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New opportunity for early on-site detection of *Plasmopara viticola* by qPCR assay

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Summary. *Plasmopara viticola,* the causal agent of grapevine downy mildew, is a widespread and significant plant pathogen. A quantitative PCR (qPCR) assay using a portable thermocycler was developed to enable rapid and early on-site detection of *P. viticola*. The internal transcribed spacer 1 (*ITS1*) region was selected as the target, and the specific primer pairs PLAV19 was designed. The assay was optimized using traditional thermocyclers, testing three different primer concentrations (100, 200, and 300 nM), and two annealing temperatures (58°C and 60°C). Optimal conditions were 200 nM primer concentration and an annealing temperature of 60°C. Under these parameters, the assay yielded a limit of detection (LoD) of 1.5 fg/µL and a limit of quantification (LoQ) of 15 fg/µL for *P. viticola* DNA (strain PLVDisspa1), showing consistent performance across both thermocyclers. Specificity tests confirmed no cross-reactivity with DNA from common grapevine-associated microorganisms, biocontrol agents, other Oomycetes and several grapevine cultivars. The PLAV19 primer set was further validated on DNA extracted from healthy, artificially inoculated, and naturally infected grapevine tissues, including samples exhibiting nonspecific leaf symptoms and latent bunch infections. Three DNA extraction protocols were evaluated to validate the extraction method, and one of these was shown to be suitable for on-field applications. The developed assay was a reliable diagnostic tool for the early detection and monitoring of *P. viticola* under field conditions, with potential applications in disease forecasting and sustainable management of grapevine downy mildew.

Keywords. Downy mildew, *Vitis vinifera*, SYBR Green, grapevine, *ITS1*, portable lab station.

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INTRODUCTION

Grapevine (*Vitis vinifera L.*), a Eurasian species, is among the most extensively cultivated plants worldwide due to its high economic significance (McGovern *et al.,* 2017). Grapevine is highly susceptible to a broad spectrum of phytopathogens, including fungi, bacteria, viruses, viroids, and phytoplasmas. One of the major threats is the Oomycete *Plasmopara viticola* Berl. & de Toni, the causal agent of downy mildew. It can affect all the green parts of vines,

causing severe yield losses and a reduction in berries quality (Toffolatti *et al*., 2018). Additionally, berries affected less early have purplish to blackish, depressed ("push"), non-fruiting spots. In this case, the presence of " brown rot" on berries can be observed (Koledenkova *et al.,* 2022).

Several studies revealed that different varieties of grapevine show different levels of resistance and susceptibility to downy mildew (Heyman *et al*., 2021; Toffolatti *et al*., 2012). Generally, the American grapevine species, *Vitis riparia*, *V. rupestris*, *V. rotundifolia* and *V. amurensis* appear to be more resistant to the disease than *V. vinifera,* perhaps due to their longer coevolution with the pathogen (Boso *et al*., 2014). However, variation has been recorded also among different varieties of *V. vinifera*, and even clones of the same variety (Yu *et al*., 2012). Importantly, on resistant varieties nonspecific small necrotic areas can be observed as symptoms of *P. viticola* infections (Sotolář *et al*., 2007). Nevertheless, the pathogen can evolve more aggressive strains, potentially leading to the manifestation of non-specific symptoms in resistant plants, and progressively diminishing their resistance (Delmas *et al.,* 2016, Gouveia *et al*., 2024).

Studies conducted through the analysis of internal transcribed spacer 1 (*ITS*), β-tubulin (*TUB*), actin (*ACT*), and cytochrome b (*cytb*) on genetic characterization of *P. viticola* isolates collected from Canada and the United States led to the identification of four distinct intraspecific clades: 1) *riparia*, found on *V. riparia* and 'Chancellor', and interspecific hybrids; 2) *aestivalis*, found on *V. aestivalis, V. labrusca, V. vinifera* and interspecific hybrids; 3) *vinifera*, found on *V. aestivalis, V. cinerea, V. vulpine* and interspecific hybrids; 4) *quinquefolia*, found on *Parthenocissus quinquefolia* (Rouxel *et al*., 2013, 2014; Fontaine *et al*., 2021). These studies laid the foundation for identifying the clades of *P. viticola*, establishing it as a species complex. In addition to its genetic complexity, the epidemiology of *P. viticola* plays a critical role in the development and spread of the disease. Indeed, *P. viticola* can undergo multiple infection cycles within a single growing season, particularly under favourable environmental conditions such as high humidity, frequent rainfall, and moderate temperatures (Khatal *et al.,* 2023). Primary infections by *P. viticola* oospores result in the formation of sporangia containing zoospores, which are dispersed by rain splash onto the leaf surface, where they germinate developing hyphae colonizing the host tissue (Burruano *et al.,* 2000; Rossi *et al.,* 2008, 2013). Sporangia formed on infected tissues give rise to secondary infections. Consequently, the early and specific detection of *P. viticola*-complex is crucial for timely deployment of effective disease management strategies to avoid yield loss.

Several methods are available for detecting *P. viticola*. Traditional techniques, such as microscopic examination of infected leaves (Toffolatti *et al*., 2007), are limited by their inability to detect and quantify low concentrations of the pathogen. In contrast, molecular methods offer higher specificity and sensitivity, allowing even quantitative detection of the pathogen. Real-time PCR and digital droplet PCR (ddPCR) were developed for the detection of several plant pathogens, such as *Apiospora marii*, *Aspergillus carbonarius*, *A. niger*, *Erwinia amylovora*, *Monilinia* spp., *P. viticola* (syn. *Diaporthe ampelina*), *Verticillium dahliae* and *V. longisporum* (Valsesia *et al.,* 2005; Palumbo *et al*., 2016; Santander *et al*., 2019; Si Ammour *et al.,* 2020; Raguseo *et al*., 2021; Wang *et al*., 2022; Yang *et al.,* 2023; Agnusdei *et al*., 2024; Sánchez-Zelaia *et al*., 2024; Fedele *et al*., 2025; Incampo *et al*., 2025; Heger *et al*., 2025; Muthukumar *et al.,* 2025). In the case of *P. viticola*, they are mainly proposed for oospores quantification in diseased, senescent grapevine leaves (Si Ammour *et al*., 2020); for oospores and sporangia detection in soil, leaf litter, and asymptomatic leaf samples (Yang *et al*., 2023); for quantify the fungicide resistance (Huang *et al.,* 2023); for investigation the relationship between oospore density in vineyard leaf litter and primary infection incidence (Fedele *et al*., 2025), and for early detection and quantification of airborne inoculum of the pathogen in spore trap (Muthukumar *et al.,* 2025).

