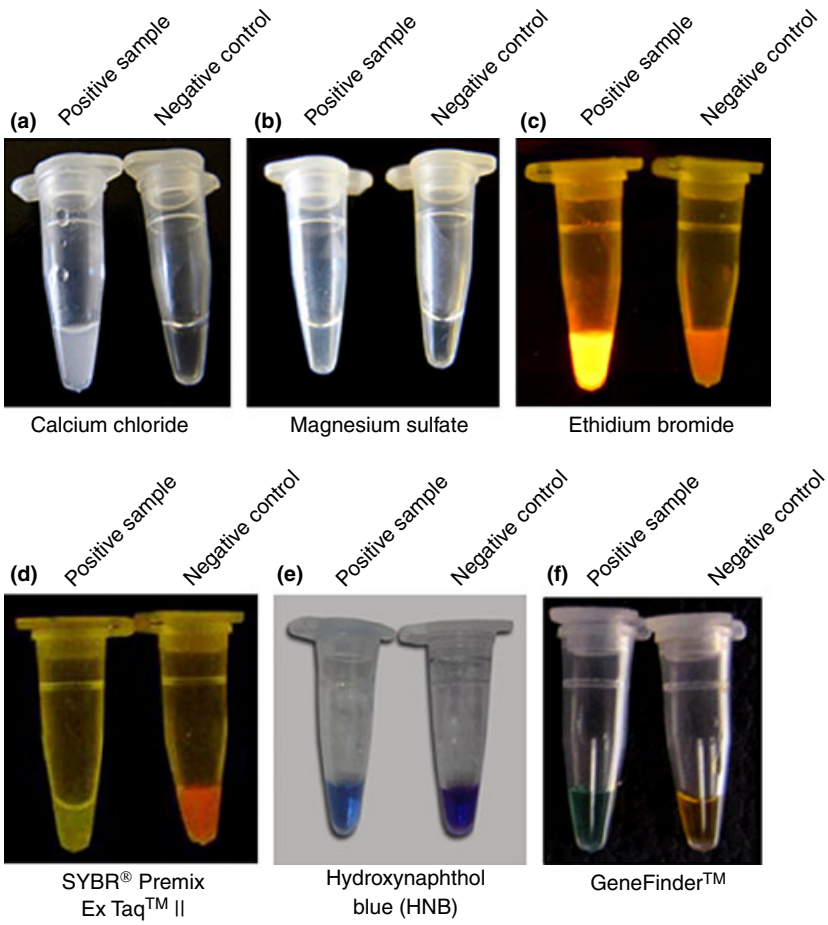

Figure 2 Details of six different visualization methods to analyse LAMP products, *Verticillium dahliae* S407525 (ND) was considered as a positive sample. Distilled water was added instead of DNA as a negative control (a) $CaCl_2$; (b) $MgSO_4$; (c) EtBr; (d) SYBR® Premix Ex Taq™ II dye; (e) HNB; and (f) GeneFinder™ dye. *Note:* The tubes contain EtBr and SYBR® Premix Ex Taq™ II were observed under UV light. HNB and GeneFinder™ dyes were observed in daylight. HNB and GeneFinder™ dyes were added to LAMP master mix, while EtBr and SYBR® Premix Ex Taq™ II were supplemented to tubes after LAMP reaction.

detection of *V. dahliae*. These experiments were repeated three times with identical results.

To determine specificity of the primers, the designed LAMP assay was challenged against DNA samples of different pathogenic and nonpathogenic fungi and bacteria (Table 1). The LAMP results were observed by direct visual inspection, and for confirmation of results, the electrophoresis was carried out. No false-positive amplification was detected for other *Verticillium* species (Fig. S3a), pathogenic and nonpathogenic fungi (Fig. S3b) and bacteria (Fig. S3c). Consequently, the newly developed LAMP assay showed high specificity in detection of *V. dahliae*.