Despite the proven reliability of molecular techniques in providing both quantitative and qualitative information, their application typically requires laboratory equipment, trained personnel, and extended processing times. In recent years, various rapid pathogen detection methods have been developed, including lateral flow devices (Immuno Strip, Pocket Diagnostic), loop-mediated isothermal amplification (LAMP) (DeShields *et al*., 2018), and chlorophyll fluorescence (Chl-F) imaging (Rodríguez-Moreno *et al*., 2008). Specifically, *P. viticola* has been successfully detected using both LAMP (Marimuthu *et al*., 2020; Kong *et al*., 2016; Douillet *et al*., 2022) and Chl-F imaging (Cséfalvay *et al*., 2009).

While these methods offer advantages in terms of speed and user-friendliness, they are limited to providing high-quality results. Consequently, there is growing interest in the development of portable quantitative PCR (qPCR) systems that enable on-field diagnostics without compromising analytical accuracy and reliability. Several portable PCR protocols have already been validated for the detection of various plant pathogens (Koo *et al*., 2013; DeShields *et al*., 2018; Nguyen *et al*., 2018). The primary objective of this study was to design and validate a rapid, sensitive, and field-deployable real-time

PCR assay, employing SYBR Green fluorescence chemistry, for the on-field early detection of *P. viticola* in samples displaying typical and atypical symptoms.

MATERIALS AND METHODS

Pathogen isolate and growing conditions

The reference isolate of *P. viticola,* PLVDisspa1 originally isolated from grapevine leaves of 'Primitivo' was used to design the primer. Other fungal (*Alternaria alternata, Aspergillus carbonarius, Aspergillus niger, Botrytis cinerea, Cladosporium fulvum, Cylindrocarpon destructans* (syn. *Ilyonectria destructans*)*, Cytospora vitis, Diplodia seriata, Erysiphe necator*, *Eutypa lata, Neofusicoccum parvum, Penicillium expansum, Phaeomoniella chlamydospora, Phaeoacremonium aleophilum* (syn. *Phaeoacremonium minimum*)*, Phomopsis viticola* (syn. *Diaporthe ampelina*)*, Rosellinia necatrix* (syn. *Dematophora necatrix*), *Trichoderma asperellum*, *T. atroviride* and *T. gamsii*) and bacterial isolates (*Bacillus amyloliquefaciens*, *B. subtilis*, *Pseudomonas syringae* pv. *syringae* and *Xylella fastidiosa* subsp. *fastidiosa ST1*) commonly associated with grapevines as well as the Oomycetes *Phytophthora infestans* and *Pythium oligandrum* were used for specificity assay. All isolates were stored in 15% of glycerol at -80°C in the culture collection of our department except for *P. viticola* and *E. necator* that were inoculated on 'Baresana' fresh leaves placed on water agar medium (0.8% agar Oxoid No. 3 L^{-1}) incubated in a growth chamber at 25°C under a 16/8 h light/dark photoperiod and routinely refreshed. Other fungal and bacterial isolates were routinely grown at 25°C under darkness, respectively on potato dextrose agar (PDA: infusion from 200 g peeled and sliced potatoes kept at 60°C for 1 h, 20 g dextrose, adjusted at pH 6.5, and 20 g agar Oxoid No. 3 per liter) and nutrient agar (NA; 8 g nutrient broth and 20 g agar Oxoid No. 3 per liter) and PD3 (Pierce disease 3, Davis, 1980).

Designs for primer sets

To identify the most suitable region for primer design, the Internal Transcribed Spacer 1 (*ITS1*) region was the target selected. The *ITS1* sequence of the isolate PLVDisspa1 was aligned with 158 *ITS1* sequences from various *P. viticola* isolates available in GenBank (https://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using Clustal Omega (version 1.2.2; https://www.ebi.ac.uk) to identify conserved regions shared across all isolates. These conserved regions were subsequently used to design a primer set using Primer3Plus version 3.3.0 (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), following the guidelines outlined in the Droplet Digital PCR Application Guide (Bio-Rad, www.bio-rad.com, last accessed April 29th, 2025). The resulting primer set, designated PLAV19, was evaluated for in silico specificity using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast; Ye *et al.,* 2012) against the non-redundant nucleotide database. The selected primers were custom synthesized by Macrogen Inc. (Seoul, South Korea).

DNA extraction

Genomic DNA (gDNA) from PLVDisspa1 and *E. necator* was obtained by collecting mycelium directly from infected leaves, followed by extraction using the CTAB protocol. gDNA of the other fungal isolates was extracted from 3-day-old cultures grown on PDA overlapped by sterile cellophane disks (De Miccolis Angelini *et al.,* 2010). The DNA of *Bacillus* spp. and *P. syringae* pv. *syringae* was extracted from a cell pellet of 2 mL of nutrient broth (NA without agar) culture, obtained after 16 h of incubation at 25°C under shaking (200 rpm) (Nigro *et al.,* 2005). The DNA of different grapevine varieties was extracted from 500 mg of leaves after their homogenization in extraction bags (Bioreba AG, Reinach, Switzerland) with the Homex apparatus (Bioreba AG), using a CTAB based protocol (Doyle and Doyle, 1987). The DNA of *Xylella fastidiosa* subsp. *fastidiosa* ST1 was kindly supplied by the researchers of the DiSSPA SELGE Official Laboratory for quarantine plant pathogens. The DNA of *P. viticola* isolates from Lombardia was kindly supplied by S.L. Toffolatti (Department of Agricultural and Environmental Sciences, University of Milan, Italy). The quality and quantity of all DNA extracts was assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), while the concentration of double-stranded DNA (dsDNA) was specifically determined using a Qubit 2.0 fluorometer (Life Technologies Ltd., Paisley, UK).

qPCR assay optimization

For the optimization of the qPCR assay, the DNA extracted from the PLAVDisspa1 strain was used. Three distinct PLAV19 concentrations (100, 200, and 300 nM) and two annealing temperatures (58°C and 60°C) were evaluated. This optimization process was performed on the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA).

Each 20 µL reaction mixture contained 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Labo-

ratories), one of three primer concentrations (100, 200, or 300 nM for each primer), 2 µL of DNA template, and ultrapure nuclease-free water to the final volume. The qPCR cycling conditions included an initial denaturation at 95°C for 3 min., followed by 35 cycles of denaturation at 95°C for 10 sec., annealing at 58°C or 60°C and extension at 72°C both for 30 seconds. A melting curve analysis was subsequently performed from 65°C to 95°C, with 0.5°C increments every 5 seconds, to assess the specificity of the PCR products. Amplification data were analysed using CFX™ Manager Software (version 3.1, Bio-Rad Laboratories). Key performance metrics, including the quantification cycle (Cq), amplification curve slope, reaction efficiency (E), relative fluorescence units (RFU), and coefficient of determination (R^2), were determined to evaluate the assay's consistency and reliability under the tested conditions. The optimization reactions were conducted in biological triplicates to ensure reproducibility, and a no-template control (NTC), consisting of ultrapure sterile water, was included in each run to monitor for potential contamination and nonspecific amplification. Cq values were determined using the baseline settings automatically assigned by the instrument. The baseline was defined over the initial cycles where no significant increase in fluorescence signal was observed, thereby excluding background noise. Resulting Cq values from technical triplicates were averaged to represent each biological replicate, and these averages were used for all subsequent statistical analyses. The resulting Cq values were exported to Excel (version 2021, Microsoft Corp., Redmond, WA, USA) for construction of the calibration curve.

Sensitivity and specificity of detections using a conventional and a portable thermocycler

The sensitivity of the qPCR assays was assessed using PLVDisspa1 DNA at an initial concentration of 1.5 ng/µL, as measured by Qubit 2.0 fluorometer (Life Technologies Ltd., Paisley, UK) with the dsDNA BR Assay kit (Thermo Fisher Scientific Inc.), followed by seven10-fold serial dilutions down to 150 ag/µL. A linear regression analysis was performed by plotting the Log_{10} of DNA concentration against both the corresponding Cq values obtained from qPCR performed using CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and Hyris bCUBE, a compact and portable real-time PCR (Generon S.p.a., Modena, Italy), in order to evaluate assay performance under both laboratory and field-compatible conditions. From this analysis, the coefficient of determination (R^2) and the slope were calculated. To assess the analytical sensitivity, the limit of detection (LoD) and limit of quantification (LoQ) were determined based on ten replicate measurements. Precision was further evaluated by performing the assay on the same samples across independent runs on different days, allowing assessment of both intra- and inter-assay variability.

Specificity of the PLAV19 primer was evaluated using DNA from a broad range of grapevine-associated pathogens, biocontrol agents other *P. viticola* isolates collected from different locations across the Apulia, Calabria and Lombardia regions, and the Oomycetes *P. infestans* and *P. oligandrum*. DNA from the *V. vinifera* cultivars 'Gaglioppo', 'Greco Bianco', 'Negroamaro', 'Nero d'Avola', 'Primitivo', 'Red Globe', 'Sugar Crisp', 'Sugar One', and 'Timco' was also used. In all amplifications, the reference strain PLVDisspa1 was used as positive control (PC) and ultrapure water as NTC.

Methods for validation of in-field DNA extractions

Three DNA extraction methods were compared. The CTAB based method (1) (Doyle and Doyle (1987), was used as laboratory control, while the other two methods (2 and 3) herein set up were carried out using the portable laboratory for qPCR (Generon S.p.a.), including specific equipment for extraction (centrifuge, dry bath, vortex) and the portable real-time Hyris bCUBE.

The protocol 2 was a chloroform-based method, in which a total of 500 mg of grapevine tissue was homogenized in extraction bags using a manual homogenizer (Bioreba AG) and 5 mL of CTAB extraction buffer. The samples were incubated for 15 min at 65°C in a heat block. Subsequently, 1 mL of chloroform was added, followed by centrifugation at 7,200 g for 5 min. The supernatant was carefully collected, and 0.6 volumes of cold isopropanol were added to precipitate nucleic acids. The mixture was incubated on ice for 5 min and then centrifuged for 10 min at 7,200 g. The resulting pellet was resuspended in 20 µL of nuclease-free water (Qiagen, Venlo, The Netherlands). The protocol 3 was performed following the same initial steps as previously described, homogenizing 500 mg of plant tissue in Bioreba extraction bags (Bioreba AG) using CTAB buffer and incubating the samples for 15 min at 65°C in a heat block. Subsequently, 1 mL of isopropanol was added to the lysate, followed by centrifugation at 7,200 g for 10 min. The supernatant was discarded, and the pellet was resuspended at 100 µL in nuclease-free water.

Validation of the molecular diagnostic method

The validation of the molecular assay was performed using DNA extracted from: i) leaf samples from artificial

inoculation; ii) leaf and bunch samples natural infected; iii) a homemade siliconized sampling tape for trap spore artificially contaminated with PLVDisspa1 sporangia; iv) leaves with atypical symptoms and berries with "brown rot" symptoms from the field; v) leaves with necrotic spots bordered of a purple line never associated to downy mildew. DNA was extracted by using all three protocols.

For the artificial contamination, symptomatic leaves were used to collect the sporangia, which were counted and diluted up to 10^6 sporangia mL^{-1}. Subsequently, asymptomatic leaves were collected, using a sterile blade, from vines cuttings maintained in greenhouses of our department and 50 mg aliquots were transferred into 2 mL Eppendorf tubes. Ten-fold dilutions up to 10^0 sporangia mL^{-1} were prepared, and 1 mL of each concentration was added to 50 mg of health leaf tissue. The homemade siliconized sampling tape (1x2 cm) for trap spore was also artificially contaminated with 80 µL of each sporangial suspension of 10-fold serial dilution (10^6 to 10^0) and allowed to dry before DNA extraction. To assess the potential interference of the sample matrix to which sporangia were added, control reactions were performed using sporangia pure extracts. While to evaluate the impact of inhibitory substances on the qPCR reaction, the final three dilutions (10^2, 10^1, and 10^0) were spiked into unripe grape samples, known to be rich in polyphenolic compounds, and subsequently subjected to extraction using the three methods. The coefficient of variation (CV) of the reactions was calculated. Leaves of cultivar Primitivo (Bari, Apulia, Italy), showing unspecific necrotic spots, and bunches of cultivar Italia (Canosa di Puglia, Apulia, Italy), showing brown rot symptoms, were collected in June 2025. Finally, the gDNA was extracted and amplified with PLAV19 using CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and Hyris bCUBE qPCR thermocycler (Generon S.p.a.).

To ensure the reliability of the protocol, both repeatability and reproducibility were evaluated. Repeatability was assessed by performing three measurements on the same sample by a single operator using the same instrument. Reproducibility was evaluated by performing the same measurements with two different operators using two equivalent instruments. For both assessments, the CV was calculated.

Statistical analysis

The Cq values obtained from the qPCR optimization tests were analysed using a two-way ANOVA (Costat software, Cohort, Monterey, CA, USA) to evaluate the effects of primer concentration (100, 200, and 300 nM) and annealing temperature (58°C and 60°C). Additionally, a separate two-way ANOVA was conducted to determine the influence of these two factors on relative fluorescence units (RFUs). To evaluate the agreement between the Cq values obtained from the CFX96™ Real-Time PCR Thermal Cycler and the Hyris bCUBE, a Pearson correlation analysis was performed using the same software. Finally, a one-way ANOVA was carried out to evaluate statistical differences in Cq values among the three DNA extraction methods.