Direct detection of *Verticillium dahliae* in PDA medium

DNA extraction from PDA medium is time-consuming and complicated. Thus, elimination of DNA purification stage can be a critical factor in saving time and money, especially in screening of large samples numbers. In this study, a straightforward and rapid procedure (approx. 5–10 min) was used on fungal biomass grown on PDA plates before direct-LAMP reaction. The results showed that mycelia from *V. dahliae* isolates yielded positive results after direct-LAMP assay (Fig. 4, lane: 1). However, the turbidity became slightly weaker, and detection time increased slightly (approx. 5–8 min) compared to standard LAMP method (data not shown). In contrast, PCR assay could not detect *V. dahliae* from samples that were treated the same way as before (Fig. 4, lane: 4). Additionally, the nested-PCR assay did not show amplification for either D or ND pathotypes. This experiment was repeated three times with identical results.

Analysis of soil samples by nested-PCR, LAMP and direct-LAMP methods

The collection of 32 *V. dahliae* naturally infested soil samples from olive orchards was analysed by nested-PCR, LAMP and direct-LAMP methods. The results showed that the direct-LAMP assay could directly detect the presence of *V. dahliae* in the soil samples. These results indicated that LAMP (run by purified DNA from soil samples) yielded better detection rate (26/32) compared

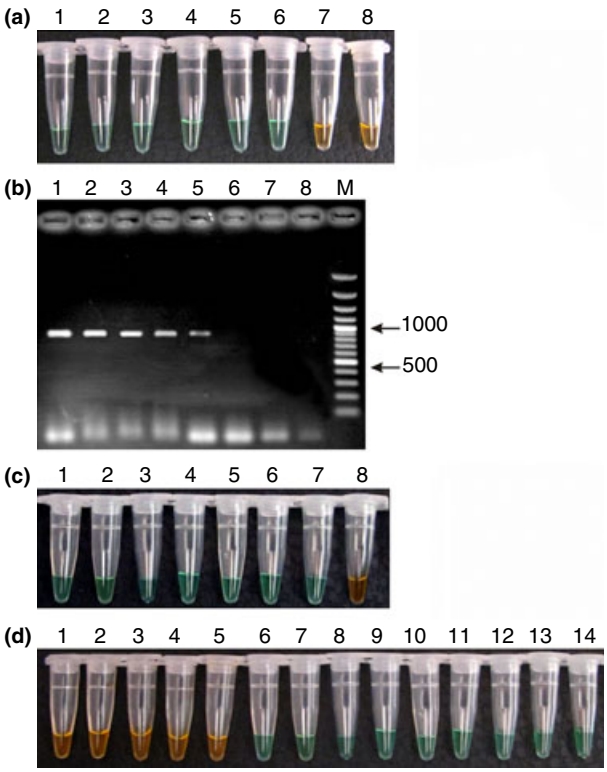

Figure 3 Sensitivity of the LAMP and nested-PCR assays, using 10-fold serial dilution of purified target DNA from *Verticillium dahliae* S407525 (ND) per reaction; (a) detecting LAMP products by adding GeneFinder™ dye, Tube 1: 50 ng DNA; Tube 2: 5 ng DNA; Tube 3: 500 pg DNA; Tube 4: 50 pg DNA; Tube 5: 5 pg DNA; Tube 6: 500 fg DNA; Tube 7: 50 fg DNA; Tube 8: negative control with distilled water; (b**)** electrophoresis of the nested-PCR products (the primers for ND were used) which were run on 1% agarose gels, Lane 1: 50 ng DNA; Lane 2: 5 ng DNA; Lane 3: 500 pg DNA; Lane 4: 50 pg DNA; Lane 5: 5 pg DNA; Lane 6: 500 fg DNA; Lane 7: 50 fg DNA; Lane 8: negative control with distilled water and Lane M: DNA size marker (100 bp); (c) The 10-fold serial dilution of purified target DNA from *V. dahliae* T1 (D)per reaction, Tube 1 to Tube 8 correspond to a diluted with 50 ng to 50 fg DNA; (d**)** detection of DNA from *V. dahliae* T1 by LAMP assay in agricultural soils artificially infested with a range of microsclerotia. Tube 1: DNA extracted from *V. albo-atrum*; Tube 2: DNA extracted from *V. longisporum*; Tube 3: DNA extracted from *V. tricorpus*; Tube 4: negative control with distilled water; Tubes 5–11 correspond to soil samples infested with 1, 5, 50, 5×10^2, 5×10^3, 5×10^4, 5×10^5 microsclerotia g^{-1} of soil, respectively; Tube 12: purified DNA from S407525 isolate; Tube 13: purified DNA from T1 isolate and Tube 14: purified DNA from agricultural soil sample.

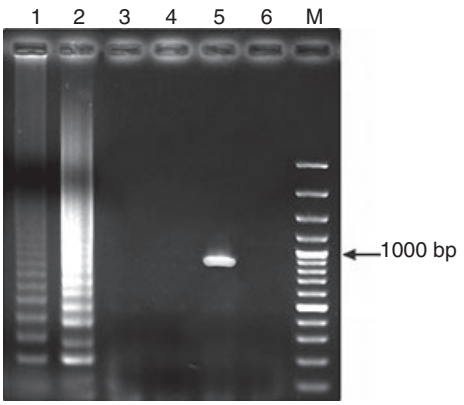

Figure 4 Direct analysis of plate-grown culture with LAMP and PCR. Fungal material was mixed into the master mix prior to incubation, *Verticillium dahliae* S407525 (ND) was used as a template, Lane 1: LAMP reaction with 2 μl supernatant from culture extract; Lane 2: LAMP reaction with purified DNA; Lane 3: negative control (distilled water) for LAMP reaction; Lane 4: nested-PCR with 2 μl supernatant from culture extract; Lane 5: nested-PCR with purified DNA; Lane 6: negative control (distilled water) for nested-PCR and Lane M: DNA size marker (100 bp).

Table 3 Detection of *Verticillium dahliae* in 32 agricultural soil samples using nested-PCR, LAMP and direct-LAMP methods and their comparative results

Methods	Nested-PCR*	LAMP	Direct-LAMP
No. of positives samples	ND 22/32 (68·75%) D 0/32 (0·00%)	26/32 (81·25%)	24/32 (75·00%)
Detection time†	7–10 h	3 h	60–80 min

*The detected ND pathotype showed the C pattern (824 bp amplified which is associated with ND isolates) for previously described markers (Collado-Romero *et al.* 2006).
†DNA extraction was considered in detection time.

to other methods. Direct-LAMP yielded slightly better results (24/32) than nested-PCR (simultaneous detection of D and ND pathotypes by purified DNA from soil samples, Mercado-Blanco *et al.* 2003) (22/32, Table 3). All soil samples positive for the nested-PCR method were also positive for the LAMP and direct-LAMP assays. On the other hand, amplification was never observed in autoclaved soil samples which always failed to yield detectable amounts of DNA (after implementing the DNA extraction procedure). The results of three methods were verified by the medium NP-10 (data not shown). These experiments were carried out using three biological replicates, and the same results were obtained for all of them.

The detection methods compared in terms of time and results indicated that the direct-LAMP could detect *V. dahliae* within a total of 60–80 min without the need for any DNA purification procedures (Table 3). The results of simultaneous detection of the D and ND *V. dahliae* pathotypes by duplex, nested-PCR (Mercado-Blanco *et al.* 2003) exhibited that the ND pathotypes were only detected in the inspected olive orchards, while D pathotypes were nonexistent. Moreover, the detected ND pathotype in soil

samples was examined for PCR pattern, and it yielded the PCR pattern C (amplification of the 824-bp marker, Collado-Romero *et al.* 2006; Dervis *et al.* 2010) (Fig. S4).

Discussion

In the present study, the LAMP assay amplified a target *V. dahliae* DNA sequence (RAPD marker) previously reported to be associated with D-pathotype isolates from cotton and olive (Mercado-Blanco *et al.* 2002), displaying high specificity and sensitivity for detection of *V. dahliae* isolates. Furthermore, no false-positive results were detected for diverse pathogenic and nonpathogenic fungi and bacteria assessed as well as other *Verticillium* species (Table 1, Fig. S3).