RESULTS

Generation of primer sets

Assessing the inter-isolate variability among all *ITS1* sequences of *P. viticola* available in GenBank (accessed on April 28th, 2025), a region was identified allowing the design of the primer set (Table 1). In detail, considering the partial *ITS1* sequence of *P. viticola* as reference (NCBI Accession No.: JF897782.1), the PLAV19 set includes the region from base 126 to base 261 (Table 1).

The results of the *in-silico* specificity analysis of the primers showed 100% identity with *P. viticola* sequences, including those from other countries such as Australia (MG552098.1), Brazil (MH310113.1), China (KM279691.1), India (ON183962.1), and the United States (MK345987.1).

The *in silico* specificity was proved against the Non-Redundant Nucleotide sequence database and in deep detail against the main grape-associated microorganisms, such as *A. alternata*, *Aspergillus* spp., *Bacillus* spp., *B. cinerea*, *C. fulvum*, *C. destructans*, *C. vitis*, *D. seriata*, *E. lata*, *N. parvum*, *P. expansum*, *P. chlamydospora, P. minimum, P. viticola, P. syringae, R. necatrix, Trichoderma* spp., *E. necator,* and *X. fastidiosa* subsp. *fastidiosa*, the other Oomycetes *P. infestans* and *P. oligandrum*, and grapevine.

Optimization of qPCR assays

The results of the qPCR optimization using the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) are presented in Figure 1.

Table 1. Primer set generated with this study.

Set	Primer		Amplicon size (bp)
	Name	Sequence (5'-3')	
PLAV19	Forward	GTAGCTTACCCTGCACCAC	136
	Reverse	TCTTCATCGATGCCAGAACC	

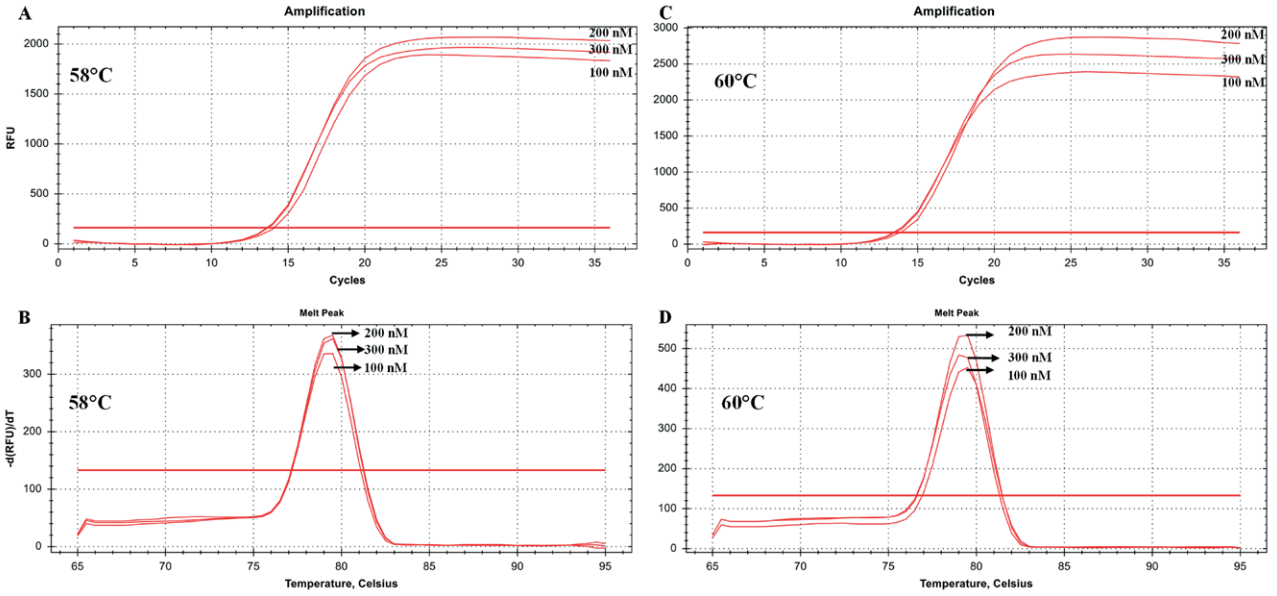


Figure 1. Optimization of qPCR conditions; in A 58°C of annealing temperature and three concentrations (100, 200, and 300 nM, in C the same concentrations at annealing temperature of 60°C. Panels B and D show the respective melt curves corresponding to the different temperatures and concentrations tested. For each tested conditions, was reported the mean of three biological replicate.

The Cq (quantification cycle) values remained consistent across all tested primer concentrations (F = 5.24x10^{-29}, p = 1.000) and annealing temperatures (ANOVA, F = 0.102 p = 0.904), with no statistically significant differences.

In contrast, analysis of RFUs revealed notable differences. At an annealing temperature of 58°C, the highest fluorescence was observed with a primer concentration of 200 nM, yielding an average RFU of approximately 2,000 (Figure 1A). At 60°C, RFU values increased further, up to 2,900 (Figure 1C). A two-way ANOVA confirmed statistically significant effects of both temperature (F = 555.17, p < 0.0001) and primer concentration (F = 129.96, p < 0.0001) on RFU values. Moreover, a significant interaction between temperature and concentration was observed (F = 9.70, p = 0.0031), indicating that the impact of primer concentration on RFU was temperature dependent. Based on these findings, the optimal qPCR conditions were determined to be a primer concentration of 200 nM and an annealing temperature of 60°C. Across all experimental conditions, the melting peak was consistently observed at 79.5°C, confirming the high specificity of the amplification reaction (Figure 1 B and D).

Sensitivity and specificity assays

A 10-fold serial dilution of PLVDisspa1 DNA, ranging from 1.5 ng μL^{-1} to 150 ag μL^{-1}, was tested using the PLAV19 primer set in qPCR assays (Figure 2).

On the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories), PLAV19 successfully detected DNA concentrations as low as 1.5 fg μL^{-1}, corresponding to a Cq value of 33.5. Across the dynamic range of 1.5 ng μL^{-1} to 1.5 fg μL^{-1}, the assay exhibited excellent linearity (R^2 = 0.998), with a slope of -3.375, corresponding to a mean PCR efficiency of 97.9%. Similarly, the Hyris bCUBE (Generon S.p.a.) demonstrated the capability to detect 1.5 fg μL^{-1} with a Cq value of 32.0. Within the same dynamic range, this platform also displayed strong linearity (R^2 = 0.992) and a slope of -3.478, equivalent to a PCR efficiency of 93.6%. Notably, the Hyris bCUBE (Generon S.p.a.) yielded slightly earlier Cq values at higher DNA concentrations. For instance, a concentration of 1.5 ng μL^{-1} was detected at a Cq of 11, compared to 13.5 on the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories). Despite this difference in amplification kinetics, the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) exhibited a light higher linearity.

The strong positive correlation observed between Cq values from the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and the Hyris bCUBE (Generon S.p.a.) (Pearson's r = 0.99, p = <0.001) highlights the comparable performance and high reproducibility of the two qPCR thermocyclers.

The analytical sensitivity of the assays was determined by calculating the LoD and LoQ. The LoD was

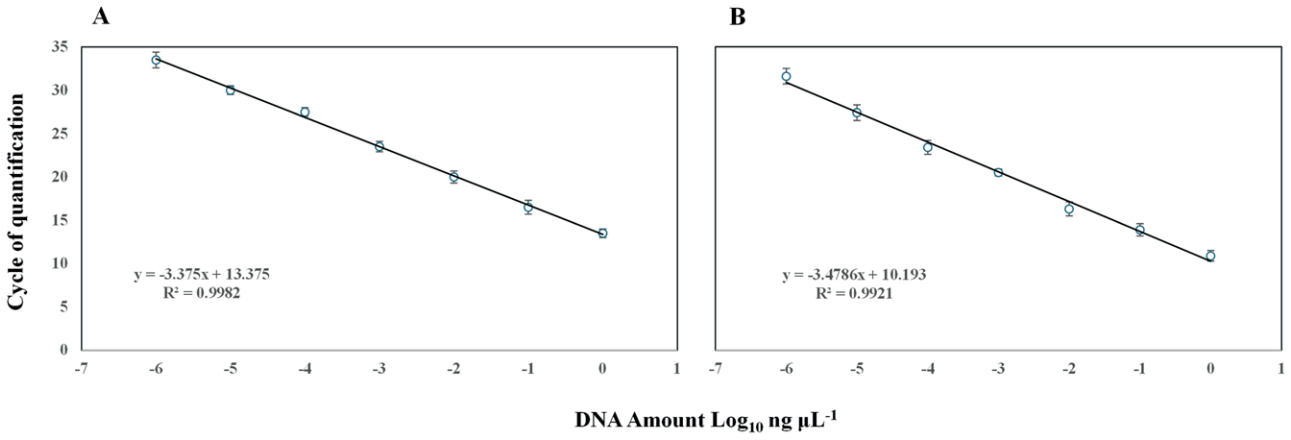


Figure 2. Linear regression analysis obtained from qPCR assays performed with different concentrations of PLVDisspa1 DNA, amplified using the PLAV19 primer. Panel A shows results from the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories), while Panel B shows results from the Hyris bCUBE system (Generon S.p.a.).

determined empirically as the lowest concentration at which the target was reliably distinguishable from the background signal and was found to be 1.5×10^{-6} (1.5 fg) for both the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and the Hyris bCUBE (Generon S.p.a.) platforms. The LoQ was calculated from ten replicate measurements at low concentrations, with all replicates exhibiting a CV below 20%, resulting in a LoQ of 1.5×10^{-5} (15 fg). This criterion ensures that concentrations at or above the LoQ can be quantified with acceptable precision and accuracy. Precision was further evaluated through intra- and inter-assay analyses. Intra-assay precision was assessed by measuring replicates of the same sample within a single run, yielding CV of 1%. Inter-assay precision was determined by comparing measurements of the same sample across independent runs performed on different days, yielding a CV of 3%. These results confirm that the assay is reproducible and robust, providing reliable quantitative measurements across the tested concentration range.

Specificity testing for both qPCR assays was performed using DNA at a concentration of 20 ng μL^{-1} from a range of fungal and bacterial species, as well as various grapevine cultivars. The results of the specificity assays were conducted on both the thermocyclers confirmed that no reaction occurred using the DNA from the panel of microorganisms different from *P. viticola* and grape genotypes (Table 2).

Reference DNA PLVDisspa1 yielded Cq values of 13.5 and 11.0 for CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and Hyris bCUBE (Generon S.p.a.), respectively. Similarly, the other 9 *P. viticola* isolates yielded Cq in the range 13.1-13.8 for CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and in the range 10.7-11.3 by using Hyris bCUBE thermocycler (Generon S.p.a.) Table 2.

Extraction protocol

Three protocols were compared. Four criteria were considered: solvents required, the total nucleic acid yield, the double-stranded DNA (dsDNA) concentration, and overall processing time. The classical CTAB method (1) Doyle and Doyle (1987) was used as reference. For protocol 2, the extraction involved several solvents, including CTAB, chloroform, and isopropanol. The total time required to extract DNA from a single sample was approximately 37 minutes, broken down as follows: 2 minutes for sample disruption using the Bioreba homogenizer (Bioreba AG), 15 minutes of incubation, 5 minutes of chloroform treatment, 5 minutes at –20°C with isopropanol, and 10 minutes of centrifugation. The total nucleic acid yield was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.), while the concentration of double-stranded DNA (dsDNA) was specifically determined using a Qubit 2.0 fluorometer (Life Technologies Ltd.). The results, summarized in Table 3, showed a total nucleic acid concentration of 350 ng μL^{-1} and a dsDNA concentration of 22 ng μL^{-1}. In the protocol 3, the use of solvents was lower, as it involved only CTAB and isopropanol. The DNA extraction process was faster, requiring a total of 27 minutes, distributed as follows: 2 minutes for sample disruption using the Bioreba homogenizer (Bioreba AG), 15 minutes of incubation, and 10 minutes of centrifugation in isopropanol. The total nucleic acid concentration was 200 ng μL^{-1}, while the double-stranded DNA (dsDNA)

Table 2. Panel of microorganism used for specificity assay.