Nested-PCR assays were previously developed for the *in planta* and in soil detection of *V. dahliae* pathotypes (D and ND) infecting olive and cotton (Mercado-Blanco *et al.* 2001, 2002, 2003; Pérez-Artés *et al.* 2005). Although these methods were both specific and sensitive, they are time-consuming, and need of appropriate equipment, posing difficulties for some diagnostic/detection laboratories. The results of this study indicated that the developed LAMP assay (limit of detection 500 and 50 fg DNA reaction^{-1} for ND and N pathotypes, respectively) (Fig. 3a,c) was 10 times more sensitive than nested-PCR assay (limit of detection 5 pg DNA reaction^{-1}) (Fig. 3b). Moreover, limit of detection of LAMP assay was evaluated by detection of microsclerotia of *V. dahliae* for both D and ND pathotypes in artificially infested soil samples. The results demonstrated that the LAMP assay detected 5 or less microsclerotia per gram of soil for D pathotype (T1 isolate, Fig. 3d). This could be considered a reliable level of sensibility as the level of microsclerotia in agricultural infested soils is much higher than 5 microsclerotia per gram of soil (Pérez-Artés *et al.* 2005). In addition, the LAMP method detected 50 or less microsclerotia g^{-1} soil for ND pathotype (S407525 isolate). Similarly, the direct-LAMP assay detected 5 and 50 microsclerotia g^{-1} soil for both D and ND pathotypes, respectively. Due to the few amount of DNA in supernatant and overwhelming number of inhibitors, decreasing the efficiency of the direct-LAMP assay, the detection time slightly increased (8–10 min). The limit of detection of nested-PCR for ND pathotype (by specific primers) corresponded to D pathotype (50 microsclerotia g^{-1} soil). This difference in detection limit between D and ND pathotypes could explain by the D associated marker is less frequent in ND pathotype which the previous study clearly demonstrated this fact (Mercado-Blanco *et al.* 2002).

Upon screening of 32 agricultural soil samples, the LAMP method yielded the best results, and the direct-LAMP showed (within 60–80 min) slightly better results compared to nested-PCR (Table 3). The rapid, simple and cost-effective direct-LAMP assay can therefore overcome limitations frequently encountered when using PCR assays for detection of *V. dahliae* and other slow-growing fungal pathogens in agricultural soils. The results of nested-PCR (Mercado-Blanco *et al.* 2003) showed that the ND pathotype was only detected in the monitored olive orchards. Further experiments revealed that these isolates produced the PCR pattern C characteristic of ND pathotype (Fig. S4) (Collado-Romero *et al.* 2006; Dervis *et al.* 2010).

The LAMP procedure here developed used as target DNA a RAPD marker earlier reported to be associated with D pathotype isolates infecting cotton and olive (Mercado-Blanco *et al.* 2002). This is quite relevant as D isolates are highly virulent and destructive in olive orchards and because this pathotype has become prevalent in many areas where olive is cultivated (Dervis *et al.* 2010; López-Escudero and Mercado-Blanco 2011; Sanei and Razavi 2012). Therefore, developing a rapid and simple method as the direct-LAMP assay for the detection of the D pathotype in soil samples could be a crucial preventive tool to help to contain the spread of the disease to new areas. Interestingly, the LAMP procedure was not able to differentiate between the D and ND isolates evaluated in this study (Table 1). This outcome supports the early finding that the D-associated marker seems to be present in (some) ND isolates as well, an observation reported after conducting nested-PCR (Mercado-Blanco *et al.* 2002). The higher sensitivity of the LAMP procedure here developed may explain why this marker is detected in both pathotypes. This lack of differentiation between D and ND isolates, while relevant from an epidemiological perspective, does not diminish the merit of the LAMP procedure as *V. dahliae* isolates could be consistently detected in soil samples and with the advantages mentioned above. Moreover, differentiation of D and ND isolates can be further implemented by developing new LAMP primers/assay from a wide range of molecular markers already available (Mercado-Blanco *et al.* 2003; Collado-Romero *et al.* 2006, 2009; Pantou *et al.* 2006; Maruthachalam *et al.* 2010) and on the basis of the LAMP set-up here reported.