Species	Isolate/ variety	Cq	
		1	2
Plasmopara viticola	PLVDisspa1	13.5 ± 0.5	11.1 ± 0.3
	PLV_puglia1	13.2 ± 0.4	10.7 ± 0.3
	PLV_puglia2	13.5 ± 0.5	11.1 ± 0.2
	PLV_puglia3	13.8 ± 0.6	11.3 ± 0.4
	PLV_calabria1	13.6 ± 0.3	11.1 ± 0.3
	PLV_calabria2	13.2 ± 0.6	10.8 ± 0.3
	PLV_calabria2	13.1 ± 0.6	10.8 ± 0.3
	CAS_A_6(PN)	13.4 ± 0.6	10.9 ± 0.3
	GH_NO	13.5 ± 0.5	11.1 ± 0.3
	LONG_A2(VI)	13.2 ± 0.4	10.7 ± 0.3
Bacterial isolates			
Bacillus amyloliquefanciens	D747_Disspa	-	-
Bacillus subtilis	QST713	-	-
Pseudomonas syringae pv. *syringae*	Pssy_1	-	-
Xylella fastidiosa subsp. *fastidiosa*	ST1	-	-
Fungal isolates			
Alternaria alternata	Alt_Disspa		-
Aspergillus carbonarius	Ac9_Disspa	-	-
Aspergillus niger	An2_Disspa	-	-
Botrytis cinerea	SAS 56_Disspa	-	-
Cladosporium fulvum	Cladsp2	-	-
Cylindrocarpon destructans	Cd_2_Disspa	-	-
Cytospora vitis	Cv1_Disspa	-	-
Diplodia seriata	Ds_Disspa	-	-
Erysiphe necator	EN_1_Disspa	-	-
Eutypa lata	EL_1_Disspa	-	-
Neofusicoccum parvum	DiSSPA_NP1	-	-
Penicillium expansum	Pspp1	-	-
Phaeomoniella chlamydospora	Pch1_Disspa	-	-
Phaeoacremonium aleophilum	PcAl_1_Disspa	-	-
Phytophthora infestans	Phy_1	-	-
Pythium oligandrum	M1	-	-
Phomopsis viticola	PhomV_1_Disspa	-	-
Rosellinia necatrix	Rn_1_Disspa	-	-
Trichoderma asperellum	ICC012	-	-
Trichoderma atroviridae	SC1	-	-
Trichoderma gamsii	ICC080	-	-
***Vitis* varieties**			
Vitis vinifera	Gaglioppo	-	-
	Greco Bianco	-	-
	Negramaro	-	-
	Nero d'Avola	-	-
	Primitivo	-	-
	Red Globe	-	-
	Sugar Crisp	-	-
	Sugar One	-	-
	Timco	-	-
Vitis rupestris × *Vitis berlandieri*	140 RU	-	-
Vitis berlandieri × *Vitis rupestris*	1103	-	-

1, CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories); 2, Hyris bCUBE (Generon S.p.a.).

Table 3. Comparison of solvents, time and amount of DNA extracted by three methods.

Extraction protocols	Solvents	Time (minutes)	Total nucleic acid (ng μL^{-1})	dsDNA (ng μL^{-1})
1	CTAB, Chloroform, Isopropanol	110	750 ± 10	65 ± 6
2	CTAB, Chloroform, Isopropanol	37	350 ± 8	22 ± 5
3	CTAB, Isopropanol	27	200 ± 5	16 ± 3

*Values are presented as mean ± standard error (SE) of three independent biological replicates, each measured in triplicate. SE reflects variability only among biological replicates and does not include technical variation.

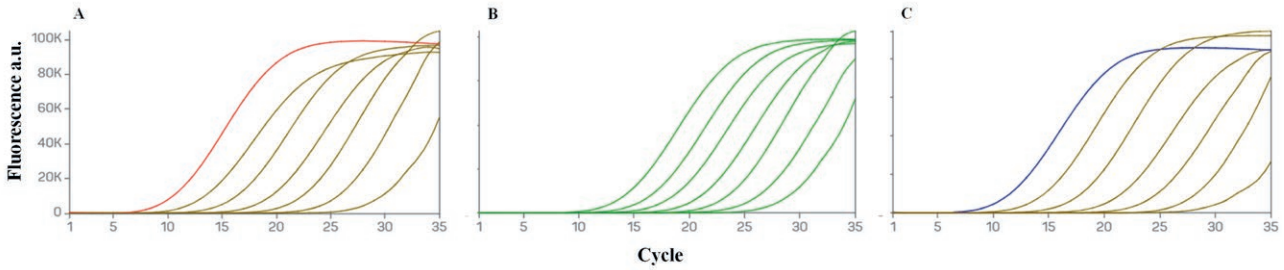


Figure 3. Validation of PLAV19 results of different sporangia suspension 10^6 to 10^0 added to leaves, extracted with protocol 1 (A), protocol 2 (B) and protocol 3 (C).

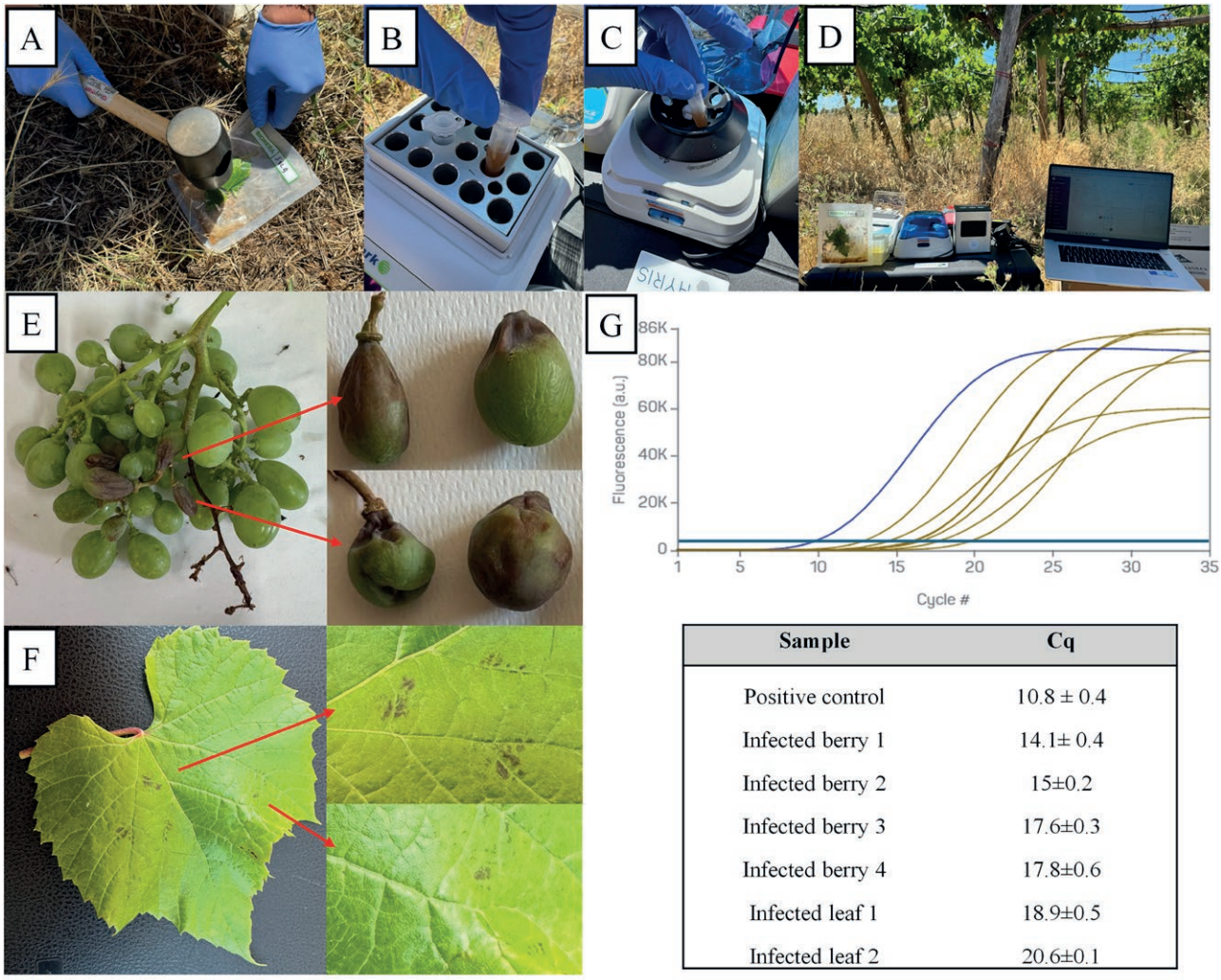
concentration was 16 ng μL^{-1}. For comparison, the control protocol (CTAB) yielded a total nucleic acid concentration of 750 ng μL^{-1} and a dsDNA concentration of 65 ng μL^{-1}.

Validation of the molecular diagnostic

To validate the diagnostic method, an initial evaluation was carried out by amplifying DNA extracted from grapevine leaves artificially inoculated with known quantities of *P. viticola* sporangia. The assay was performed using the portable thermocycler Hyris bCUBE (Generon S.p.a.) to determine the limit of detection (LoD) for sporangia, comparing the performance of the three DNA extraction protocols.

The results showed that the quantification cycle (Cq) values were consistent across all extraction methods (Figure 3). Notably, each protocol achieved a detection limit of 10^0 sporangia, indicating equivalent sensitivity regardless of the extraction protocol employed. Statistical analysis confirmed the absence of significant differences among the protocols ($F = 0.020$, $P = 0.980$), supporting the reliability and comparability of the three extraction protocols for the sensitive detection of *P. viticola* sporangia. Furthermore, analysis of CV was performed to assess the potential interference of sample matrices. Compared to the controls, all three extraction methods showed a CV of approximately 2%, confirming the absence of significant interference from components external to the DNA of *P. viticola* sporangia. Similarly, testing the potential impact of polyphenolic compounds from unripe grapes yielded comparable CV values (~2%) across all three methods.

These results enabled the selection of the most rapid DNA extraction method (3) suitable for early in-field detection of *P. viticola*, optimizing both efficiency and practicality for field applications. Similar results were noted for DNA extracted from strips added with different concentrations of sporangia. PLAV19 was capable of detecting up to 10^0 sporangia on the strip. Moreover, during the validation phase using samples collected from various vineyards across Apulia, Calabria and Lombardia regions, the assay demonstrated robust performance. Notably, it successfully detected *P. viticola* in leaf samples exhibiting unspecific symptoms of downy mildew, as well as in berries with brown rot (Figure 4 E and F). Figure 4 G illustrates the corresponding Cq values obtained from field-collected samples, further confirming the assay's sensitivity and reliability under real-world conditions. Leaves with necrotic spots bordered by purple line, a smptoms never associated to downy mildew, confirmed to be always negative to the test. Furthermore, method repeatability and reproducibility were assessed by calculating the CV%. The CV for repeatability was around 1%, while for reproducibility it was approximately 3%, indicating very low variability and confirming the robustness of the method.



Sample	Cq
Positive control	10.8 ± 0.4
Infected berry 1	14.1± 0.4
Infected berry 2	15±0.2
Infected berry 3	17.6±0.3
Infected berry 4	17.8±0.6
Infected leaf 1	18.9±0.5
Infected leaf 2	20.6±0.1

Figure 4. Validation assay of PLAV19 with naturally infected samples. From A to D steps are carried out in the field. In E symptoms on berries (brown rot); in F unspecific symptoms on leaves. In G the corresponding Cq values detected in qPCR assay with Hyris bCUBE (Generon S.p.a.) thermocycler on berries and leaves.

DISCUSSION

This study focused on the development of a portable SYBR Green-based qPCR molecular diagnostic assay for the on-site detection of *P. viticola* and to our knowledge it is the first for downy mildew detection and quantification in grapevines.

The PLAV19 primer set, targeting specifically the *ITS1* region of *P. viticola* within the base 126 to 261, was designed and although statistical analysis revealed no significant differences in quantification cycle (Cq) values across the tested conditions, significant differences in RFUs were observed in relation to both primer concentrations and temperatures resulting 200 nM primer concentration and 60°C the annealing temperature the best condition to be used.

Both *in-silico* analysis and *in vivo* specificity assay confirmed the reliability and usability of the primer set PLAV19, enlarging so the ones available for *P. viticola* detection and quantification. Their specificity was proved also for additional grapevine cultivars ('Gaglioppo', 'Greco Bianco', 'Negroamaro', 'Nero d'Avola', 'Primitivo', 'Red Globe', 'Sugar Crisp', 'Sugar One', and 'Timco') as well as for other important pathogens of grapes such as *A. carbonarius* responsible for Ochratoxin A contamination of wine (Pollastro *et al.*, 2005) and fungi reported as Grapevine Trunk diseases causal agents (Gramaje *et al.*, 2018). The PLAV19 specificity was proved for the first time also against important bacterial pathogens i.e. *X. fastidiosa* subsp. *fastidiosa* (Cornara *et al.*, 2025) and *P. syringae* pv. *syringae* (Gerin *et al.*, 2019), as well as several biocontrol agents currently

applied against Grapevine Trunk Disease (*Trichoderma* spp.), bunch roots (*Bacillus* spp.), and downy mildew (*P. oligandrum*). All reactions were terminated at 35 Cq to achieve an optimal balance between sensitivity, specificity, and rapid processing time suitable for field monitoring applications.