DNA extracted from soil samples may contain a wide range of inhibitor substances (e.g., humic acid, polysaccharides, fulvic acid, metal ions.) which can disrupt performance of PCR assays (Watson and Blackwell 2000). These inhibitors could reduce amplification efficiency as well as sensitivity for detection and quantification of *V. dahliae* in soil samples (Pérez-Artés *et al.* 2005; Bilodeau *et al.* 2012). Although Kermekchiev *et al.* (2009) have developed new mutants of *Taq* and *Klentaq* DNA polymerases displaying high tolerance to PCR inhibitors,

making it possible to eliminate DNA extraction steps prior to PCR, some important disadvantages of PCR procedures still remain for some laboratories. These include the need for thermocyclers as well as a complete electrophoresis set-up for PCR product verification, besides the fact that less sensitivity to low DNA concentration in soil samples is not a rare phenomenon. Consequently, it is worth to emphasize the advantages offered by the LAMP assay and that the developed direct-LAMP protocol can be viewed as a simple, powerful and consistent approach for detecting soil pathogens in the absence of sophisticated equipment.

Sharp fluctuations in the concentration of *Taq* polymerase inhibitors depending on soil source make it extremely difficult to develop a standard DNA extraction procedure effective for all soil samples before running PCR and/or q-PCR protocols (LaMontagne *et al.* 2002; Kermekchiev *et al.* 2009). As DNA purification stage is time-consuming, labour and capital-intensive, it would be desirable to eliminate this step prior to LAMP reaction. Previous studies have suggested that LAMP assay shows a significant tolerance to inhibiting substances derived from soil and plant materials (Tani *et al.* 2007; Tomlinson *et al.* 2010; Niessen *et al.* 2012). The developed direct-LAMP assay worked successfully without a DNA purification step (samples were prepared with straightforward protocol that took about 20 min), and more importantly, decreasing the cost, time, labour and avoiding the use of environmentally dangerous reagents (i.e. EtBr).

Early detection of *V. dahliae* by screening and monitoring new orchards and nurseries is a key preventive measure for the effective management of Verticillium wilt of olive, helping to difficult the spread of the disease to new areas (López-Escudero and Mercado-Blanco 2011). The LAMP methodology requires only basic equipment (a simple heater block), and end-point results can be detected with naked eye. In the LAMP visualizing system, adding calcium chloride ($CaCl_2$) to the LAMP reaction produces a stable and cloudy turbidity (Fig. 2a) (Almasi *et al.* 2013a). Adding $CaCl_2$ to the LAMP reaction allowed visual detection of LAMP products without adding extra dyes. Moreover, this modification did not diminish either sensitivity or specificity of the reaction. To enhance the output of LAMP reactions, several dyes have been applied (Goto *et al.* 2010; Almasi *et al.* 2012; Ahmadi *et al.* 2013). The results demonstrated that addition of GeneFinder™ (Fig. 2f) and HNB (Fig. 2e) in the LAMP assay is a simple and effective strategy for the easy and rapid visual detection of *V. dahliae* in soil samples.

In summary, the LAMP method was successfully developed for detection of *V. dahliae* in PDA and soil samples, showing superior sensitivity, specificity, simplicity and user-friendly handling compared with PCR-based procedures. The direct-LAMP methodology did not require costly or sophisticated equipment. It is also minimally affected by soil inhibitors, and results are readily observable by the naked eye. It would be possible to scale up the simplified extraction method for routine screening of pathogens in large samples for phytopathological diagnosis purposes. Unquestionably, LAMP characteristics turn it into a suitable alternative to plating, PCR and real-time PCR methods to accelerate decision-making processes in disease management frameworks. Although the developed LAMP assay showed high sensitivity for *V. dahliae* isolates (5 microsclerotia g^{-1} soil), further studies need to be carried out to improve its specificity for accurate targeting of known *V. dahliae* pathotypes and strains, as well as to discriminate among *Verticillium* spp. currently described (Inderbitzin *et al.* 2011) infecting a wide range of important crops worldwide.

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Conflict of interest

There is no conflict of interest in the submission of this manuscript, and all authors of this manuscript agreed with publication.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Analysis of LAMP product for detection of *Verticillium dahliae* strains.

Figure S2 Using the BLASTN algorithm for testing the results of sequencing for *Verticillium dahliae* RAPD marker T1 (D) and S407525 (ND) isolates.

Figure S3 Specificity of the LAMP assay for *Verticillium dahliae*.

Figure S4 Identification of the PCR pattern of *Verticillium dahliae* ND isolates from agricultural soil samples.

Data S1 The results of sequencing for the T1 (D) and S407525 (ND) isolates (between the F3 and B3 primers) that the outer LAMP primer (F3, Table 2) was used for sequencing.