DNA extraction methods recover a crucial role into a qPCR assay. Indeed, if the quality and quantity of DNA extraction was low, the qPCR can yield false negative results, since the quantity of possible inhibitors can alter sensibility in the qPCR assays (Nourrisson *et al.,* 2020). In this study, three DNA CTAB-based extraction methods were evaluated. Two simplified CTAB-based methods herein proposed for the first time were compared to the reference protocol detailed by Doyle and Doyle (1987) with the aim of identify the most suitable in the balancing the following four criteria: solvents required, total nucleic acid yield, double-stranded DNA (dsDNA) concentration, and overall processing time, respect to the performance characteristics as detailed in EPPO PM7/98 and PM7/7 (2021; 2024). As expected, the standard CTAB method yielded the highest concentrations of total nucleic acids and of dsDNA but required multiple handling steps, laboratory-specific materials, and the use of solvents, such as chloroform particularly and isopropanol. Unsing method 2 in which the modification affects the processing time (37 minutes) a halved amount of total nucleic acid and a third amount of dsDNA was obtained, but non-beneficial in respect to the solvents used. In protocol 3, the main modification consists in removal the chloroform step by adding isopropanol directly to the lysate and removing it by a 10 min-centrifugation. It proved to be the most suitable for field applications (less than 30 minutes) and the most ecofriendly avoiding the use of the potential carcinogenic solvent chloroform. Additionally, it is chipper comparing the commonly used total DNA extraction procedures CTAB- or Kit- based and proposed in qPCR for *P. viticola* sporangia, oospores and mycelium detection in different matrices as leaves, litter, air and spore-trap (Valsesia *et al*., 2005; Piccolo *et al*., 2012; Si Ammour *et al*., 2020; Huang *et al*., 2023; Yang *et al*., 2023; Fedele *et al*., 2025; Muthukumar *et al*., 2025). The total amount of DNA and dSDNA resulted in about a quarter of the reference protocol, resulting in quite aligned with the concentration commonly used in qPCR for *P. viticola* and so not requiring the additional step of DNA dilution usually made in lab (i.e. Fedele *et al*., 2025). Two different extraction methods from grape leaves (Xin *et al*., 2003) and from air samples collected by the spore trap (Rogers *et al*., 2009) were proposed by Kong *et al*. (2016) in *P. viticola* LAMP based detection, with the first one certainly easy to be applied on-site and the second one longer and requiring lab-equipment. Also, Marimuthu *et al*. (2020) extract DNA according to the long-used CTAB protocol proposed by McDermott *et al*. (1994) confirming several limitations to the on-field application. Differently, a simple rapid DNA extraction procedure based on cell lysis in potassium hydroxide (KOH), was compared with the CTAB protocol described in Douillet *et al*. (2022) in both LAMP and ddPCR. In our work, two different Thermal cyclers the CFX96™ Real-Time PCR Thermal Cycler (Bio-Rad Laboratories) and the portable Hyris bCUBE (Generon S.p.a.) never used in the qPCR reported for *P. viticola* detection and quantification were compared. Both systems demonstrated high linearity, with R^2 values of 0.998 and 0.992, respectively, across a dynamic range from 1.5 ng μL^{-1} to 1.5 fg μL^{-1}, resulting better performing to the LAMP and ddPCR (Douillet *et al*., 2022). The analytical sensitivity expressed as limit of detection (LoD) for both thermocyclers was established at 1.5 fg μL^{-1}, corresponding to mean Cq values of 33.5 for the CFX96™ and 32.0 for the Hyris bCUBE. No potential unspecific target until the 35 Cq were also observed. The Cq values obtained using the CFX96™ and the Hyris bCUBE showed a Pearson's r = 0.99 (p <0.001) indicating an extremely strong and statistically significant positive correlation between the results obtained with the two thermal-cyclers. The LoD value is on average 10 times lower than that reported for LAMP based portables (Marimuthu *et al*., 2020; Kong *et al.,* 2016; Douillet *et al*., 2022) being so the technique more sensible for pathogen detection on-field. The LoQ was calculated at 15 fg, these findings are consistent with the sensitivity levels reported for other qPCR assays developed for the detection of fungal pathogens such as *Monilia* spp. and *Verticillium* spp. (Raguseo *et al*., 2021; Wang *et al*., 2022), and also for the oomycete *P. viticola* (Valsesia *et al.,* 2005; Si Ammour *et al*., 2020; Yang *et al*., 2023; Huang *et al*., 2023; Fedele *et al*., 2025; Muthukumar *et al*., 2025) but to our knowledge it is the first time for a portable qPCR for this pathogen. Moreover, the CV were examined to assess whether the presence of sample matrices could interfere with the qPCR assays. Across all three extraction methods, the CV remained around 2% relative to the controls, indicating that components external to the *P. viticola* sporangia DNA did not significantly affect the reaction. Similarly, the inclusion of polyphenolic-rich unripe grape tissue did not alter the CV, which remained consistently low (~2%), further supporting the robustness of the extraction methods against potential matrix-related inhibition. Overall, precision evaluated through both intra- and inter-assay analyses yielded CVs below 20%, confirming that the proposed

assay is reproducible and robust, providing reliable quantitative measurements across the tested concentration range. The demonstrated high specificity, combined with a detection sensitivity of 1.5 fg (Cq 32), ensures excellent assay performance. Stopping amplification at Cq 35 led to complete the reaction in 75 min improving the on-field portability of the assay. Considering the performance herein obtained using the DNA extracts with the three methods in terms of analytical sensitivity, analytical specificity and risks of false-positive and false-negative no statistical differences in the qPCR results were observed. Consequently, the protocol identified as protocol 3 is proposed for on-site applications.

The availability of portable diagnostic methods represents a valuable opportunity to accelerate pathogen monitoring and support more effective and sustainable plant disease management strategies.

For *P. viticola*, the use of molecular tools enables the early detection of airborne inoculum such as sporangia, oospores and mycelium and is also proposed for monitoring population variability in terms of fungicide resistance (Massi *et al*., 2021). This early warning capability is crucial for implementing targeted proactive and reactive protective measures, thereby minimizing the impact of the disease on vineyards, the reliance on chemical treatments and consequently reducing the risk of acquire resistance to fungicides. The molecular method herein developed can be integrated with existing forecasting models to enhance the accuracy of *P. viticola* prevention strategies (Puelles *et al*., 2024). Our results lay the foundation for the implementation of reliable and portable diagnostic tools, offering an effective solution for real-time *P. viticola* monitoring also integrating spore trap air sampling and so providing real evidence of pathogen presence essential for verifying model accuracy, calibration, and updating. Additionally, the portable qPCR herein presented proved to be applied on necrotic non sporulated spots frequently detected on some resistant grapevine genotypes in Apulian and Calabrian vineyards, confirming the usefulness of this device in detection and quantification of this important Oomycete also in unusual conditions, and resulting useful at evaluating the risk of infection and of overcoming genetic resistance in the new grapevine genotypes. These findings corroborate the requiring of knowledge in these new scenarios opening new epidemiological questions.

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